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MICROBIAL LABORATORY GUIDANCE MANUAL

FOR THE FINAL LONG TERM 2 ENHANCED SURFACE WATER TREATMENT RULE

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ACRONYMS

CFU	Colony-forming unit
CNFG	Confluent growth
DAPI	4, 6-diamidino-2-phenylindole
DIC	Differential interference contrast
DCTS	LT2/Stage 2 Data Collection and Tracking System
EPA	United States Environmental Protection Agency
FA	Fluorescence assay
FITC	Fluorescein isothiocyanate
GWUDI	Ground water under the direct influence [of surface water]
ICR	Information Collection Rule
IDC	Initial demonstration of capability
IESWTR	Interim Enhanced Surface Water Treatment Rule
IFA	Immunofluorescence assay
IMS	Immunomagnetic separation
IPR	Initial precision and recovery
IPT	Initial proficiency testing
L	Liter
LT2 Rule	Long Term 2 Enhanced Surface Water Treatment Rule
LT2ESWTR	Long Term 2 Enhanced Surface Water Treatment Rule
mL	Milliliter
MPC	Magnetic particle concentrator
MPN	Most probable number
MS	Matrix spike
MS/MSD	Matrix spike/matrix spike duplicate
μm	Micrometer
NA-MUG	Nutrient agar (NA) with 4-methylumbelliferyl-beta-D-glucuronide (MUG)
NELAC	National Environmental Laboratory Accreditation Conference
nm	Nanometer
NPDWR	National Primary Drinking Water Regulations
NTU	Nephelometric turbidity unit
OPR	Ongoing precision and recovery
OPT	Ongoing proficiency testing
PBMS	Performance-based measurement system
PT	Proficiency testing
PWS	Public water system
QA	Quality assurance
QAP	Quality assurance plan
QC	Quality control
RSD	Relative standard deviation
SDWA	Safe Drinking Water Act
SOP	Standard operating procedure
TNTC	Too numerous to count
UV	Ultraviolet

SECTION 1: INTRODUCTION

The Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR or LT2 Rule; Reference 5.1) requires public water systems (PWSs) that use surface water or groundwater under the direct influence of surface water to monitor their source water (influent water prior to treatment plant) for *Cryptosporidium*, *E. coli*, and turbidity for a limited period [40 CFR § 141.701(a) and (c-h)]. In support of the monitoring requirements specified by the rule, three documents have been developed to provide guidance to the affected PWSs and the laboratories that support them:

- *Source Water Monitoring Guidance Manual for Public Water Systems for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)*. This guidance manual for PWSs affected by the rule provides information on laboratory contracting, sample collection procedures, and data evaluation and interpretation. This guidance manual also provides information on grandfathering requirements for *Cryptosporidium* and *E. coli* data.

- *Microbial Laboratory Guidance Manual for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)* (this document). The goal of this manual is to provide *Cryptosporidium* and *E. coli* laboratories analyzing samples in support of the LT2 Rule with guidance and detailed procedures for all aspects of microbial analyses under the rule to maximize data quality and consistency.

- *Users' Manual for the LT2/Stage 2 Data Collection and Tracking System (DCTS)*. This manual provides PWSs and laboratories with instructions on the entry, review, and approval of electronic data using the LT2/Stage 2 DCTS, and for generating reports using the system.

All of these manuals, as well as responses to frequently asked questions and an online microscopy module to assist analysts with identification of *Cryptosporidium* oocysts, are available at http://www.epa.gov/safewater/disinfection/lt2/compliance.

This guidance document is provided to help implement the LT2 Rule. This guidance document does not, however, substitute for the LT2 Rule or the analytical methods approved for use under the rule. The material presented is intended solely for guidance and does not alter any regulatory or analytical method requirements not altered by the LT2 Rule itself.

Sections 1 and 2 of the microbial laboratory LT2 manual provide LT2 background information and guidance on issues that apply to both *Cryptosporidium* and *E. coli* laboratories. Section 3 provides guidance specific to *Cryptosporidium* analyses for the LT2 Rule, and Section 4 provides guidance for *E. coli* analyses performed in support of the LT2 Rule.

1.1 Background

The LT2 Rule is a National Primary Drinking Water Regulation that requires monitoring, reporting, and public notification for all PWSs that use surface water sources. The rule requires additional treatment techniques for some systems, based on *Cryptosporidium* monitoring results and the treatment currently provided (40 CFR § 141.711 — 141.712). The LT2 Rule was developed to improve control of microbial pathogens in drinking water, including specifically the protozoan *Cryptosporidium*, and to address risk trade-offs between control of microbial pathogens and control of disinfection byproducts.

1.2 LT2 Rule Microbial Monitoring Requirements

Filtered PWSs serving a population of at least 10,000 people, and some filtered wholesale PWSs serving populations less than 10,000 people, are required to collect and analyze source water samples for *Cryptosporidiu*m, *E. coli* and turbidity for a minimum of 2 years [40 CFR § 141.701(a)(1)]. Unfiltered PWSs serving a population of at least 10,000 people, and some unfiltered wholesale PWSs serving populations less than 10,000 people, are required to collect and analyze source water samples for *Cryptosporidium* for a minimum of 2 years [40 CFR § 141.701(a)(2)]. Small, filtered systems (PWSs that serve fewer than 10,000 people) are required to monitor their source water for *E. coli* for a minimum of 1 year. A subset of small filtered systems would then be required to collect and analyze source water samples for *Cryptosporidium* over a 1 or 2-year period if they exceed *E. coli* trigger levels [40 CFR § 141.701(a)(3-4)]. Small, unfiltered systems are required to monitor their source water for *Cryptosporidium* instead of *E. coli*. [40 CFR 141.701(a)(6)].

Monitoring requirements for each system size, and the schedule for each stage of monitoring, are described in **Table 1-1**. Detailed guidance for sample collection during the LT2 Rule, and procedures for sample collection, documentation, and shipment, are provided in the *Source Water Monitoring Guidance Manual for Public Water Systems for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)* (at http://www.epa.gov/safewater/disinfection/lt2/compliance).

Cryptosporidium samples must be analyzed by a laboratory approved for analysis under the Laboratory Quality Assurance Evaluation Program for the Analysis of *Cryptosporidium* in Water (Section 3.2 of this manual, below) or a laboratory approved for *Cryptosporidium* analysis by an equivalent State laboratory certification program. However, at the time of publication, there were no equivalent State programs. Samples must be analyzed using EPA Method 1622/1623 [40 CFR § 141.704(a) and part 141.705] (References 5.2 and 5.3).

E. coli samples must be analyzed by certified laboratories using methods approved under the LT2 Rule for surface water monitoring [40 CFR § 141.704(b)] (See Section 4 of this manual for details). Under the LT2 Rule, turbidity samples must be analyzed by a party approved by the State (usually a certified operator or a professional engineer) using methods approved in 141.74(a)(Reference 5.4)1)[40 CFR §141.704(c)].

Table 1-1. Summary of LT2 Rule Monitoring Requirements (Initial Round of Monitoring)

SCHEDULE	Monitoring begins	Monitoring duration	Monitoring parameters and sample frequency requirements		
			Cryptosporidium	***E. coli***	**Turbidity**
***SCHEDULE 1**: Large systems serving ≥100,000 *	*October 1, 2006*	*2 years* [a]	*Minimum 1 sample/month* [b]	*Minimum 1 sample/month* [c]	*Minimum 1 sample/month* [c]
***SCHEDULE 2**: Large systems serving ≥50,000 and <100,000 *	*April 1, 2007*	*2 years* [a]	*Minimum 1 sample/month* [b]	*Minimum 1 sample/month* [c]	*Minimum 1 sample/month* [c]
***SCHEDULE 3**: Large systems serving ≥10,000 and <50,000 *	*April 1, 2008*	*2 years* [a]	*Minimum 1 sample/month* [b]	*Minimum 1 sample/month* [c]	*Minimum 1 sample/month* [c]
***SCHEDULE 4:** Small filtered systems (serving fewer than 10,000 *)*	*October 1, 2008*	*1 year* [a,d]	*See next row* §	*Every two weeks*	*N/A*
Small unfiltered systems (serving fewer than 10,000) and § *Small filtered systems exceeding E. coli* trigger levels [d] *or that elect to proceed directly to Cryptosporidium monitoring or that fail to conduct E. coli monitoring*	April 1, 2010	1 year [b], 2 *Cryptosporidium* samples per month, or 2 years [e], 1 *Cryptosporidium* sample per month		N/A	N/A

*** Wholesale systems must comply with the requirements based on the population of the largest system in the combined distribution system [40 CFR § 141.700(b)(1)]**

[a] PWSs may be eligible to use (grandfather) data collected prior to the applicable monitoring start date if certain requirements are met [40 CFR § 141.707(a)(1)]

[b] PWSs monitoring for *Cryptosporidium* may collect more than one sample per month if sampling is evenly spaced over the monitoring period [40 CFR § 141.701(a)(7)]

[c] Unfiltered systems serving ≥10,000 are not required to perform *E. coli* monitoring or turbidity but to conduct source water monitoring that includes *only Cryptosporidium* sampling [40 CFR § 141.701(a)(2)]

[d] Filtered systems serving fewer than 10,000 people must monitor for *Cryptosporidium* only if their *E. coli* annual mean concentrations is greater than 10 *E. coli*/100 mL for systems using lakes/reservoirs or is greater than 50 *E. coli*/100 mL for systems using flowing streams [40 CFR § 141.701(a)(4)]

[e] Small systems collecting one sample per month for 2 years are still required, where applicable, to meet the treatment technique implementation deadlines in 40 CFR § 141.713 (c). The same treatment compliance dates apply to the PWS regardless of which *Cryptosporidium* sampling frequency is used (i.e., selecting the 2 year *Cryptosporidium* sampling frequency does not extend *Cryptosporidium* treatment compliance deadlines).

N/A = Not applicable. No monitoring required.

1.3 Use of *Cryptosporidium* Data

Two types of *Cryptosporidium* data are collected under the LT2 Rule: *Cryptosporidium* occurrence data from the analysis of field samples, and method performance data from the analysis of matrix spike (MS) samples. The use of occurrence data from field samples is discussed in Section 1.3.1; the use of method performance data from MS samples is discussed in Section 1.3.2.

1.3.1 *Cryptosporidium* Monitoring Sample Data

The concentration of *Cryptosporidium* oocysts in source water samples analyzed during the LT2 Rule will be used to calculate a mean *Cryptosporidium* concentration for a PWS and classify the PWSs into a treatment requirements "bin" (40 CFR § 141.710). These bin classifications are provided in **Table 1-2.** The treatment bin classification established for each PWS will be used to determine whether additional treatment is needed. PWSs in Bin 1 are not required to implement additional treatment. PWSs in Bins 2 - 4 will be required to implement increasing levels of treatment and source water protection to address their higher risk for high *Cryptosporidium* source water concentrations.

Table 1-2. Bin Classifications for Filtered Systems

Average Sample Concentration	Bin classification
Cryptosporidium <0.075 oocyst/L or no monitoring requirement	Bin 1
0.075 oocysts/L ≤ *Cryptosporidium* < 1.0 oocysts/L	Bin 2
1.0 oocysts/L ≤ *Cryptosporidium* < 3.0 oocysts/L	Bin 3
Cryptosporidium ≥ 3.0 oocysts/L	Bin 4
PWSs that serve fewer than 10,000 people and NOT required to monitor for *Cryptosporidium*	Bin 1

The method used to average individual sample concentrations to determine a PWS's bin classification depends on the number of samples collected and the length of the sampling period.
For a filtered PWS monitoring for *Cryptosporidium*, bin classification would be based on the following calculations of bin concentration:

- For PWSs that collect at least 48 samples during a two year monitoring period, the *Cryptosporidium* bin concentration is equal to the arithmetic mean of all sample concentrations [40 CFR § 141.710(b)(1)]
- For PWSs that collect at least 24 samples, but not more than 47 samples, during a two year monitoring period, the *Cryptosporidium* bin concentration is equal to the highest arithmetic mean (average) of all sample concentrations in any 12 consecutive months in the monitoring period [40 CFR § 141.710(b)(2)]
- For PWSs serving fewer than 10,000 people and monitoring *Cryptosporidium* for only one year (i.e., collect 24 samples in 12 months), the bin concentration is based on the arithmetic mean of all sample concentrations [40 CFR § 141.710(b)(3)]

- For systems with plants operating only part of the year that monitor fewer than 12 months per year, the bin concentration is based on the highest arithmetic mean of all sample concentrations during any year of *Cryptosporidium* monitoring [40 CFR § 141.710(b)(4)]
- For filtered PWSs in which sampling frequency varies, systems must first calculate a monthly average concentration for each month of monitoring. Systems must then use these monthly average concentrations, rather than individual sample concentrations, in the calculation of bin concentration as described in the four examples above [40 CFR § 141.710(b)(5)]

Unfiltered systems must calculate the arithmetic mean of all *Cryptosporidium* samples concentrations [40 CFR § 141.712(a)(1)]. For unfiltered systems in which sampling frequency varies, systems must first calculate a monthly average for each month of monitoring. Systems must then use these monthly average concentrations, rather than individual sample concentrations, in the calculation of the mean *Cryptosporidium* level [40 CFR § 141.712(a)(3)]

In all cases, the bin concentration is calculated using individual sample concentrations. These concentrations are calculated as "number of oocysts detected / volume (in L) analyzed." Individual sample concentrations are not calculated as "oocysts detected / 10 L," nor are bin concentrations calculated as the "sum of the oocysts detected / the sum of the volumes analyzed." As a result, each sample has an equal weight on the final bin concentration. In cases where no oocysts are detected, the number of oocysts used to calculate the sample concentration is "0." There should be no adjustment of the number of oocysts detected based on recovery or oocyst viability.

1.3.2 *Cryptosporidium* Matrix Spike Data

During LT2 Rule *Cryptosporidium* monitoring, PWSs are required to analyze, at a minimum, one MS sample for every 20 field samples from their source water [Section 9.1.8 of Method 1622/1623]. For all PWSs, the first MS sample should be collected and analyzed during the first sampling event under the monitoring program per Section 9.1.8 in EPA Method 1622.1623. Details on MS sample requirements are provided in Section 3.3.12.

Based on this requirement, the following PWS categories must 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.

Although MS sample results will not be used to adjust *Cryptosporidium* recoveries at any individual source water, the results will be used collectively to assess overall recovery and variability for EPA Method 1622/1623 in source water.

1.4 Use of *E. coli* and Turbidity Data

E. coli and turbidity data are being collected by PWSs on Schedules 1-3 during LT2 Rule monitoring to confirm or refine the levels of *E. coli* that would indicate high *Cryptosporidium* concentrations in a source water.

Small, filtered systems are permitted to monitor for *E. coli*, rather than more expensive *Cryptosporidium* analyses. Only those small filtered systems with *E. coli* levels that exceed the trigger level are required to monitor for *Cryptosporidium* to determine bin placement [40 CFR § 141.701 (a)(4)]. Based on the data from the Information Collection Rule (ICR) and Information Collection Rule Supplemental Survey (ICRSS), the following *E. coli* trigger levels were set:

- A mean of 50 *E. coli*/100 mL for flowing stream–type source waters
- A mean of 10 *E. coli*/100 mL for reservoir/lake source waters

Small filtered PWSs that are monitoring for *E. coli* must collect at least one sample twice per month. Individual sample concentrations are then averaged to determine a mean *E. coli* level. A PWS's mean *E. coli* level is compared to the *E. coli* trigger levels above to determine if the PWS is required to monitor for *Cryptosporidium*.

The indicator data from large PWS monitoring will be reviewed and, if appropriate, guidance on alternate indicator trigger values will be issued to States prior to when small PWSs begin monitoring. States are allowed to approve alternative approaches to indicator monitoring for small PWSs [40 CFR § 141.701(a)(5)].

SECTION 2: GENERAL MICROBIAL LABORATORY QUALITY ASSURANCE

All laboratories analyzing *Cryptosporidium* and *E. coli* samples for the LT2 Rule should adhere to defined quality assurance (QA) procedures to ensure that analytical data generated under the rule are scientifically valid and are of known and acceptable quality. Detailed quality control (QC) requirements and recommendations specific to *Cryptosporidium* and *E. coli* analyses are discussed in Sections 3 and 4 of this manual, respectively. Two QA issues that apply to both analyses—quality assurance plans and sample temperature monitoring—are discussed below, in Sections 2.1 and 2.2.

2.1 Quality Assurance Plans

As specified in both the Lab QA Program for *Cryptosporidium* laboratories (http://www.epa.gov/safewater/disinfection/lt2/, Section 3.2 of this manual, Reference 5.5) and the Laboratory Certification Manual (Chapter III, page III-4, Reference 5.6) for *E. coli* laboratories, each laboratory should operate a formal QA program and document the scope of this program through a QA plan.

The laboratory's QA plan should be a stand-alone document. However, some information can be incorporated into the document by reference, including laboratory standard operating procedures (SOPs), analytical methods, and quality control (QC) and calibration notebooks. Laboratories currently certified for coliform analysis under the drinking water laboratory certification program may use their current QA plan; however, this plan should be updated to address the specific requirements for LT2 Rule monitoring. Topics that should be addressed in the QA plan are outlined below. Details on LT2 *Cryptosporidium* requirements are provided in Section 3 of this manual; details on LT2 *E. coli* analyses are provided in Section 4.

For *Cryptosporidium* laboratories, this QA plan should be available for review during a laboratory's on-site audit, as part of the EPA's *Cryptosporidium* Laboratory Quality Assurance Evaluation Program (Section 3.2 of this manual). For *E. coli* laboratories, this QA plan should be available for review during recertification audits as part of the National Primary Drinking Water Regulations. If the laboratory also performs turbidity testing, then the QA plan should address turbidity requirements.

The following items should be addressed in each QA plan:

1. Laboratory organization and responsibility
   - Include a chart showing the laboratory organization and line authority, including QA Managers
   - List the key individuals who are responsible for ensuring the production of valid measurements and the routine assessment of QC measurements
   - Specify who is responsible for internal audits and reviews of the implementation of the QA plan and its requirements

2. Personnel
   - List each analyst's academic background and water analysis experience
   - List each analyst's training on the method
   - Describe training available to keep personnel up to date on methods and regulations

3. Facilities
   - Arrangement and size of laboratory
   - Bench space
   - Storage space
   - Lighting
   - Air system
   - Lab reagent water system
   - Waste disposal system
   - Safety considerations. The laboratory should address biosafety in the laboratory when handling or processing *Cryptosporidium* samples and organism controls. Guidance on laboratory biosafety for *Cryptosporidium* is provided in **Appendix A** of this guidance manual.

4. Field sampling procedures (with SOP used by laboratory or sent to PWS clients)
   - Describe how samples are collected, including sample containers, sample storage, transport times, and sample temperature
   - Describe sample identification and information recording system

5. Laboratory sample handling procedures
   - Describe sample storage conditions
   - Describe the laboratory's sample tracking system; specify procedures used to maintain the integrity of all samples, i.e., logging, tracking samples from receipt by laboratory through analysis to disposal
   - Describe sample acceptance criteria

6. Equipment
   - Specifications for each piece of equipment used for *Cryptosporidium*, *E. coli* analyses, and/or turbidity
   - Calibration procedures, frequency, standards for each piece of equipment used for *Cryptosporidium*, *E. coli* analyses, and/or turbidity
   - Quality control records for each piece of equipment used for *Cryptosporidium*, *E. coli* analyses, and/or turbidity
   - Preventative maintenance and schedules, documentation for each piece of equipment used for *Cryptosporidium*, *E. coli* analyses, and/or turbidity

7. Supplies
   - Laboratory glassware and plastic ware acceptance conditions
   - Chemicals, reagents, dyes and culture media acceptance conditions
   - Chemicals, reagents, dyes, and culture media storage conditions
   - Filters acceptance conditions
   - Description of the laboratory's reagent tracking system

8. Laboratory practices (may reference SOP)
   - Preparation of reagent-grade water
   - Glassware washing and preparation
   - Sterilization procedures

9. Analytical procedures
   - Describe all reference methods used
   - State that the analytical methods described in this manual will be followed
   - Identify available SOPs

10. Quality control (QC) checks
    - Confirmation/ verification procedures, frequency
    - Sterility controls
    - Replicate analyses; frequency
    - QC samples, source; frequency
    - Positive and negative controls, proficiency testing (PT) samples, source; frequency
    - Spiked field samples
    - Between-analyst deviation

11. Data reduction, verification, validation, and reporting
    - Data reduction (conversion of raw data to *Cryptosporidium* oocysts/L and/or *E. coli*/100 mL)
    - Procedures to ensure the accuracy of data transcription and calculations
    - Validation (ensuring that QC steps associated with a field result are acceptable)
    - Reporting, including procedures and format for reporting data to utilities/EPA

12. Corrective actions
    - Define the laboratory response to unacceptable results from PT or QC samples and from internal QC checks
    - Identify persons with responsibility to take corrective action
    - Describe how the actions taken and the effectiveness of the actions taken will be documented

13. Recordkeeping
    - Describe how records are to be maintained (e.g. electronically, hard copy, etc.)
    - Describe length of time records are to be kept (see archive requirements, Sections 3.8 and 4.8 of this manual)
    - State where records are to be stored

The laboratory QA plan should be concise, but responsive to the above listed items. Additional guidance on developing QA plans is available in "Guidance on Quality Assurance Project Plans (QAPP) (G-5)," (EPA/240/R-02/009, December 2002), which is available as a download from http://www.epa.gov/quality/qa_docs.html#noneparqt.

However, the goals of a lab QA plan in general are different from the goals of the Guidance on QAPP, and not all of the issues that should be addressed for laboratory QA during the LT2 Rule are covered by this guidance (i.e., laboratory sample handling and record keeping). However, some of the concepts presented in the QAPP guidance that typically are not included in laboratory QA plans may aid the laboratory in updating their QA plan to address specific LT2 requirements.

2.2 Sample Temperature Monitoring

Cryptosporidium oocysts present in a sample can degrade and *E. coli* present in a sample can grow or die off, biasing analytical results. *Cryptosporidium* and *E. coli* samples for LT2 Rule monitoring are stored and maintained between 1°C and 10°C to reduce biological activity. This is specified in Section 8.0 of the December 2005 versions of EPA Method 1622/1623 for *Cryptosporidium* samples and at 40 CFR § 704(b)(3) and Chapter V, Section 6.3, of the Laboratory Certification Manual (Reference 5.6) for *E. coli* samples.

Samples for all analyses should remain above freezing at all times. This is a requirement in Section 8.0 of the December 2005 versions of EPA Method 1622/1623. Although not a significant concern for 10 L water samples, this is a greater concern for *Cryptosporidium* filters and 120 or 250 mL *E. coli* samples that are shipped off-site with coolant materials, such as wet ice, blue ice, or gel packs. The sample collection protocols discussed in the *Source Water Monitoring Guidance Manual for Public Water Systems for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)* provide sample packing procedures for *E. coli* and *Cryptosporidium* samples. Utility personnel should follow these procedures to ensure that samples remain at acceptable temperatures during shipment.

It is recommended that utilities practice the collection, packing, and shipping protocols prior to monitoring to ensure acceptable temperatures upon receipt at the laboratory.

Because *Cryptosporidium* samples collected for the LT2 Rule must meet the QC criteria in the methods [40 CFR § 704 (a)], and because these QC criteria include receipt of samples at ≤20°C and not frozen, laboratories must reject LT2 *Cryptosporidium* samples that are received at >20°C or frozen unless the sample was collected the same day it was received. This is discussed further in Section 3.3.11 in this manual. In these cases, the PWS must re-collect and re-ship the sample.

E. coli samples that are received at >10°C or frozen, or *E. coli* samples that the laboratory has determined exceeded 10°C or froze during shipment, must be rejected. After receipt, *E. coli* samples must be stored at the laboratory between 0°C and 10°C, and not frozen, until processed [40 CFR § 141.704(b)(3)].

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

- **Temperature sample.** One option, for *Cryptosporidium* filtered samples (not for 10 L bulk samples) and *E. coli* 120 and 250 mL samples, is for the PWS to fill a small, inexpensive sample bottle with water and pack this "temperature sample" next to the field sample. The temperature of this extra sample volume is measured upon receipt to estimate the temperature of the field sample. Temperature sample bottles are not appropriate for use with 10 L bulk samples because of the potential effect that the difference in sample volume may have in temperature equilibration in the sample cooler. Example product: Cole Parmer cat. no. C-06252-20 or equivalent.

- **Temperature vial.** A similar option is to use a thermometer that is securely housed in a liquid-filled vial. Temperature vials are not appropriate for use with 10 L bulk samples for the reasons stated above. Unlike temperature samples, the laboratory does not need to perform an additional step to monitor the temperature of the vial upon receipt, but instead just reads the thermometer. Example product: Eagle-Picher Sentry Temperature Vial 3TR-40CS-F or 3TR-40CS or equivalent.

- **iButton.** Another option for measuring the sample temperature during shipment and upon receipt is a Thermocron® iButton. An iButton is a small, waterproof device that contains a computer chip to record temperature at different time intervals. The information is then downloaded from the iButton onto a computer. The iButton should be placed in a temperature sample in the cooler, rather than placed directly in the cooler, where it may be affected by close contact with the coolant. Again, this option is not appropriate for use with 10 L bulk samples. Example product: Thermocron® iButtons or equivalent.

- **Stick-on temperature strips.** Another option is for the laboratory to apply a stick-on temperature strip to the outside of the sample container upon receipt at the laboratory. This option does not measure temperature as precisely as the other options, but provides an indication of sample temperature to verify that the sample temperature is acceptable. This option is appropriate for use with both 10 L bulk samples and field-filtered samples, but not for use with *E. coli* samples. Example product: Cole Parmer cat. no. C-90316-00.

- **Infrared thermometers.** Another option is for the laboratory to measure the temperature of the surface of the sample container or filter using an infrared thermometer. The thermometer is pointed at the sample, and measures the temperature without coming in contact with the sample volume. This option is appropriate for use with both 10 L bulk samples and field-filtered samples. Example product: Cole Parmer cat. no. EW-35625-10.

As with other laboratory equipment, all temperature measurement devices should be calibrated routinely to ensure accurate measurements. See the EPA Lab Certification Manual (Reference 5.6) for more information.

All temperature readings should be documented and archived as required by the analytical methods.

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SECTION 3: GUIDANCE FOR *CRYPTOSPORIDIUM* LABORATORIES

Cryptosporidium analyses conducted in support of the LT2 Rule must be performed using EPA Method 1622 or EPA Method 1623 [40 CFR § 141.704(a)]. Guidance on the use of these methods during the LT2 Rule is provided in this section of the manual.

3.1 LT2 Rule *Cryptosporidium* Sample Analysis Requirements

LT2 Rule requirements of particular significance are summarized in Sections 3.1.1 through 3.1.6, below, and discussed in more detail along with guidance in the remainder of Section 3.

3.1.1 Approved Laboratories

Systems must have *Cryptosporidium* samples analyzed by a laboratory that has passed a quality assurance (QA) evaluation under EPA's Laboratory Quality Assurance Evaluation Program for Analysis of *Cryptosporidium* in Water (Reference 5.5) or a laboratory that has been approved for *Cryptosporidium* analysis by an equivalent State laboratory approval program [40 CFR § 141.705(a)]. However, at the time of publication of this guidance document there were no equivalent State programs for approval of *Cryptosporidium* laboratories. Details on the elements of the Lab QA Program QA evaluation are provided in Section 3.2. It is suggested that each approved laboratory establish and maintain a relationship with another approved laboratory and develop a protocol to provide back-up analyses to clients if needed during LT2. A list of approved laboratories is posted at http://www.epa.gov/safewater/disinfection/lt2/.

3.1.2 Revised *Cryptosporidium* Method

The LT2 Rule requires that samples collected under the rule be analyzed using the December 2005 version of Methods 1622/1623. EPA proposed the use of the April 2001 versions of EPA Methods 1622/1623 in the LT2 Rule. However EPA requested and received comments on the use of updated versions to consolidate several method-related changes EPA believes are necessary to address LT2 Rule monitoring requirements. Methods 1622/1623 were revised and the December 2005 version of these methods (References 5.2 and 5.3) are included as **Appendix B** and **Appendix C** of this guidance manual. These changes include the following:

- Increased flexibility in matrix spike (MS) and initial precision and recovery (IPR) requirements. The requirement that the laboratory must analyze an MS sample on the first sampling event for a new PWS has been changed to a recommendation; the revised method allows the IPR test to be performed across four different days, rather than restrict analyses to 1 day.
- Clarification of some method procedures, including the spiking suspension vortexing procedure; the buffer volumes used during immunomagnetic separation (IMS); requiring (rather than recommending) that laboratories purchase HCl and NaOH standards at the normality specified in the method; and the use, or not, of methanol during slide staining in Section 14.2 of Method 1622/1623 is per manufacturer's instructions.

- Addition of recommendations for minimizing carry-over of debris onto microscope slides after IMS and information on microscope cleaning. Clarification of the actions to take in the event of QC failures.
- A change in the sample receipt temperature requirements to "≤20°C, and not frozen," and additional guidance on sample storage (between 1°C and 10°C) and shipping procedures based on time of day of collection. The revision includes suggested options for monitoring sample temperature during shipment and/or upon receipt at the laboratory.
- Addition of the requirement for examination using differential interference contrast (DIC) microscopy to the analyst verification procedure.
- Addition of an approved method modification using the Pall Gelman Envirochek™ HV filter. This approval is based on an interlaboratory validation study demonstrating that three laboratories, each analyzing reagent water and a different source water, met all method acceptance criteria for *Cryptosporidium* (but not *Giardia;* however, individual laboratories are permitted to demonstrate acceptable performance for *Giardia* in their laboratory).
- Incorporation of detailed procedures for concentrating samples using an IDEXX Filta-Max® foam filter. (A method modification using this filter was already approved by EPA in the April 2001 version of the methods.)
- Addition of BTF EasySeed™ irradiated oocysts and cysts as acceptable materials for spiking routine QC samples. EPA approved the use of EasySeed™ based on side-by-side comparison tests of method recoveries using EasySeed™ and live, untreated organisms.
- Removal of the Whatman Nuclepore CrypTest™ cartridge filter. Although a method modification using this filter was approved by EPA in the April 2001 versions of the methods, the filter is no longer available from the manufacturer, and so is no longer an option for sample filtration.
- Addition of BTF EasyStain™ monoclonal antibody stain as an acceptable reagent for staining in Methods 1622/1623. The product was validated through an interlaboratory validation study using the Pall Envirochek™ HV filter.
- Addition of portable continuous-flow centrifugation (PCFC) as a filtration/concentration technique for the detection of *Cryptosporidium* in Methods 1622/1623. The product met all method acceptance criteria for *Cryptosporidium* using 50 L source water samples (but not *Giardia;* however, individual laboratories are permitted to demonstrate acceptable performance for *Giardia* in their laboratory).

3.1.3 Minimum Sample Volume Analysis Requirements

Under LT2 Rule *Cryptosporidium* sample volume requirements [40 CFR § 141.704(a)(1)], PWSs are required to analyze, at a minimum, either

- 10 L of sample, *or*
- 2 mL of packed pellet volume, *or*
- As much volume as two filters can accommodate before clogging (this condition applies only to filters that have been approved by EPA for nationwide use with EPA Method 1622/1623—the Pall Gelman Envirochek™ and Envirochek™ HV filters, or the IDEXX Filta-Max® foam filter)

The LT2 Rule sample volume analysis requirement of 10 L (rather than 10.0 or 10.00 L) accommodates the potential for imprecisely filled sample containers or filters. Therefore, sample volumes of 9.5 L and higher would meet the LT Rule requirements. Sample volumes should be recorded to the nearest 0.25 L when using a graduated carboy instead of a flow meter to measure volume.

Systems may analyze larger volumes than 10 L, and larger volumes analyzed should increase analytical sensitivity, provided method performance is acceptable. EPA prefers systems to analyze similar sample volumes throughout the monitoring period. However, data sets including different sample volumes will be accepted, provided the system analyzes the minimum sample volume requirements noted above.

Matrix spike samples must be collected from the same location as the field sample and the volume analyzed must be within 10 percent of the volume analyzed for the field sample (Section 9.5 of Method 1622/1623). It is suggested that the same volume of sample be collected for both the field and the matrix spike sample to ensure the volumes analyzed are within 10 percent. Additional guidance on matrix spike samples is provided in Section 3.3.12 of this manual.

Additional guidance on sample volume and sample collection issues is provided in the *Source Water Monitoring Guidance Manual for Public Water Systems for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)*, available for download from http://www.epa.gov/safewater/disinfection/lt2/

3.1.4 Spiking Suspensions Requirements for Spiked Quality Control Samples

Flow cytometer–counted spiking suspensions must be used for ongoing precision and recovery (OPR) and matrix spike (MS) samples [40 CFR § 141.704(a)(3)]. The use of flow cytometer–counted spiking suspensions is a recommendation in EPA Method 1622/1623, and is a requirement in the LT2 Rule. Spiking suspensions are discussed in more detail in Section 3.3.1, below.

3.1.5 Acceptable Sample Results

Cryptosporidium sample results reported under the LT2 Rule must be generated at an approved laboratory and meet the quality control (QC) requirements specified in EPA Method 1622/1623. These requirements include, but are not limited to, sample temperature requirements, minimum frequencies for ongoing precision and recovery (OPR), method blank, and matrix spike samples; acceptable OPR and method blank results; holding time requirements; and staining control frequency and results. A checklist for these requirements is provided as **Appendix D**. Guidance on implementing *Cryptosporidium* method QC requirements is provided in Section 3.3, below.

3.1.6 *Cryptosporidium* Oocyst Counts to Report

Sample examination using EPA Method 1622/1623 includes an immunofluorescence assay using fluorescein isothiocyanate (FITC) as the primary antibody stain, 4',6-diamidino-2-phenylindole (DAPI) staining to detect nuclei, and differential interference contrast microscopy (DIC) to detect internal structures. Hoffman Modulation Contrast (HMC) optics may be a suitable alternative to DIC, provided that the laboratory demonstrates acceptable analyst performance and acceptable microscope capability to the technical auditor during the on-site laboratory audit conducted prior to laboratory approval through the Lab QA Program. *Cryptosporidium* oocysts to be reported using Section 15 of EPA Method 1622/1623 are defined as the following:

- Those determined by brilliant apple green fluorescence under UV light, size (4 to 6 μm), and shape (round to oval)

- Excluding any atypical organisms detected by FITC, DAPI, and DIC (e.g., those possessing spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.)

The oocyst counts for a sample, based on the above definition and appropriate magnification and examination procedures per Method 1622/1623, and the sample volume analyzed, will be used to calculate the oocyst concentration for each sample during the LT2 Rule.

In a field sample, all organisms that meet the above definition must be counted and assigned to the appropriate categories. This reporting requirement cannot be met unless all *Cryptosporidium*-like organisms in a field sample are examined first under the FITC filter, then under the DAPI filter, and finally using DIC. If no organisms meet the above definition, the lab should report zero oocysts. The number listed in the summary row, "Total FA number", on the *Cryptosporidium* examination results form and the volume examined (L) should be the minimum information reported to the utility (see Section 3.7 of this manual for details regarding the LT2/Stage 2 Data Collection and Tracking System).

3.2 Laboratory Quality Assurance Evaluation Program

The objectives of the Lab QA Program are to evaluate laboratories' competency to reliably measure for the occurrence of *Cryptosporidium* in surface water using EPA Method 1622/1623. Each laboratory participating in the program will be required to complete the following steps to be qualified through this program:

- Complete an application (including a self-evaluation and initial demonstration of capability)
- Perform initial proficiency testing (IPT)
- Participate in an on-site evaluation
- Perform ongoing proficiency testing (OPT) every four months

Information on the Laboratory QA Program is available at http://www.epa.gov/safewater/disinfection/lt2/ and is summarized below, in Sections 3.2.1 - 3.2.7.

3.2.1 Application

Applications for the program (**Appendix E**) are available on the website, http://www.epa.gov/safewater/disinfection/lt2/, and may also be requested from the following address:

Cryptosporidium Laboratory Quality Assurance Coordinator
c/o CSC Water Programs
6101 Stevenson Avenue
Alexandria, VA 22304

EPA reviews each application to verify that the laboratory has submitted the following information:

- A completed self-evaluation checklist
- Resumes of laboratory personnel
- Standard operating procedures for each method version

- Initial demonstration of capability (IDC) data, which consist of the following:
  - Acceptable initial precision and recovery (IPR) test results
  - Acceptable method blank result analyzed with IPR test
  - Acceptable matrix spike/matrix spike duplicate (MS/MSD) results with results from the unspiked matrix sample collected and analyzed at the same time
- Table of contents from the laboratory's quality assurance plan
- Documentation of personnel training and list of samples analyzed and duration of time using the method
- Example of client data reporting form
- A statistical summary of percent recoveries for all OPR and MS samples analyzed over the past six months.

Completed applications should be submitted to *Cryptosporidium* Laboratory Quality Assurance Coordinator, c/o CSC Water Programs, at the address listed above.

3.2.2 Personnel Qualifications and Training

As part of approval, EPA will determine whether laboratory personnel are qualified to analyze *Cryptosporidium* samples for LT2 Rule monitoring. Suggested personnel qualifications for the Lab QA Program are provided in **Table 3-1**. Each laboratory should have at least one principal analyst.

Table 3-1. Suggested Laboratory QA Program Personnel Qualifications

Position	Education	Experience with Crypto and IFA Microscopy	Experience Using Method 1622/1623	Number of Samples Analyzed Using Method 1622/1623
Principal Analyst	BS/BA in Microbiology or closely related field	1 year continuous	6 months	100 (50 if approved as an analyst during Information Collection Rule [ICR])
Analyst	2 years college in Microbiology or equivalent	6 months continuous	3 months	50 (25 if approved as an analyst during ICR)
Technician	No minimum requirement	No minimum required	3 months performing specific parts of procedures	50 (25 if approved as an analyst during ICR)

During the on-site evaluation (Section 3.2.4 of this manual), EPA will review laboratory records to verify that the personnel performing EPA Method 1622/1623 analyses are qualified to do the analyses required under LT2. For new staff that is added after the on-site evaluation, the laboratory should send a letter to EPA providing the following information on the new staff member:

- Resume including education
- Number of samples analyzed using EPA Method 1622/1623
- Number of months of experience
- Verification that analyst training followed the laboratory's training SOP

In addition, the following steps should be completed by new personnel as part of their training prior to analyzing samples for LT2 (specify in the laboratory's training SOP):

- Review laboratory SOPs for analysis of samples using Method 1622/1623
- Observation of an experienced analyst performing the method
- Performance of the method while being observed by an experienced analyst
- Acceptable performance of a set of IPR samples using blind spikes
- Analysis of as many MS/MSD samples as possible
- Repeated study of microscopy module

It is also helpful for trainees to examine and characterize oocysts that are live and inactivated to note the differences between the two states and variations which may occur in the staining and morphological quality of the oocysts. Comparing the quality of the organisms between live and inactivated oocysts may help define and troubleshoot any problems that may occur during processing and eliminate the possibility that the staining or morphological differences are due to the inactivation techniques used for the organisms.

After the initial training is successfully completed, a trainee should analyze as many Ongoing Precision and Recovery (OPR) samples as possible. In addition, the trainee should analyze non LT2 samples using the same method to gain experience. To optimize the experience gained in the required 3 month period and 50 samples analyzed, the trainee should complete the analysis of 20 MS/MSD sets from a variety of source waters. At a minimum, it is recommended that samples analyzed to gain experience include different matrices that may demonstrate possible interferences with processing and/or examination.

After the initial training is successfully completed, a trainee may assist with LT2 samples with an experienced analyst (i.e., both trainee and analyst may sign the laboratory bench sheet and slide examination form) to gain experience with a variety of source waters. It should be noted that any such assistance with processing should be supervised closely enough to proactively eliminate processing mistakes and preserve the analysis. The trainee may re-examine a slide after complete examination by an experienced analyst. The experienced analyst's microscopy results should be used for reporting LT2 results. When a trainee successfully completes the required experience and number of samples, documentation supporting the completion should be sent to the EPA and the trainee's status will be changed to analyst or technician. The troubleshooting in Section 9.7.5 of Method 1622/1623 may be used during analyst training also. Proficiency of skill in photomicrography and development of an analyst photo library is encouraged.

3.2.3 Initial Proficiency Testing

After the laboratory's application has been reviewed and accepted, EPA will send the laboratory a set of eight initial proficiency testing (IPT) samples, which consist of a suspension of *Cryptosporidium* oocysts in a concentrated matrix. Laboratories will resuspend these spikes in reagent water to produce simulated source water samples, and analyze the samples using the version of EPA Method 1622/1623 that the laboratory plans to use for routine *Cryptosporidium* analyses.

Laboratory IPT data will be evaluated against the mean recovery and precision (as relative standard deviation [RSD]) criteria that EPA has established for IPT samples. If a laboratory fails the IPT criteria twice, EPA recommends that the laboratory

- Receive additional training in performing the method (discussed further in Section 3.2.6 of this manual)

- Repeat their IPR analyses until acceptable and submit the results to EPA

If the laboratory does not pass the third IPT following additional training, they may consult with the EPA regarding the level and type of training undertaken. After satisfying these requirements, the laboratory may re-apply for IPT samples.

3.2.4 On-Site Evaluation

Each laboratory that passes the IPT is eligible to participate in an on-site evaluation next, which consists of two concurrently performed assessments: a data and QA evaluation and a technical evaluation.

3.2.4.1 Data and QA Evaluation

During the data and QA evaluation, laboratory documentation will be evaluated to verify compliance with QA program requirements. The evaluation will cover the following:

- Equipment and personnel records
- Data recording procedures, based on field sample data and quality control sample data
- Quality control test frequency and acceptability
- Quality assurance plans
- Standard operating procedures

To ensure consistency and thoroughness for all audits, the data auditor uses a detailed checklist (**Appendix F**) to evaluate specific factors under each of these categories.

To prepare for the on-site evaluation, the laboratory can use the checklists provided with the program application to perform a self-audit.

3.2.4.2 Technical Evaluation

During the technical evaluation, laboratory sample processing and analysis using EPA Method 1622/1623 will be evaluated. The laboratory will be assessed on its capabilities including the following:

- Sample processing and analyses
- Microscopy

To ensure consistency and thoroughness for all audits, the technical auditor uses a detailed checklist (**Appendix F**) to evaluate specific factors under each of these categories.

3.2.5 Approval and Ongoing Proficiency Testing

Laboratories will be approved after they have submitted an acceptable application, passed the IPT and passed the on-site evaluation, as summarized in steps 1-3 in **Figure 3-1**. Laboratories that are approved will also receive a set of three ongoing proficiency testing (OPT) samples approximately every four months that should be analyzed in the same manner as the IPT samples. EPA will evaluate the precision and recovery data for OPT samples to determine if the laboratory continues to be qualified under the Laboratory QA Program. Laboratories must successfully evaluate the OPT samples to maintain EPA approval for the LT2.

If a laboratory fails to meet the precision or recovery criteria for a set of OPT samples, the laboratory will be shipped a second set of samples. If the laboratory's next set of OPT data are acceptable, no further action is required.

If a laboratory fails the next set of OPT samples (two sets of OPTs in a row), it is recommended that the laboratory receive additional training and examine laboratory control charts. If the laboratory continues to fail OPT samples, EPA will reevaluate the laboratory's approval for this program.

3.2.6 Additional Training

Additional training can be received at another approved laboratory or through training opportunities supported by the EPA. Laboratories also can consult universities, vendors, websites, and microscope service companies for possible training opportunities. EPA has produced an online microscopy module detailing the characterization of *Giardia* and *Cryptosporidium*. Viewing this module from the LT2 website, http://www.epa.gov/safewater/disinfection/lt2/, is open to all analysts as a supplement to one-on-one microscopy training. Practice samples may be performed including method blanks, OPR samples, MS samples, and non-LT2 samples. It is recommended that samples analyzed to gain additional practice include different matrices which may demonstrate possible interferences with processing and/or examination.

3.2.7 Notifying Utilities of Change in Laboratory Status

Two actions are necessary if a laboratory is disapproved:

- The laboratory should notify clients
- EPA will remove the laboratory from the approved list (http://www.epa.gov/saftewater/disinfection/lt2/index.html)

If a laboratory receives notice that they have been "disapproved," the laboratory will no longer be able to analyze samples under LT2 until they have been re-approved [141.705(a)]. The laboratory should immediately notify their clients of their status change and cease analyzing samples for LT2. As suggested in Section 3.1.1 of this manual, laboratories should have a protocol in place to divert samples to another approved laboratory in the event back-up analyses are needed.

All samples being processed by the laboratory at the time of the disapproval are considered acceptable, provided all QC and holding time requirements (EPA Methods 1622 and 1623) are met. Analysis of these samples should be completed by the laboratory. However, no new LT2 sample analyses may be initiated unless/until the laboratory is re-approved.

3.3 *Cryptosporidium* Method Quality Control

During the LT2 Rule, *Cryptosporidium* samples must meet the quality control (QC) requirements listed in EPA Methods 1622/1623. The requirements discussed in this guidance manual are based on the December 2005 versions of EPA Methods 1622/1623 (References 5.2 and 5.3). Section 3.1 in this manual included QC for samples, Section 3.2 included QC for laboratories, and this section covers QC for the method.

Sections 3.3.1 through 3.3.13 provide guidance on the implementation of the QC requirements specified in the December 2005 version of EPA Method 1622/1623. Routine QC requirements that must be verified internally by the laboratory before reporting LT2 Rule monitoring results are summarized in **Table 3-2**. QC guidance for method modifications and use of multiple method variations are covered in 3.3.14. This

guidance is provided to help implement the QC requirements in the methods and does not substitute for, or alter, the method requirements.

Figure 3-1. Process for Receiving and Monitoring Laboratory Approval

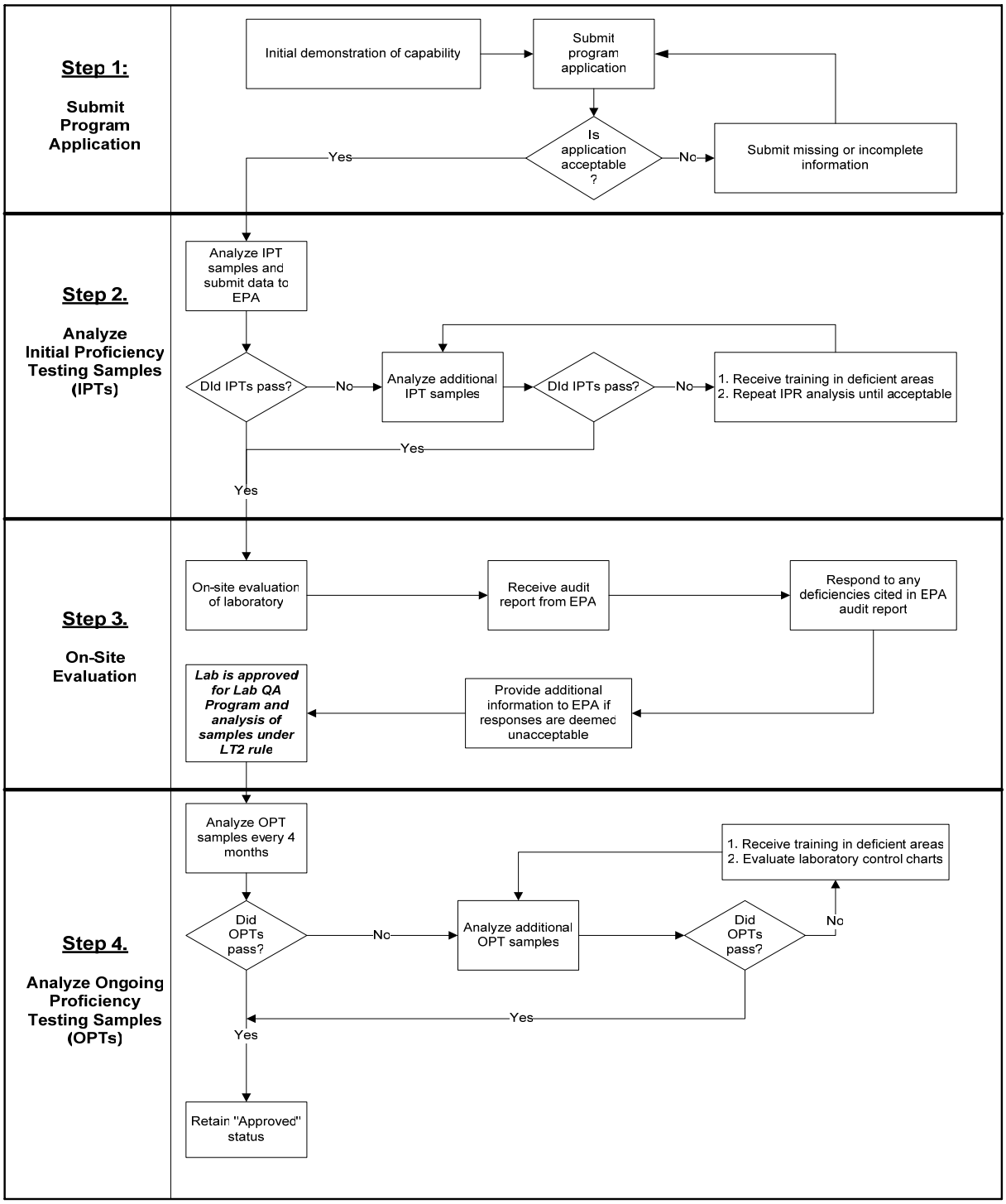


Table 3-2. Summary of Routine QC Requirements

QC sample or procedure	Matrix	Number of samples	Frequency	Purpose	Control Charts
IDC	Reagent water and source water	8	Once	To demonstrate control over the analytical system; consists of IPR set, Method blank ,and MS/MSD as a requirement of Lab QA Program	No
IPR	Reagent water	4	Initial use of method	To establish initial control over the analytical system and demonstrate acceptable method performance (recovery and precision)	
Method Blank	Reagent water	1	Each IPR and OPR set	To demonstrate the absence of contamination throughout the analytical process	
OPR	Reagent water	1	At least each week samples are processed or every 20 samples, whichever is more frequent	To demonstrate ongoing control of the analytical system and verify continuing method performance (recovery and precision)	Required
MS	Source water	2	For each source water - initial sampling and every 20 samples	To determine the effect of the matrix on (oo)cyst recoveries; must be accompanied by an unspiked field sample collected at the same time as the MS sample	Recommended
Positive staining control	none	1	Process each time samples are stained; examine each microscope session	To demonstrate ongoing control of the staining process and performance of reagents and microscope	No
Negative staining control	none	1	Each time samples are stained	To demonstrate the absence of contamination through staining process	
Verification of Analyst Performance	Reagent Water	N/A	Monthly	Refine consistency of organism characterizations between analysts	

3.3.1 *Cryptosporidium* Spiking Materials

During LT2 *Cryptosporidium* monitoring, laboratories must analyze samples spiked with *Cryptosporidium* oocysts to assess ongoing laboratory and method performance in accordance with method QC requirements. These ongoing spiked sample analyses include initial precision and recovery samples (IPRs), ongoing precision and recovery samples (OPRs), matrix spike samples, and positive staining controls (Sections 3.3.2, 3.3.4, 3.3.12, and 3.3.8 of this manual, respectively). Flow cytometer–counted spiking suspensions must be used for the IPR, OPR and matrix spike samples [40 CFR § 141.704(a)(3)], and those suspensions must be used within the noted expiration (Section 8.3 of Method 1622/1623). The laboratory should spike samples according to the procedures provided in Section 11.4 of EPA Method 1622/1623 or according to the procedures provided by the spiking suspension vendor.

The commercial staining kits contain positive control organisms which may be used for routine positive staining control slides. These organisms are typically treated by various inactivation techniques and need to be evaluated for appropriate FITC fluorescence, DAPI-stained nuclei, and internal morphology. If an analyst notes that the appropriate features are not present, e.g., the majority of oocysts are DAPI negative or exhibit weak nuclei staining, it is suggested that a different vendor of positive control organism be utilized. Laboratories are encouraged to use the online microscopy module for comparison (http://www.epa.gov/safewater/disinfection/lt2/). Laboratories may also obtain positive staining control organisms from the vendors listed below and in Method 1622/1623 Section 7.10 which may be live or inactivated.

Sources of flow cytometer–counted *Cryptosporidium* spiking suspensions for use with routine, spiked *Cryptosporidium* QC samples include the following:

1. Wisconsin State Laboratory of Hygiene
   Flow Cytometry Unit
   http://www.slh.wisc.edu/
   2601 Agriculture Drive
   Madison, WI 53718
   Phone: (608) 224-6260
   Fax: (608) 224-6213

   The Wisconsin State Laboratory of Hygiene prepares and distributes live *Cryptosporidium parvum* oocysts and *Giardia intestinalis* cysts that have not been treated to reduce viability.

2. BioTechnology Frontiers (BTF)
   http://www.btfbio.com
   Unit 1, 35-41 Waterloo Road
   North Ryde NSW 2113
   Australia
   Phone: +61 2 8877 9150
   Fax: +61 2 8877 9101
   Email: contact@btfbio.com

   BTF prepares and distributes *Cryptosporidium parvum* oocysts and *Giardia intestinalis* cysts that have been irradiated to inactivate the organisms. ***Note:*** Irradiated, flow cytometer–counted spiking suspensions may be used for routine laboratory QC samples, including initial precision and recovery (IPR) samples, ongoing precision and recovery (OPR) samples, and matrix spike (MS) samples. In accordance with EPA Method 1622/1623, irradiated organisms may not be used for interlaboratory validation studies performed to seek nationwide approval of modified versions of the methods.

3.3.2 Initial Precision and Recovery Test

The initial precision and recovery (IPR) test required by EPA Method 1622/1623 consists of four reagent water samples spiked with ~100 to 500 oocysts and is used to demonstrate acceptable performance with the method. Section 9 of EPA Method 1622/1623 also requires the IPR to be performed for each method modification (additional guidance on QC when using multiple method variations is provided in Section 3.3.13 of this manual).

The results of the four analyses are used to calculate the mean percent recovery and the relative standard deviation (RSD) of the recoveries for *Cryptosporidium* (Section 3.6.3 of this manual). For EPA Method

1622/1623, the mean *Cryptosporidium* recovery should be from 24 percent to 100 percent and the RSD of the four recoveries should be less than or equal to 55 percent. Characterization of the first three *Cryptosporidium* oocysts and three *Giardia* cysts must be reported on the slide examination form for each IPR sample following Section 9.4.5 in EPA Method 1622/1623.

3.3.3 Method Blank Test

The method blank test required in Section 9.6 of EPA Method 1622/1623 consists of analysis of an unspiked reagent water sample to demonstrate freedom from contamination. The method requires that one method blank sample must be analyzed each week or every 20 field and matrix spike samples, whichever is more frequent (Section 9.1.7 in Method 1622/1623). A week is defined as any 168 hour (7 day) period that begins with the processing of the OPR. If more than one method variation will be used for filtration and/or another technique which exposes samples to different apparatus and/or reagents, a separate method blank is required for each variation.

Method blank samples should be analyzed before any field samples in a batch are processed to verify acceptable performance. If one or more *Cryptosporidium* oocysts (as defined in Section 13 of Method 1622/1623) are found in a blank, the method blank is unacceptable and analysis of additional samples is halted until the source of contamination is eliminated and a blank shows no evidence of contamination. Troubleshooting the problem and repeating the method blank to bring the analytical system under control is required before proceeding with sample analysis. If the repeated method blank is acceptable and field samples can be processed within holding times, no replacement samples are necessary.

Note: *If oocysts are detected in the method blank, analysis of additional samples is halted until the source of contamination is eliminated.*

3.3.4 Ongoing Precision and Recovery Test

The ongoing precision and recovery (OPR) in Section 9.7 of EPA Method 1622/1623 entails analysis of a reagent water sample spiked with ~100 to 500 oocysts to demonstrate ongoing acceptable performance. One OPR sample should be analyzed each week or every 20 field and MS samples, whichever is more frequent (Section 9.1.7 of Method 1622/1623). A week is defined as any 168 hour (7 day) period that begins with the processing of the OPR (Section 9.1.7 of Method 1622/1623). If more than one method variation will be used for filtration and/or another technique, a separate OPR may be required for each variation.

OPR samples should be analyzed before any field samples in a batch are processed to verify acceptable performance. OPR *Cryptosporidium* recovery should be from 11 percent to 100 percent to be considered acceptable (Section 9.7.3 of Method 1622/1623). Characterization of the first three *Cryptosporidium* oocysts and three *Giardia* cysts must be reported on the slide examination form as per Section 9.7.1.2 of EPA Method 1622/1623. If the OPR *Cryptosporidium* recovery is not acceptable, no samples may be processed. Troubleshooting the problem and repeating the OPR to bring the analytical system under control is required before proceeding with sample analysis. If the repeated OPR is acceptable and field samples can be processed within holding times, no replacement samples are necessary.

Note: *Ongoing precision and recovery results should be 11 percent to 100 percent.*

3.3.5 Ongoing Precision and Recovery Control Charts

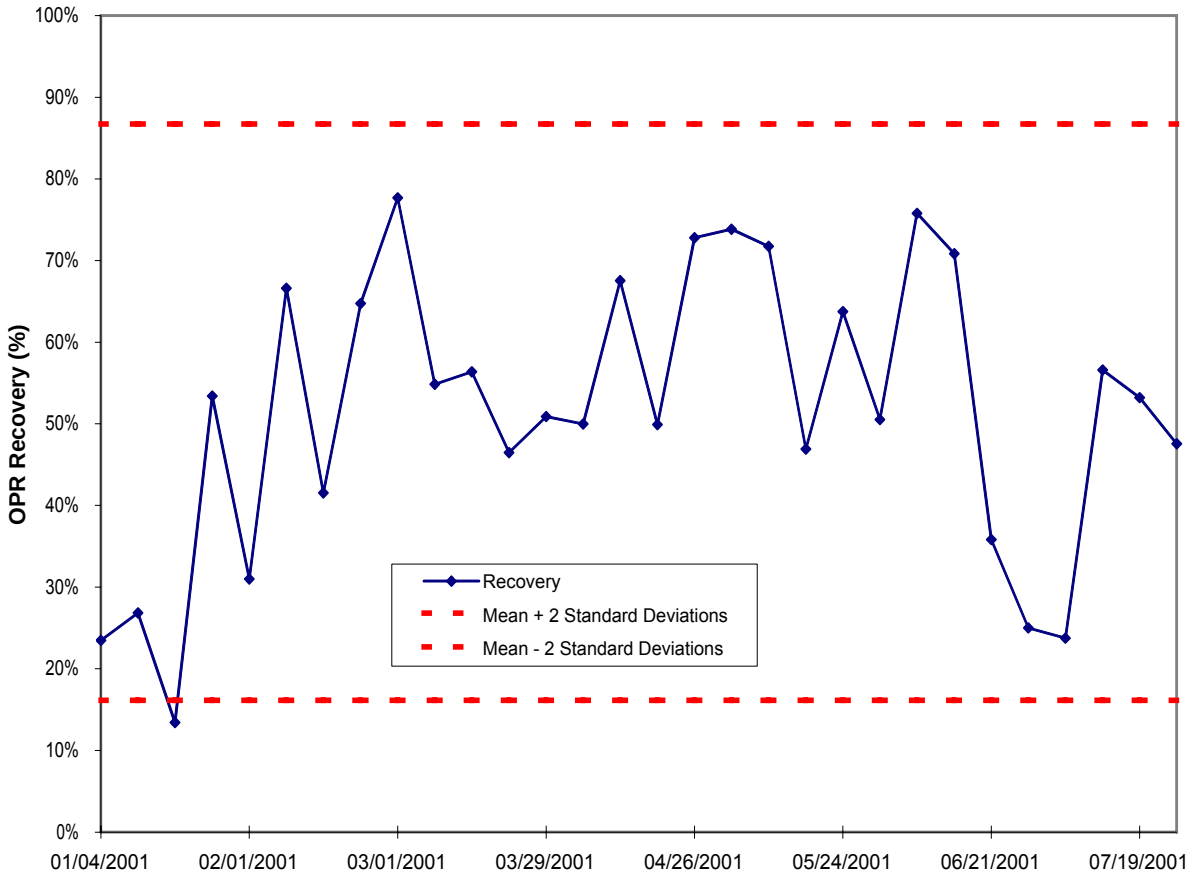
As noted in Section 9.7.6 of the December 2005 version of EPA Method 1622/1623, laboratories should maintain a quality control (QC) chart of OPR recoveries, graphically displaying the results of continuing performance. The control chart should be developed using the most recent 20 to 30 test results.

The control chart is developed by plotting percent recovery of each OPR sample over time (Figure 3.2). Based on the mean of the recoveries ($\bar{x}$) on the chart, the upper and lower control limits should be established as follows

- Upper control limit = $\bar{x}$ + 2 standard deviations
- Lower control limit = $\bar{x}$ - 2 standard deviations

After each 5 to 10 new recovery measurements, new control limits should be recalculated using the most recent 20 to 30 data points. Control charts can be used to track the laboratory's performance and determine if any trends in recovery are occurring. Control charts can also be used to compare performance of different method variations, different analysts, and/or other changes implemented by the laboratory. If recovery measurements fall outside the control limits, laboratories should take corrective action, investigating potential causes of the outlying result. The troubleshooting guidance for OPR failures provided in Section 9.7.5 of EPA Method 1622/1623 is also useful for investigating the cause of acceptable, but outlying, OPR measurements identified through the use of control charts.

Figure 3-2. Ongoing Precision and Recovery Control Chart Example



3.3.6 Quality Control Batches

All LT2 *Cryptosporidium* samples must be associated with an acceptable OPR and method blank sample as stated in Section 9 of the EPA Method 1622/1623. LT2 samples are associated with QC samples through a "QC batch." A QC batch consists of an OPR and method blank and a maximum of 20 field and MS samples that are *eluted, concentrated, and purified* in the same week as the OPR and method blank samples using the same reagents (e.g., eluting solution). 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 processed in a week, the OPR and method blank samples are associated with the field and MS samples which are *eluted, concentrated, and purified* using the same reagents as the OPR and MB and processed prior to the next OPR/MB. The next QC batch may begin concurrently with the processing of the last of the 20 samples associated with the previous OPR/MB. A field sample and its associated MS sample should be analyzed in the same "QC batch". QC samples do not need to be analyzed necessarily during weeks in which no field samples are analyzed unless analytical practice would be beneficial for the laboratory.

3.3.7 Holding Time Requirements

During *Cryptosporidium* analyses for the LT2 Rule, sample processing should be completed as soon as possible by the laboratory. The laboratory should complete sample filtration (if sample is received in bulk), elution, concentration, purification, and staining the day the sample is received whenever possible. However, the laboratory is permitted to split up the sample processing steps if processing a sample

completely in one day is not possible. If this is necessary, sample processing can be halted after filtration, application of the purified sample onto the slide, or staining.
The following holding times must be met for samples analyzed by EPA Method 1622/1623 during the LT2 Rule:

- **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 slide in one work day. It is critical that these steps be completed in one work day to minimize the time that any target organisms present in the sample sit in eluate or concentrated matrix. This process ends with the application of the purified sample on the slide for drying. Follow the stain manufacturer's instructions regarding the drying procedure to use. Drying options include: on the lab bench, on the lab bench with air gently moving around the slides, on a slide warmer between 35°C and 42°C, in a humid chamber or incubator between 35°C and 42°C, or in the refrigerator. The slides must be completely dried before staining and stored to maintain the dried state until stained. Storage of dried slides consists of storing on the lab benchtop (protected), in a desiccator box in the refrigerator, or in a desiccator box on the lab bench (if the lab is humid).

- **Staining.** The sample must be stained within 72 hours of application of the purified sample to the slide including drying time.

- **Examination.** Although immunofluorescence assay (FA) and 4',6-diamidino-2-phenylindole (DAPI) and differential interference contrast (DIC) microscopy examination and confirmation 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 confirmation of samples. However, if fading/diffusion of FITC or DAPI staining is noticed, the laboratory should reduce this holding time. In addition, the laboratory may adjust the concentration of the DAPI staining solution so that fading/diffusion does not occur per Section 14.6 in Method 1622/1623.

The laboratory also may evaluate the use of another mounting medium (alternatives are provided in Section 3.8.2 of this manual, below).

LT2 Rule requirement:	*Each sample must meet the QC criteria for the methods. Per EPA Method 1622/1623, samples must be processed or examined within each of the holding times specified in Section 8.2 of the method.*

A breakdown of the holding times for each set of steps is provided in **Table 3-3**

Table 3-3. Method 1622/1623 Holding Times (adapted from Table 1 EPA Method 1622/1623)

Sample Processing Step	Maximum Allowable Time between Breaks *(samples should be processed as soon as possible)*
Collection	**Up to 96 hours are permitted between sample collection and initiation of elution**
Filtration	
Elution	**These steps must be completed in 1 working day**
Concentration	
Purification	
Application to slide	
Staining	**Up to 72 hours are permitted from application of the purified sample to the slide to staining**
Examination	**Up to 168 hours (7 days) are permitted between sample staining and examination**

3.3.8 Staining Controls

Positive staining controls are used to verify that the FITC and DAPI stains are fluorescing appropriately. Positive staining controls are 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 samples. The analyst examines several fields of view to verify that the stain is fluorescing at the appropriate intensity and uniformity. Each analyst must characterize a minimum of 3 *Cryptosporidium* oocysts on the positive staining control slide before examining field sample slides per Section 15.2.1.1 in Method 1622/1623. 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.

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 solution.

The analyst should indicate on each *Cryptosporidium* slide examination form whether the positive staining control and negative staining control were acceptable.

LT2 Rule requirement:	*Each sample must meet the QC criteria for the methods. Per EPA Method 1622/1623, positive and negative staining controls must be acceptable (Section 15.2.1).*

3.3.9 Examination Preparation

To help the analyst identify the target analyte during field sample slide examination, each analyst must characterize a minimum of three *Cryptosporidium* oocysts on the positive staining control slide before examining field sample slides. This characterization must be performed by each analyst during each

microscope examination session. FITC examination must be conducted at a minimum of 200X total magnification, DAPI examination must be conducted at a minimum of 400X, and DIC examination and size measurements must be conducted at a minimum of 1000X.

Size, shape, and DIC and DAPI characteristics of the three *Cryptosporidium* oocysts must be recorded by the analyst in a microscope log (Section 15.2.1.1 of Method 1622/1623).

3.3.10 Verification of Analyst Performance

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 encourage comparison and discussion among analysts to continually refine their microscopy skills. At least monthly when microscopic examinations are being performed, the laboratory shall prepare at least one slide containing 40 to 200 oocysts. More than 50 percent of the oocysts must be DAPI positive and undamaged under DIC. Another option is to order prepared slides from Wisconsin State Laboratory of Hygiene, Flow Cytometry Unit (http://www.slh.wisc.edu/, (608) 224-6260).

For laboratories with multiple analysts, each analyst shall determine the DAPI category (DAPI negative, DAPI positive intense internal blue staining, and DAPI positive with 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 analysts at the same time, i.e. each analyst determines the category independently, then the differences in the DAPI and DIC categorizations among analysts are discussed and resolved, and these resolutions documented. This round-robin approach with all analysts may encourage further discussion among analysts and lead to more consistent characterizations. Alternatively, organism coordinates may be recorded for each analyst to locate and categorize the organisms at different times. Differences among analysts must still be discussed and resolved.

Laboratories should be aware that both FITC and especially DAPI fluorescence may fade during the process of analyst verification. Therefore, DAPI comparisons should be performed first and the UV light shutter should remain closed except for a few seconds during observation by the analyst. Repeat comparisons with new FITC organisms, again taking care to keep the shutter closed whenever the oculars are not being used.

Each analyst shall also 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 the DAPI and DIC characterization so that fading will not influence counts. The total number of oocysts enumerated by each analyst must be within ±10 percent of each other. If the number is not within this range, the analysts must identify the source of any variability between analysts' examination criteria, prepare a new slide, and repeat the performance verification.

Laboratories with only one analyst should maintain a protozoa library and compare the results of slide examinations to photographs of oocysts and cysts and interfering organisms to verify that examination results are consistent with these references. These laboratories may also perform repetitive counts of a single verification slide for FITC and DAPI. These laboratories are encouraged to coordinate with other laboratories to share slides and compare counts.

Analyst verification serves as an on-going and consistent training venue for new and experienced analysts. In addition to the monthly verification of analyst performance, other training options exist at universities, microscope service companies, and existing laboratories. EPA has produced an online microscopy module detailing the characterization of *Giardia* and *Cryptosporidium*. Viewing this module

from the LT2 website, http://www.epa.gov/safewater/disinfection/lt2/, is open to all analysts as a supplement to one-on-one microscopy training.

3.3.11 Acceptance Criteria for Receipt of Field Samples

Cryptosporidium samples for LT2 Rule monitoring should be stored between 1°C and 10°C to reduce biological activity and so they cannot freeze. This is specified in Section 8.0 of the December 2005 versions of EPA Method 1622/1623. Because *Cryptosporidium* samples collected for the LT2 Rule must meet the QC criteria in the methods, and because these QC criteria include receipt of samples at ≤20°C and not frozen, laboratories must reject LT2 *Cryptosporidium* samples that are received at >20°C or frozen. In these cases, the PWS must re-collect and re-ship the sample.

Several options available to measure sample temperature upon receipt at the laboratory and, in some cases, during shipment, are provided in Section 2.2 of this manual.

LT2 Rule requirement:	*Each sample must meet the QC criteria for the methods. Per EPA Method 1622/1623, samples not received on the day of collection must be received at the laboratory at ≤20°C and not frozen (Section 8.1 of the method).*

3.3.12 Matrix Spike Samples

The matrix spike (MS) in EPA Method 1622/1623 (Section 9.5.1 of the December 2005 version) entails analysis of an extra bulk water sample spiked with ~100 to 500 oocysts in the laboratory to determine the effect of the source water matrix on the method's oocyst recovery. The laboratory should analyze an MS sample when samples are first received from a PWS location for which the laboratory has never before analyzed samples to identify potential method performance issues with the matrix.

LT2 Rule requirement:	*Each sample must meet EPA Method 1622/1623 requirements [40 CFR § 141.704(a)], which include the following: (1) The MS and field sample must be collected from the same sampling location by splitting the sample stream or collecting the samples sequentially; (2) The volume of the MS sample analyzed must be within 10 percent of the volume of the field sample analyze); and (3) The MS and field sample must be analyzed by the same procedure (Section 9.5.1 of Method 1622/1623).*

3.3.12.1 Matrix Spike Frequency

For all PWSs, the first MS sample should be collected and analyzed during the first sampling event under the monitoring program per EPA Method 1622/1623 (Section 9.1.8 of Method 1622/1623). 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. The laboratory and PWS should evaluate the MS recoveries, as well as other attributes of sample processing and examination, and work together to determine whether sample filtration and processing procedures are working acceptably or need to be re-evaluated. Matrix spike samples may be analyzed more frequently than one every 20 field samples to better characterize method performance in the matrix.

Based on this requirement, the following PWS categories must 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 must be analyzed.

3.3.12.2 Matrix Spike Samples Associated with Field-Filtered Samples

Matrix spike samples must be collected as bulk samples and spiked in the laboratory prior to filtration. The volume of the MS sample must be within 10 percent of the volume of the associated field sample. PWSs that field-filter 10 L samples may field filter the monitoring sample, but must 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 may be filtered in the field. The remaining 10 L of source water for MS analysis may be collected in bulk and shipped to the laboratory. The laboratory will 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 must be collected as usual [40 CFR § 141.704(a)(2)(ii)].

3.3.12.3 Matrix Spike Control Charts

As with the OPR samples and described in Section 9.5.1.4 of the December 2005 version of EPA Methods 1622/1623, 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 suggested that the precision assessment be maintained across all MS samples as well as stratified by source. The control chart across all MS samples should be developed when at least 5 MS samples have been completed. It is the laboratory's decision as to the maximum number of samples used to determine precision assessments. For individual sources completing only two MS samples, precision estimates should be calculated using percent difference instead of standard deviation. If more MS samples are completed per source, the laboratory should generate precision assessments using the mean and standard deviation.

The control chart is developed by plotting percent recovery of each matrix spike sample versus time. Based on the mean of the recoveries ($\bar{x}$) on the chart, the upper and lower control limits should be established as follows:

- Upper control limit = $\bar{x}$ + 2 standard deviations
- Lower control limit = $\bar{x}$ - 2 standard deviations

An example of a control chart (using OPR data, not MS data) is provided in **Figure 3-2**. Control charts can be used to track the laboratory's performance and determine if any trends in recovery are occurring. Control charts can also be used to compare performance of different method variations, different analysts, and other changes implemented by the laboratory along with performance in different matrices. If recovery measurements fall outside the control limits, laboratories may take corrective action, investigating potential causes of the outlying result.

3.3.13 QC Guidance for Method Modifications and Use of Multiple Method Variations

EPA Methods 1622/1623 are performance-based methods and, therefore, allow method modifications if a laboratory can meet applicable QC criteria (EPA Method 1622/1623 [Section 9.1.2]). **Table 3-4** presents examples of changes to EPA Method 1622/1623 that EPA considers to be "routine," "occasional," and "substantive," and what QC steps are necessary to demonstrate acceptability before implementing these changes.

Table 3-4. Recommended QC for Different Types of Changes to EPA Method 1622/1623

"Routine" Changes	
Using new lots of the same method component, such as • new lots of filters • IMS kits • stains • other reagents including reagent water	**Verification of reagent acceptability, per routine OPRs/method blanks, is recommended.**
Changing to new equipment that meets existing specifications in the method (e.g., pumps or centrifuges)	
"Occasional" Changes	
Using different equipment, reagents or procedures for which specifications are not included in the method, such as • lab shaker • IMS magnets • Leighton tubes • slides • mounting medium • centrifuge speeds • slide drying procedures • staining procedure using the same brand of reagents • vortex speeds during IMS • microscope	**Initial precision and recovery, method blank, matrix spike/matrix spike duplicate, and unspiked field sample are recommended.**
"Substantive" Changes	
Changing to a different filter	**New demonstration of acceptable performance is required through initial precision and recovery, and method blank. In addition, matrix spike/matrix spike duplicate, and unspiked field sample is strongly recommended.**
Changing flow rate (e.g., using a flow rate of 4 L/min with the Envirochek™ HV version of Method 1623, rather than the 2 L/min flow rate specified in the method)	
Changing sample volumes (e.g., processing 50 L samples rather than 10 L samples)	
Changing to a different Filta-Max® concentration or elution procedure: • Standard wash station and concentrator tube • Stomacher and concentrator tube • Stomacher and centrifugation	
Incorporating the use of multiple filter membranes in the Filta-Max® concentrator tube	
Changing to a different antibody staining kit	
Incorporating additional rinses and transfers to reduce carryover from IMS to the slide	
Changing vendors for spike organisms	
Changing laboratory space or location	

3.3.13.1 Making a Substantive Change to the Method

Substantive changes to Method 1622/1623 as described in Table 3-4 essentially comprise a new variation on the method. A method variation is the complete set of sample processing components (including the filter, IMS, and stain) and sample processing procedures (including filtration, concentration, purification, and staining) used to process a water sample for examination. Per EPA Method 1622/1623 (Section 9.1.2), if a laboratory intends to switch completely from one method variation to another, then the laboratory should demonstrate acceptable QC as outlined in Table 3-4 using the new method variation before implementing this procedure for the analysis of field samples. If the laboratory demonstrates

acceptable initial laboratory performance and implements the new method variation and discontinues use of the old method variation, then the laboratory must demonstrate acceptable ongoing laboratory performance (through the OPR, method blank, and OPT tests) using the new variation.

Guidance on requirements for initial and ongoing demonstrations of acceptable laboratory performance for different method variations is provided in Sections 3.3.14.2 of this manual. The examples of substantive changes given in Table 3-4 have been shown to be effective in multiple laboratories with different source water matrices. Guidance to demonstrate acceptability of substantive changes that are novel is discussed in Section 3.3.13.3 of this manual.

3.3.13.2 Using Multiple Method Variations

Per EPA Method 1622/1623 (Section 9.1.2), if a laboratory intends to use multiple method variations (that differ through a substantive change) concurrently, then the laboratory demonstrates acceptable *initial* laboratory performance (through the IDC test per the Lab QA Program) using each method variation before implementing this procedure for the analysis of LT2 Rule samples. In addition, the laboratory demonstrates acceptable *ongoing* laboratory performance (through the OPR, method blank, and OPT tests) for each substantive method change for all but the following method variations:

- **Antibody staining kits.** If a laboratory alternates among more than one antibody staining kit, the laboratory performs positive and negative staining controls for each antibody kit for each batch of slides for which the kit is used and should alternate between the kits for ongoing demonstrations of acceptable laboratory performance. MS samples should be processed using the same method variation as the associated field sample, regardless of the method variation used to demonstrate ongoing acceptable laboratory performance.

- **Additional rinses and transfers.** If the laboratory uses additional rinses and transfers for some samples to reduce carryover from IMS onto the slide, the laboratory should use this procedure (which may reduce recoveries) to demonstrate acceptable ongoing laboratory performance on the same percentage of OPR samples as percentage of field samples analyzed with this variation. MS samples should be processed using the same method variation as the associated field sample, regardless of the method variation used to demonstrate ongoing acceptable laboratory performance.

- **Multiple membranes for Filta-Max® concentration.** If the laboratory uses multiple membrane filters in the Filta-Max® particle concentrator for some samples, the laboratory should use multiple memebrane filters to demonstrate acceptable ongoing laboratory performance on at least the same percentage of OPR samples as the percentage of field samples analyzed with multiple membranes. MS samples should be processed using the same method variation (and same number of membranes) as the associated field sample, regardless of the method variation used to demonstrate ongoing acceptable laboratory performance.

- **Multiple sample volumes.** See discussion in Section 3.3.14.

3.3.13.3 Substantive Change Acceptability through Alternate Test Procedure (ATP) or Tier 2 validation

Novel changes with new kinds of equipment or reagents cannot be evaluated using the QC tests and criteria described above because experiments with more than one water matrix or a study designed to compare the new procedure with an established test procedure are necessary for maintaining the same level of data quality. EPA's Microbiological ATP Protocol (Reference 5.7) describes a process for

conducting QC acceptance criteria-based studies and for conducting side-by-side comparisons to demonstrate comparability with an EPA-approved method. For example, the ATP protocol would be useful for evaluating new elution systems or new staining or immunomagnetic separation techniques.

The Tier 2 validation is used if nationwide approval of a modification is sought (Section 9.1.2.1.2 of Method 1622/1623). A Tier 2 validation includes analysis of an IPR set, method blank, MS/MSD, and field sample at a minimum of 3 laboratories and compares the results to the methods' QC criteria.

3.3.14 Guidance on QC for Different Sample Volumes

A laboratory with multiple PWS clients representing a range of sample volumes is not responsible for performing QC tests at all of the volumes. However, if the laboratory does analyze both 10 L and 50 L sample volumes for clients—or any volumes in between—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 below, in Sections 3.3.14.1 through 3.3.14.3.

3.3.14.1 Initial Precision and Recovery Tests for Different Sample Volumes

A laboratory with multiple PWS clients representing a range of sample volumes should successfully perform the IPR and method blank test, as well as successfully analyze matrix spike/matrix spike duplicate (MS/MSD) and initial proficiency testing (IPT) samples (for the Lab QA Program's initial demonstration of capability [IDC]), at the largest (most challenging) volume. The laboratory should demonstrate acceptable performance for these tests using spikes of no greater than 500 oocysts.

3.3.14.2 Ongoing Precision and Recovery Tests and Method Blank Tests for Different Sample Volumes

A laboratory demonstrates ongoing acceptable performance at both extremes of the volume spectrum by performing OPRs and method blanks at a volume consistent with the highest sample volume submitted by clients (e.g. 50 L) as well as OPRs and method blanks at a volume consistent with the lowest sample volume submitted by clients (but not less than 10 L). Labs analyzing multiple sample volumes, should demonstrate acceptable performance in a manner representative of the sample volumes they process i.e. at the same percentage as volumes in field samples submitted to the laboratory. Frequency of OPRs with different volumes should be consistent with the frequency of samples with different volumes, e.g. if half of the samples received have 50 L volumes and half have 10 L volumes, then half of the OPRs should be performed with 50 L and half with 10 L. Laboratories should work with their PWS clients to attempt to schedule clients with different sample volume sizes for different periods during the week, so the field samples can be batched with QC samples of comparable volume.

3.3.14.3 Ongoing Proficiency Tests for Different Sample Volumes

Laboratories with multiple PWS clients representing a range of sample volumes are not required to analyze OPT samples at each sample volume. The laboratory should notify EPA of the sample volume most representative of the LT2 samples processed and perform the OPT test using this volume.

3.4 Sample Collection Procedures

Several options are available to the PWS for collecting untreated surface water samples for *Cryptosporidium* analysis, including the following.

- On-site filtration of water samples from pressurized or unpressurized sources using the Pall Gelman Envirochek™ or Envirochek™ HV capsule 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 analysis.

Detailed procedures for each of these options, as well as packing and shipping the samples from the PWS to the *Cryptosporidium* analysis laboratory, are provided as appendices in the *Source Water Monitoring Guidance Manual for Public Water Systems for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)* (Reference 5.15).

As noted in the PWS guidance manual, EPA recommends that the laboratory and PWS conduct at least one practice sampling and analysis event prior to starting official LT2 monitoring. Based on previous experiences in the Information Collection Rule (ICR) and ICR Supplemental Surveys, unanticipated problems are often encountered during the first sampling event, but are addressed in subsequent events. Rather than risking sampling problems during official LT2 monitoring, the PWS and laboratory can identify and resolve any problems by conducting the practice sampling and analysis.

The contract laboratory is often involved with PWSs regarding sampling issues including scheduling, multiple sources, location, and replacement samples. These issues are detailed in the *Source Water Monitoring Guidance Manual for Public Water Systems for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)*; however, an overview is below.

- Scheduling: PWSs are encouraged to work with the contract laboratory to establish a schedule that will comply with LT2 Rule requirements and is mutually acceptable to the PWS and the laboratory.

- Sampling Location: PWSs are required to collect source water samples for the LT2 Rule from the plant intake prior to chemical treatment, unless approved by the State to collect the source water sample after chemical treatment [40 CFR § 141.703(b)(2)]. Systems that recycle filter backwash water must collect source water samples prior to the point of filter backwash water addition [40 CFR § 141.703(c)]. All *Cryptosporidium*, *E. coli*, and turbidity source water samples collected under LT2 Rule requirements should be collected from the same appropriate sampling location.

- Multiple Sources: The use of multiple sources during monitoring must be consistent with routine operational practice [40 CFR § 141.703(e)]. If there is a tap prior to treatment where sources are combined, the sample must be collected there. If not, the PWS must pursue one of the following options:
  - collect manually from each source prior to treatment and composite into one sample in the same proportion as used by the plant at the time of collection
  - collect manually from each source prior to treatment, analyze separately, and calculate a weighted average of the analysis results.

- Replacement Samples: Certain situations may dictate the need for a replacement sample, i.e. the PWS is unable to report a valid *Cryptosporidium* analytical result for a scheduled sampling date. The following list details some of the possible situations:
  - sample not collected during required time frame due to extreme conditions or situations that may pose a danger to the sampler
  - sample is lost or contaminated

- laboratory exceeds analytical method holding time
- sample receipt temperature fails criteria
- volume requirements not met (field and/or MS sample)
- QC samples fail acceptance criteria
- problems encountered during processing
- failure of an approved laboratory to analyze the sample

The PWS must 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 must 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 [40 CFR § 141.702(b)(2)].

3.5 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. For EPA Methods 1622/1623, original data, including microscope examination counts and notes, must be recorded. The data may be recorded on bench sheets (**Appendix G**) and slide examination forms (**Appendix H**).

Data should be recorded in ink and a single line drawn through any change with an initialed, dated correction entered next to it. Data files may also be microfiche or electronic. Electronic data should be backed up by a protected tape or disk or hard copy. Under the LT2 Rule, monitoring data (both initial and second round of monitoring) must be kept until 3 years after bin classification for filtered systems or determination of mean *Cryptosporidium* levels for unfiltered systems [40 CFR § 141.722(a)]. Although it is the PWS's responsibility to meet LT2 Rule data storage requirements for compliance monitoring samples, the PWS may contract this work to the laboratory.

As laboratories perform *Cryptosporidium* analyses during the LT2 Rule, the following data recording practices should be followed:

- 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 technicians and analysts are performing the method

The minimum data elements that should be recorded for *Cryptosporidium* samples during the LT2 are discussed in detail below. 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 were met. Standardized bench sheets and *Cryptosporidium* examination results forms are available for download as Appendices G and H on the LT2 website http://www.epa.gov/safewater/disinfection/lt2/compliance_mlmanual.html.

Sampling records provided by the PWS with the sample should include the following information, at a minimum:

- Public water system 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 phone number (or alternate contact for laboratory if problems are encountered)
- 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)

* *Note: These three elements are used to identify the LT2 sample for tracking sample collection, analysis, reporting, and use.*

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 for Public Water Systems for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)*. This manual is available for download from http://www.epa.gov/safewater/disinfection/lt2/compliance.html.

Upon receipt of the sample at the laboratory, laboratory personnel should record, at a minimum, the information in **Table 3-5.** Laboratories should immediately notify utilities of any deficiencies requiring a resample.

Table 3-5. Sample Receipt Data Elements to Record in the Laboratory

Public water system 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 laboratory
Volume filtered (if sample is filtered in the field)
Name of laboratory person receiving the sample
Temperature of sample upon receipt
Any deficiencies (deficiencies may include but are not limited to: exceeded sample holding time, transport temperature exceeded 20°C, or sample leaked during transport)

Laboratories analyzing samples for *Cryptosporidium* using EPA Method 1622/1623 in support of the LT2 Rule should record the primary elements required to calculate the final concentrations and percent recoveries for matrix spike (MS), ongoing precision and recovery (OPR), and proficiency test samples. These primary data elements are provided in **Table 3-6**, and should be recorded on the EPA Method 1622/1623 bench sheet (**Appendix G**) and slide examination form (**Appendix H**).

Table 3-6. Primary Data Elements to Record for Calculations

Estimated number of oocysts spiked (MS and OPR samples), based on information provided by the flow-cytometry laboratory with the spiking suspension
Sample volume spiked, in L (MS, OPR and proficiency test samples)
Sample volume filtered, to nearest 1/4 L
Number of filters used (if the filter clogged)
Pellet volume after concentration, to the nearest 0.1 mL
Total volume of resuspended concentrate, in mL
Volume of the resuspended concentrate transferred to IMS, in mL
Number of subsamples analyzed
Total number of oocysts detected in the sample

To determine that all method QC requirements were met and that the samples were analyzed by qualified personnel according to the requirements of the Laboratory QA Program for the Analysis of *Cryptosporidium* the laboratory should record the elements in **Appendices G and H**. These appendices include, but are not limited to, the key QC data elements in Table 3-7.

Table 3-7. Key QC Data Elements to Record

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 completed within 168 hours (7 days) of sample staining)
Person (PWS or Lab employee) performing filtration
Analyst performing elution
Analyst performing IMS
Analyst performing sample staining
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 obtained or allow problems with sample results and laboratory performance to be identified. This additional information includes the following:

- Information on the version of EPA Method 1622/1623 used to perform the analysis including filter type, elution procedure, concentration procedure, IMS system used, detection kit used, and source of oocysts for spiking suspensions
- Lot numbers of reagents and materials used during the analysis, including the filter, elution buffer, IMS system, detection kit, and spiking suspension
- FITC, DAPI, and DIC information of all oocysts detected in the field samples using the slide examination form

This information should be recorded on the EPA Method 1622/1623 bench sheet (**Appendix G**) and *Cryptosporidium* slide examination form (**Appendix H**), as appropriate.

Size, shape, and DIC and DAPI characteristics of the three *Cryptosporidium* oocysts on the positive staining control slide (Section 15.2.1.1 of Method 1622/1623) must be recorded by the analyst on a microscope log.

3.6 Calculations for EPA Methods 1622/1623

During LT2 Rule monitoring, field sample results will be reported using the LT2/Stage 2 Data Collection and Tracking System (DCTS) described in Section 3.7 of this manual. The DCTS will reduce the data elements entered by the laboratory to yield final sample results in oocysts/L. The DCTS will also verify that LT2 Rule *Cryptosporidium* sample volume analysis requirements were met and calculate MS recoveries. Sections 3.6.1 through 3.6.3 are guidance for laboratories to perform the same type of calculations as the DCTS for oocyst concentrations, matrix spike recoveries, and OPR recoveries. The laboratory may choose to report the total oocysts and volume analyzed, along with the oocysts/L, and other primary data elements listed in **Table 3-6** to the PWS. The PWS may then use that report during the DCTS data review process discussed in Section 3.7.2 of this manual. For QC samples, the laboratory calculates recoveries for OPR and MS samples to report to their PWS and to maintain QC control charts as discussed in Sections 3.3.5 and 3.3.12.3 also in this manual.

3.6.1 Calculating Oocyst Concentrations

To calculate the concentration of *Cryptosporidium* in a sample, reported as oocysts/L, the following information is needed:

- Number of oocysts detected in the sample (recorded as a primary measurement from the slide examination form)
- Volume analyzed

Using these two data elements, the final concentration should be calculated as:

$$\text{final concentration (oocysts/L)} = \frac{\text{oocysts detected in the sample}}{\text{volume analyzed (L)}}$$

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 more than one filter was used, and all of the volume filtered through the multiple filters is processed through the remainder of the method, then the volume examined is simply the sum of the volumes filtered through each of the filters used.

If <100% of the volume filtered was processed through the remainder of the method, then additional calculations are needed to determine the volume analyzed. This is discussed in Section 3.6.1.1 through 3.6.1.3 of this manual.

3.6.1.1 Determining Volume Analyzed when Less than 100% of Sample Was Examined

When <100% of the sample filtered is processed through the remainder of the method and examined (such as when the volume filtered yields >2 mL of packed pellet volume after centrifugation), then the volume analyzed should be determined using the following equations to determine the percentage of the sample that was examined.

$$\text{percent examined (expressed as a decimal)} = \frac{\text{total volume of resuspended concentrate transferred to IMS (mL) (see Section 3.6.1.2 of Method 1622/1623)}}{\text{total volume of resuspended concentrate (mL)}}$$

$$\text{volume analyzed (L)} = \text{percent examined} \times \text{sample volume filtered (L)}$$

3.6.1.2 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. You 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.

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

3.6.1.3 Example Calculation

Example. A 10 L field sample was filtered and processed, producing a packed pellet volume of 2.7 mL. The laboratory transferred 20 mL of the total resuspended concentrate (27 mL) to IMS and examination (because a minimum of 2 mL of pellet is required for analysis if 10 L cannot be examined or 2 filters did not clog [see Section 3.1.3 of this manual]). The laboratory detected 20 oocysts during examination. The following calculations were performed to determine the volume analyzed and final concentration.

$$\text{total volume of resuspended concentrate (mL) required} = \frac{2.7\ \text{mL}}{0.5\ \text{mL}} \times 5\ \text{mL} = 27\ \text{mL}$$

$$\text{percent examined} = \frac{20\ \text{mL}}{27\ \text{mL}} = 0.74\ (74\%)$$

$$\text{volume analyzed (L)} = 0.74 \times 10\ \text{L} = 7.4\ \text{L}$$

$$\text{final concentration (oocysts/L)} = \frac{20\ \text{oocysts}}{7.4\ \text{L}} = 2.7\ \text{oocysts/L}$$

3.6.2 Matrix Spike Recovery Calculations

To determine the percent recovery for a matrix spike (MS) sample the following information is needed:

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

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

This calculation assumes that the same sample volume was examined for both the field and MS samples. If the sample volumes examined are different, you should calculate the number of oocysts per L for both the field and MS samples before calculating percent recovery. If both a matrix spike (MS) and a matrix spike duplicate (MSD) are analyzed, then the mean recovery and relative percent difference should be calculated and compared to the acceptance criteria in Tables 3 and 4 of the December 2005 version of EPA Method 1623. The percent recovery for each sample should be calculated as described above to determine the mean recovery.

To calculate the mean percent recovery, you should calculate the percent recovery for each sample, as described above, and then use the following formula:

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

To calculate the relative percent difference (RPD), the absolute value (without sign) of the difference between the number 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. You should calculate the number counted per L before calculating the RPD if volumes analyzed are different.

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

Example. The laboratory prepared both the MS and MSD by spiking two 10 L samples with 100 oocysts each. The laboratory detected 45 oocysts in the MS sample and 50 oocysts in the MSD. In the 10 L unspiked field sample only 2 oocysts were detected. To determine the percent recovery for each sample and the mean recovery and relative percent difference of the MS and MSD, the following calculations were performed. For both the MS and MSD, as well as the unspiked field sample, the entire 10 L sample was filtered and 100% of the sample was examined.

$$\text{MS percent recovery} = \frac{45 \text{ oocysts} - 2 \text{ oocysts}}{100 \text{ oocysts}} \times 100\% = 43\%$$

$$\text{MSD percent recovery} = \frac{50 \text{ oocysts} - 2 \text{ oocysts}}{100 \text{ oocysts}} \times 100\% = 48\%$$

$$\text{mean recovery} = \frac{43\% + 48\%}{2} = 45.5\%$$

$$\text{RPD} = \frac{|45 \text{ oocysts} - 50 \text{ oocysts}|}{((45 \text{ oocysts} + 50 \text{ oocysts}) / 2)} \times 100\% = 10.5\%$$

3.6.3 OPR Sample Calculations

The percent recovery of an OPR sample should be calculated using the following formula:

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

Example: The laboratory prepared the OPR sample by spiking 50 L with 150 oocysts. The entire sample was filtered and examined. The laboratory detected 76 oocysts.

$$\text{percent recovery} = \frac{76 \text{ oocysts}}{150 \text{ oocysts}} \times 100\% = 50.7\%$$

OPR recoveries are compared to the limits for ongoing recovery in Tables 3 and 4 of the December 2005 version of EPA Method 1623. These recoveries are tracked over time using control charts to assess precision, as discussed in Section 3.3.5, above.

3.7 Electronic Data Reporting

During the LT2 Rule, laboratories will report *Cryptosporidium* data to their PWS clients electronically through EPA's LT2/Stage2 Data Collection and Tracking System (DCTS). The DCTS is a web-based application that allows laboratory users to enter or upload data, then electronically "release" the data to the PWS for review, approval, and submission to EPA and the State. Although ownership of the data resides with the PWS throughout this process, the DCTS increases the ease and efficiency of the data entry and transfer process from one party to another by transferring the ability to access the data from the laboratory to the PWS to EPA and the State, and ensuring that data cannot be viewed or changed by unauthorized parties. A summary of the data entry, review, and transfer process through the DCTS is provided in **Table 3-8**, below.

The data reporting process is described in more detail below, in Sections 3.7.1 through 3.7.3, and is also described in detail in the *Users' Manual for the LT2/Stage 2 Data Collection and Tracking System (DCTS)*. The DCTS users' manual also provides detailed information on the PWS user registration process. Information on the DCTS and a downloadable users' manual are available at http://www.epa.gov/safewater/disinfection/lt2/.

Table 3-8. LT2/Stage 2 Data Collection and Tracking System Data Entry, Review, and Transfer

Laboratory actions • Laboratory posts analytical results to the DCTS • DCTS reduces data and checks data for completeness and compliance with LT2 Rule requirements • Laboratory Principal Analyst confirms that data meets quality control requirements • Laboratory "releases" results electronically to the PWS for review • Laboratory user cannot edit data after it is released to the PWS	EPA does not have access to data
⬇	
PWS actions • PWS reviews electronic data through the DCTS • PWS cannot edit data - only review data and either return to laboratory to resolve errors or submit to EPA • PWS "releases" data back to the laboratory if it has questions • If no questions, PWS submits data to EPA as "approved" or "contested" (indicating that samples have been correctly analyzed, but that the PWS contends that they are not valid for use in LT2 binning) • If the PWS does not review the sample result by the deadline for submitting it to EPA (no later than 10 days after the end of the first month following the month when the sample was collected) the sample result status in the DCTS is automatically changed to "approved" to prevent a monitoring violation report from generating.	
⬇	
EPA and State actions • EPA and State users cannot edit data - only review data • EPA and State review data through the DCTS and approve results where appropriate • Contested results - If EPA/the State rejects the PWS explanation for the contested sample, the sample is marked "EPA approved" in the DCTS - If EPA/the State accepts the PWS explanation for the contested sample, the sample is invalidated and the PWS must resample	

3.7.1 Data Entry/Upload

The analyst or another laboratory staff member enters a subset of the data recorded at the bench (Section 3.5 of this manual) into the DCTS, either by entering the data using web forms or by uploading data in XML format (see the DCTS users' manual). In accordance with 40 CFR § 141.706(e)(1), this information includes the following:

- PWS ID
- Facility ID
- Sample collection date
- Sample type (field or MS)
- Sample volume filtered (L), to nearest ¼ L

- Was 100% of filtered volume examined?
- Number of oocysts counted
- For samples in which less than 10 L is filtered or less than 100% of the sample volume is examined, the laboratory also must enter or upload the number of filters used and the packed pellet volume
- For samples in which less than 100% of sample volume is examined, the laboratory also must report the volume of resuspended concentrate and volume of this resuspension processed through immunomagnetic separation
- For matrix spike samples, the laboratory also must report the sample volume spiked and estimated number of oocysts spiked; these data are not required for field samples

By entering *Cryptosporidium* data into the system, the laboratory acknowledges that the following QC requirements were met including: all holding times, sample condition on receipt, results of associated method blank, OPR, and positive and negative staining controls. The DCTS allows for replacement samples to be entered and marked as replacement samples. See the DCTS users' manual for guidance.

After the information has been entered or uploaded into the data system, the system will reduce the data to yield final sample results, in oocysts/L, verify that LT2 Rule *Cryptosporidium* sample volume analysis requirements were met for samples in which less than 10 L were analyzed, and calculate MS recoveries.

The laboratory's Principal Analyst under the Lab QA Program is generally responsible for verifying the quality and accuracy of all sample results in the laboratory. If inaccuracies or other problems are identified, the Principal Analyst discusses the sample information with the analyst or data entry staff and resolves the issues before the data are released for PWS review.

If no inaccuracies or other issues are identified, the laboratory approves the reported data for "release" to the PWS for review (EPA does not receive the data at this point). When the data are approved, the rights to the data are transferred electronically by the system to the PWS, and the data can no longer be changed by the laboratory.

3.7.2 PWS Data Review

After the laboratory has released *Cryptosporidium* data electronically to the PWS using the DCTS, the PWS will review the results. The PWS user cannot edit the data, but if the PWS has an issue with the sample result, such as if the PWS believes that the sample collection point ID or collection date is incorrect, the PWS can release the results back to the laboratory for issue resolution. In addition to noting the reason in the DCTS for the return of the data to the laboratory, the PWS may also contact the laboratory verbally to discuss the issue.

If the PWS determines that the data are accurate, the PWS releases the results to EPA (and the State, if applicable) as "approved" results. If the PWS determines that the data are accurate, but believes that the data are not valid for LT2 binning purposes, the PWS can release the results to EPA and the State as "contested." Contested samples are those that have been correctly analyzed, but that the PWS contends are not valid for use in LT2 binning, and have been submitted to EPA for evaluation.

3.7.3 EPA/State Review

After the PWS has released the results as approved or contested, they are available to EPA and State users to review through the DCTS. EPA and State users cannot edit the data. EPA or State users approve results where appropriate. Pursuant to 141.702(a)(2), resampling must occur whenever EPA or the State rejects results or indicates agreement with a PWS action to contest a result.

3.8 Data Archiving

The PWS is required to keep all original, hardcopy monitoring results associated with LT2 sample analyses (both initial and second round of monitoring) for 3 years after bin classification for filtered systems or determination of mean *Cryptosporidium* level for unfiltered systems [40 CFR § 141.722(a)]. Although it is the PWS's responsibility to meet LT2 Rule data storage requirements for compliance monitoring samples, including MS samples, the PWS may contract this work to the laboratory.

3.8.1 Hardcopy Data

The following data should be archived:

- Bench sheets and slide examination forms for all LT2 monitoring samples, including both field samples and MS samples
- Bench sheets and slide examination forms for all OPR samples and method blank samples, and records of the 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 OPT samples

As part of the Lab QA Program, the laboratory also should maintain the same documentation for their IPR and IPT data for each method variation used for LT2 samples.

3.8.2 Slides

Although not required, laboratories also may want to archive slides and/or take photographs of slides to maintain for clients. Slides should be stored in the dark between 1° and 10°C and not frozen, and in the appropriate type of chamber for the mounting medium used.

As an alternate to the DABCO/glycerol mounting medium, currently specified in EPA Method 1622 and 1623, laboratories may wish to evaluate the use of the elvanol mounting medium, which hardens, and may be useful for archiving slides. EPA recommends quality control assessment before changing the mounting medium currently used by the laboratory including initial precision and recovery, method blank, matrix spike/matrix spike duplicate, and unspiked field samples. Reagents for the mounting medium include the following:

- 8.0 g elvanol (polyvinyl alcohol (PVA))—MP Biomedical cat. no. 151937, Solon, Ohio, or equivalent
- 48.0 g (40 mL) glycerol
- 10% NaN_3 (sodium azide)
- DABCO—Sigma-Aldrich, cat no. D-2522, or equivalent

- Tris buffer—Dissolve 1.2 g Tris (Fisher cat. no. BP152) in 95 mL reagent water, adjust pH to 8.5 with 1 N HCl

To prepare the medium, you should use the following procedure:

- Add 48.0 g (40 mL) glycerol to 8.0 g elvanol and stir.
- Add 49.0 mL of reagent water and 1.0 mL 10% NaN_3 and stir. Let stand 4 hours at room temperature.
- Add DABCO in Tris buffer (4.75 g of DABCO in 100 mL Tris buffer, adjusted to pH 8.5 with conc. HCl) and stir.
- Place mixture in a boiling water bath until the mixture becomes homogenous.
- Centrifuge mixture at 2000XG for 10 minutes. Centrifugation of entire mixture in one tube is preferable.
- Dispense 3- to 5 mL aliquots of the mixture into tubes and store at 0°C to 10°C.

If the mounting medium sets up in the tube during storage, re-heat in boiling water bath or microwave for a short time to restore mixture to liquid state.

Make sure to test any new reagent first on QC samples to verify that the mounting medium performs properly before using the medium on any monitoring samples.

Commercially prepared mounting media for archiving slides are also available. For example, Waterborne Inc. produces an archiving mounting medium (product no. M102) that is fade-retardant and self-sealing. Vector Laboratories produces both an anti-fade mounting medium and an archiving anti-fade mounting medium (Vectashield® Mounting Medium and HardSet™ Mounting Medium, product no. H-1000 and H-1400).

3.9 Equipment, Supplies, Reagents, and Standards

See Sections 6 and 7 of EPA Method 1622/1623 for details on the materials needed to perform the *Cryptosporidium* analyses specified in the methods.

SECTION 4: GUIDANCE FOR *E. COLI* LABORATORIES

The Long-Term 2 Enhanced Surface Water Treatment (LT2) rule requires that large filtered PWSs (those serving ≥10,000) perform *E. coli* and turbidity analyses on source water samples [40 CFR § 141.701(a)]. The *Cryptosporidium*, *E. coli*, and turbidity data will be analyzed to confirm or refine the relationship between *Cryptosporidium* and *E. coli* levels in source waters and refine the previously established trigger levels for *E. coli*. To reduce costs, small filtered PWSs (those serving <10,000) will use *E. coli* monitoring to determine the need for implementing more expensive *Cryptosporidium* monitoring or improved treatment.

Turbidity measurement requirements and recommendations are addressed in the *Source Water Monitoring Guidance Manual for Public Water Systems for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)* (http://www.epa.gov/safewater/disinfection/lt2/). Laboratories performing *E. coli* analyses under LT2 must be certified under the Drinking Water Laboratory Certification program (described below) to analyze drinking water compliance samples. The procedures required of a laboratory to become certified are described in the *Manual for the Certification of Laboratories Analyzing Drinking Water*, 5th Edition (Reference 5.6). This manual can also be found online at http://www.epa.gov/safewater/labcert/labindex.html.

LT2 compliance monitoring differs from monitoring currently being conducted under the Surface Water Treatment Rule (SWTR) and Total Coliform Rule (TCR). It is important for both PWSs and laboratories to note the differences in monitoring requirements so that there is no confusion surrounding the samples that are taken and analyzed for these three regulations.

- LT2 requires samples to be analyzed for *E.coli* while the SWTR requires that samples be analyzed for fecal coliforms.

- LT2 requires all *E. coli* analyses to be quantitative [40 CFR 141.704(b)], while the TCR requires only presence/absence analyses.

- LT2 allows the use of mTEC medium (*Standard Methods* 9213 or EPA Method 1103.1) and m-ColiBlue 24® to be used for the detection of *E. coli* in source water samples. These methods are not approved for use in analysis of samples under the SWTR.

- The holding time for LT2 samples is 30 hours, with a possible extension to 48 hours for samples analyzed by the Colilert reagent version of *Standard Method* 9223B if approved by the State [40 CFR 141.704(b)]. This extended holding time is for use only with LT2 samples; SWTR samples are still required to be analyzed within the 8 hour holding time.

Compliance monitoring for LT2 is in addition to, and does not replace, the monitoring mandated under SWTR and TCR

4.1 Laboratory Certification Program

Since 1978, the U.S. Environmental Protection Agency has implemented a certification program for laboratories performing drinking water analyses for compliance with regulations issued pursuant to the Safe Drinking Water Act and subsequent National Primary Drinking Water Regulations (NPDWR). All laboratories analyzing drinking water compliance samples must be certified for the analyses they perform [40 CFR 141.28]. The laboratory certification process and detailed specifications for certification are described in the *Manual for the Certification of Laboratories Analyzing Drinking Water*, referred to as "laboratory certification manual", 5th Edition (Reference 5.6). This manual can be found online at: http://www.epa.gov/safewater/labcert/labindex.html. Laboratories performing *E. coli* analyses for LT2 monitoring must be certified by EPA, the National Environmental Laboratory Accreditation Conference (NELAC), or the State to conduct total or fecal coliform analysis in drinking water under 40 CFR 141.74 using the same *E.coli* technique that they are certified to use for coliform analysis in drinking water [40 CFR § 141.705(b)]. Approved *E. coli* methods and references are provided in **Table 4-1** [40 CFR § 141.704(b)].

EPA notes that this approach deviates from the approach typically used in its Laboratory Certification program in that the latter program is based on certification for the specific method (not simply the same technique) being used in compliance monitoring. EPA strongly encourages all laboratories using these *E. coli* methods to seek certification for those methods as soon as is practical.

Table 4-1. Approved *E. coli* Methods for LT2 Rule

***E. coli* Methods Approved for LT2 Rule**	***Method Format***	***Method Citation***
Standard Methods 9221B.1/9221F (LTB-EC-MUG)	multiple tube fermentation/ most probable number	*Standard Methods for the Examination of Water and Wastewater* (Reference 5.8)[2]
Standard Methods 9223B (Colilert®/Colilert-18®)	multiple tube/multiple well	*Standard Methods for the Examination of Water and Wastewater* (Reference 5.8)[2]; IDEXX Laboratories, Inc. (Reference 5.9)
Standard Methods 9222B/9222G[1] (mEndo/LES-Endo-NA-MUG)	membrane filtration, two step	*Standard Methods for the Examination of Water and Wastewater* (Reference 5.8)[2]
Standard Methods 9222D/9222G (mFC-NA-MUG)	membrane filtration, two step	*Standard Methods for the Examination of Water and Wastewater* (Reference 5.8)[2]
Standard Methods 9213D/ EPA Method 1103.1 (mTEC)	membrane filtration, one step	EPA Method 1103.1 (Reference 5.10); *Standard Methods for the Examination of Water and Wastewater* (Reference 5.8)[2]
EPA Method 1603 Modified mTEC	membrane filtration, one step	EPA Method 1603 (Reference 5.11)
EPA Method 1604 MI medium[1]	membrane filtration, one step	EPA Method 1604 (Reference 5.12)
m-ColiBlue24® Broth[1]	membrane filtration, one step	Hach Company (Reference 5.13)

[1] If high levels of non-*E. coli* total coliforms interfere with the ability to accurately enumerate *E. coli* despite additional dilutions, an alternate method should be used (i.e., SM 9222D/9222G, SM 9213D/EPA Method 1103.1, EPA Method 1603, SM 9221B.1/9221F, and SM 9223B)

[2] 18th, 19th, or 20th Editions of *Standard Methods for the Examination of Water and Wastewater* may be used.

4.2 Summary of LT2 Rule *E. coli* Methods

The methods approved under the LT2 Rule were developed by EPA, voluntary consensus standards bodies (VCSB) (i.e., American Public Health Association (APHA), American Water Works Association (AWWA), and Water Environment Foundation (WEF) who jointly publish *Standard Methods for the Examination of Water and Wastewater*, referred to as "*Standard Methods*"), and commercial vendors with methods submitted to the EPA Office of Water Alternate Test Procedure (ATP) process. For several procedures, an EPA Method, VCSB method, and/or a commercially available method (submitted to the ATP program) are approved.

Laboratories should obtain a copy of the methods approved for LT2 monitoring prior to seeking certification for these methods. The method summaries provided below only offer a brief overview of the methods and are not meant to provide details of the methods. Copies of analytical methods may be obtained from the citations listed in **Table 4-1**. Copies of analytical methods published by EPA are available for a nominal cost through the National Technical Information Service (NTIS); U.S. Department of Commerce; 5285 Port Royal Road; Springfield, VA 22161. Copies of the EPA methods may also be downloaded from the EPA Office of Research and Development; National Exposure Research Laboratory (NERL)-Cincinnati Microbiology home page at www.epa.gov/microbes/. All other methods must be obtained from the publisher. Publishers for all methods are included in **Table 4-1;** addresses for publishers can be found in Section 5.

E. coli sample analyses performed under the LT2 Rule must be quantitative; presence/absence *E. coli* results are unacceptable. The analytical methods approved under LT2 are for enumeration of *E. coli* in ambient water, and the results are reported as number of *E. coli* per 100 mL water. Public Water Systems monitoring for *E. coli* under the LT2 Rule should collect and analyze at least 100 mL of sample to ensure sufficient volume for sample analysis. In order to obtain quantitative results for the source water samples for LT2 monitoring, the laboratory will need to select the appropriate configuration of tubes/filtrations and dilutions/volumes to account for the quality, character, consistency, and anticipated *E. coli* density of the water sample. See Section 4.5 below for guidance on sample volume and dilutions for LT2 sample analyses.

To assess the comparability of results obtained with individual methods, it is suggested that side-by-side tests be conducted across seasons of the year with water samples routinely tested in accordance with the most current *Standard Methods for the Examination of Water and Wastewater* or EPA alternate test procedure (ATP) guidelines. The methods summarized below are approved for the analysis of *E. coli* samples under the LT2 Rule [40 CFR § 141.704(b)].

4.2.1 Most Probable Number (MPN) Methods

4.2.1.1 *Standard Methods* 9223B: Colilert® and Colilert-18®

Colilert® and Colilert-18® tests are chromogenic/fluorogenic enzyme substrate tests for the simultaneous determination of total coliforms and *E. coli* in water. All tests must be conducted in a format that provides quantitative results, such as the multiple tube or multiple well formats, e.g., Quanti-tray® (51 well analysis) and Quanti-tray® 2000 (97 well analysis). Using multiple-tube procedures, laboratories will need to employ an appropriate tube and dilution configuration of the sample as needed. After the appropriate sample dilutions/volumes are added, the tubes or trays are incubated for 18 hours when using Colilert-18® or 24 hours when using Colilert®. Each tube or well is then compared to the reference color "comparator" available from the manufacturer. A yellow color greater or equal to the comparator indicates the presence of total coliforms in the sample. The tube or well is then checked for fluorescence under long-wavelength UV light. A yellow well with fluorescence greater than or equal to the

comparator is positive for *E. coli*. The most probable number (MPN) value is determined by the number of positive tubes or wells using MPN tables provided by the manufacturer. *E. coli* densities are then calculated and reported as MPN/100 mL (discussed in Section 4.6.1 of this manual below).

4.2.1.2 *Standard Methods* 9221B.1/9221F: LTB-EC-MUG

The multiple-tube fermentation (MTF) method uses multiple tubes and serial dilutions/volumes in a two-step procedure to determine *E. coli* densities in water. In the first step a series of tubes containing lauryl tryptose broth (LTB) are inoculated with undiluted sample and/or dilutions/volumes of the sample and mixed. After incubation, tubes are examined for growth (turbidity) and gas, which constitute a positive presumptive test for coliforms, which include *E. coli*. *Note*: Lactose broth may be used in lieu of LTB, if at least 25 parallel tests are conducted between this broth and LTB using the water samples normally tested, and this comparison demonstrates that the false-positive rate and false-negative rate for total coliform using lactose broth is less than 10 percent.

Growth from all presumptive tubes (showing any amount of gas, growth or activity within 48±3 hours of incubation) is transferred to tubes containing EC-MUG broth for *E. coli* enumeration. After incubation, tubes that exhibit turbidity and fluorescence are considered to be positive for *E. coli*. The density of *E. coli* is determined from the number of positive tubes using the MPN table provided in SM 9221C. *E. coli* densities are reported as MPN/100 mL (See Section 4.6.3 of this manual for guidance on determining the MPN). *Note:* there is no requirement to run the completed phase on 10 percent of all total coliform-positive tubes on a seasonal basis.

4.2.2 Membrane Filtration (MF) Methods

Note: When the MF method has not been used previously to test ambient water with high turbidity, large number of non-coliform bacteria, or samples that may contain organisms stressed by chlorine, a parallel test should be conducted with a multiple-tube technique to demonstrate applicability and comparability of results.

4.2.2.1 *Standard Methods* 9222B/9222G: mEndo/LES-Endo—NA-MUG and *Standard Methods* 9222D/9222G: mFC — NA-MUG

These membrane filter methods are two-step MF procedures that provide a direct count of *E. coli* in water. First, a sample is filtered through a 0.45 μm, white gridded membrane filter. The filter is then placed on a pad saturated with mEndo broth or a plate containing mEndo or LES-Endo agar and incubated. Pink to red colonies with a metallic (golden-green) sheen on mEndo (or LES-Endo) are total coliforms. If initial determination of fecal coliforms is desired, mFC media can be substituted for mEndo/LES-Endo. Blue colonies on the filter are fecal coliforms.

In the second step of these methods, the filter is transferred to nutrient agar containing MUG (NA-MUG) medium and incubated. Sheen colonies from mEndo/LES-Endo or blue colonies from mFC that fluoresce under a long-wavelength UV light after incubation on NA-MUG are positive for *E. coli*. *E. coli* densities are reported as CFU/100 mL. Guidance on determining the number of *E.coli*/100 mL is discussed below in Section 4.6.2.

If high levels of total coliforms interfere with the ability to accurately enumerate *E. coli* despite additional dilutions, an alternate method (e.g., SM 9213D, EPA Method 1603 or 9223B) should be used.

4.2.2.2 *Standard Methods* 9213D/EPA Method 1103.1: mTEC

The mTEC agar method is a two-step MF procedure that provides a direct count of *E. coli* in water. In this method, a water sample is filtered through a 0.45 µm white gridded membrane filter, the filter is placed on mTEC agar (a selective primary isolation medium), and the plate is incubated at 35°C ± 0.5°C for 2 hours to resuscitate injured or stressed bacteria and then at 44.5°C ± 0.2°C for 22-24 hours in a water bath. Following incubation, the filter is transferred to a pad saturated with urea substrate medium. After 15 minutes, all yellow, yellow-brown, or yellow-green colonies are counted as *E. coli*. *E. coli* densities are reported as CFU/100 mL.

4.2.2.3 EPA Method 1603: Modified mTEC

The modified mTEC agar method is a single-step MF procedure that provides a direct count of *E. coli* in water. This is a modification of the standard mTEC media (SM 9213D). In this method, a water sample is filtered through a 0.45 µm white gridded membrane filter, the filter is placed on modified mTEC agar, incubated at 35°C ± 0.5°C for 2 hours to resuscitate injured or stressed bacteria, and then incubated for 22-24 hours in a 44.5°C ± 0.2°C water bath. Following incubation, all red or magenta colonies are counted as *E. coli*. *E. coli* concentrations are reported as CFU/100 mL.

4.2.2.4 EPA Method 1604: MI Medium

The MI medium method is a single-step MF procedure used to simultaneously enumerate total coliforms and *E. coli* in water. In this method, a water sample is filtered through a 0.45 µm white gridded membrane filter, the filter is placed on an MI medium and incubated. Following incubation, all blue colonies under ambient light are counted as *E. coli*. These colonies can be fluorescent or non-fluorescent under long-wavelength ultraviolet light. *E. coli* concentrations are reported as CFU/100 mL.

If high levels of total coliforms interfere with the ability to accurately enumerate *E. coli* despite additional dilutions, an alternate method (e.g., SM 9213D, EPA Method 1603 or 9223B) should be used.

4.2.2.5 m-ColiBlue24® Broth

This broth method is a single-step MF test for enumerating total coliforms and *E. coli* in water. In this method, a water sample is filtered through a 0.45 µm white gridded membrane filter. The filter is placed on a plate containing an absorbent pad saturated with m-ColiBlue24® broth and incubated. Following incubation, all blue colonies are counted as *E. coli*. *E. coli* concentrations are reported as CFU/100 mL.

If high levels of total coliforms interfere with the ability to accurately enumerate *E. coli* despite additional dilutions, an alternate method (e.g., SM 9213D, EPA Method 1603) should be used.

4.3 Quality Assurance/Quality Control for *E. coli* Analyses

The *Manual for the Certification of Laboratories Analyzing Drinking Water* (Reference 5.6) describes criteria and procedures that should be considered when a laboratory undergoes the certification process. This manual contains elements that are required by federal regulations as well as criteria that are recommended by EPA for laboratory certification. Additional QA/QC specifications beyond those found in the laboratory certification manual may be found in *Standard Methods*, Section 9020 (Reference 5.8).

Each laboratory should have a Quality Assurance plan describing the QA program and QC activities necessary to meet the laboratory's specific needs. The QA Plan for LT2 monitoring must meet the specifications of the laboratory's certifying authority and the method used. The QA plan should address the following issues: personnel policies, equipment and instrument specifications, specifications for supplies, analytical methods and QC measures, standard operating procedures (SOPs), documentation

specifications, performance evaluation samples, internal and external lab audits, and corrective actions. For more suggestions on what items should be included in a QA plan, see the Laboratory Certification Manual, Chapter 3.

Chapter V, Critical Elements of Microbiology, of the Laboratory Certification Manual details both required and recommended criteria for a laboratory that is analyzing water samples for microbes. *E. coli* sample results reported under the LT2 Rule should meet the quality control (QC) specifications set forth in the Laboratory Certification Manual and the methods listed in **Table 4-1** above.

4.3.1 Quality Control Specifications Applicable to LT2 Samples

4.3.1.1 Holding Time and Temperature Requirements for Field Samples

Source water samples are dynamic environments and, depending on sample constituents and environmental conditions, *E. coli* present in a sample can grow or die off, biasing analytical results. Samples that are not analyzed immediately after sample collection during LT2 Rule monitoring must be chilled to reduce biological activity, and preserve the state of source water samples between collection and analysis. Samples for *E. coli* analyses must be maintained between 0°C and 10°C if they are shipped [40 CFR 141.704(b)(3)]. Samples should not be allowed to freeze.
For best results, samples should be analyzed as soon as possible after collection. Due to the need by some utilities to ship samples overnight to an off-site laboratory for analysis, the holding time for LT2 *E. coli* samples is 30 hours. If the State determines on a case-by-case basis that analyzing an *E. coli* sample within 30 hours is not feasible, the State may authorize the holding of an *E. coli* sample for up to 48 hours between collection and initiation of analysis. *E. coli* samples held between 30 to 48 hours must be analyzed by the Colilert® reagent version of Standard Method 9223B [40 CFR § 141.704(b)(2)]. The *E. coli* holding time established for source water monitoring under the LT2 Rule does not apply to *E.coli* sample holding time requirements that have been established under other programs and regulations.

Given the importance of maintaining sample temperatures for *E. coli*, laboratories should establish acceptance criteria for receipt of *E. coli* samples transported to their laboratory. Several options are available to measure sample temperature upon receipt at the laboratory and, in some cases, during shipment; these are provided in Section 2.2 of this manual.

4.3.2 Quality Control Specifications for *E. coli* Methods

In addition to the overall QC specifications set forth in the Laboratory Certification Manual and in the analytical methods, laboratories analyzing samples for LT2 Rule *E. coli* analyses must meet method-specific incubation time and temperature requirements.

4.3.2.1 Incubation Time/Temperature Specifications for MPN Methods

The required incubation times and temperatures for MPN methods are provided in **Table 4-2**.

Table 4-2. Incubation Time and Temperature Specifications for MPN Methods

Method	Media	Incubation Time/Temperature
Standard Methods 9223B	Colilert®	24 - 28 hours at 35°C ± 0.5°C
	Coliert-18®	18 - 22 hours at 35°C ± 0.5°C
Standard Methods 9221B/9221F	LTB	24 ± 2 hours at 35°C ± 0.5°C and 48 ± 3 hours at 35°C ± 0.5°C
	EC-MUG	24 ± 2 hours at 44.5°C ± 0.2°C

4.3.2.2 Incubation Time and Temperature Specifications for MF Methods

The required incubation times and temperatures for MF methods are provided in **Table 4-3**.

Table 4-3. Incubation Time and Temperature Specifications for Membrane Filter Methods

Method	Media	Incubation Time/Temperature
Standard Methods 9222B/9222G	mENDO→NA-MUG	22 - 24 hours at 35°C ± 0.5°C→ 4 hours at 35°C ± 0.5°C
	Les-ENDO→NA-MUG	22 - 24 hours at 35°C ± 0.5°C→ 4 hours at 35°C ± 0.5°C
Standard Methods 9222D/9222G	mFC→NA-MUG	22 - 26 hours at 44.5°C ± 0.2°C→ 4 hours at 35°C ± 0.5°C
Standard Methods 9213D/EPA Method 1103.1	mTEC agar	2 hours at 35°C ± 0.5°C → 22 - 24 hours at 44.5°C ± 0.2°C
EPA 1603	Modified mTEC	2 hours at 35°C ± 0.5°C → 22 - 24 hours at 44.5°C ± 0.2°C
EPA 1604	MI agar	24±2 hours at 35°C ± 0.5°C
m-ColiBlue24® Broth	m-ColiBlue24® Broth	24 hours at 35°C ± 0.5°C

4.4 Sample Collection Procedures

PWSs are required to collect source water samples for the LT2 Rule from the plant intake prior to chemical treatment, unless approved by the State to collect the source water sample after chemical treatment [40 CFR § 141.703(b)(2)]. Systems that recycle filter backwash water must collect source water samples prior to the point of filter backwash water addition [40 CFR § 141.703(c)]. All *Cryptosporidium*, *E. coli*, and turbidity source water samples collected under LT2 Rule requirements must be collected from the same appropriate sampling location. Detailed guidance on sample collection procedures for *E. coli* samples for on-site analysis and for shipment to an off-site laboratory for analysis are provided in the *Source Water Monitoring Guidance Manual for Public Water Systems for the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule)*. This manual is available for download from http://www.epa.gov/safewater/disinfection/lt2/.

4.5 Sample Volume and Dilution Guidance

Because *E. coli* analyses will be performed on source waters, rather than finished drinking waters, multiple sample volumes/dilutions may be necessary to assess high *E. coli* levels. Initially all laboratories should consider analyzing four sample volumes (100, 10, 1.0, and 0.1 mL) for all methods except MTF. For MTF methods, the PWS should initially consider analyzing five sample volumes of 10, 1.0, 0.1, 0.01, and 0.001 mL. Sample volumes may need to be adjusted based on confounding conditions (e.g., high turbidity, heavy rainfall, etc.).

If the PWS has historical data demonstrating that *E. coli* levels are consistently low, they may drop the smaller sample volumes. If there has been a substantial rainfall in the 24 hours prior to sample collection causing runoff, sample volumes may need to be adjusted in order to obtain valid results and avoid data that are above the analytical range of the method. This could also be a concern if the PWS eliminates the 100 mL sample volume due to historical data indicating that values tend to be very high and the *E. coli* levels are significantly lower than anticipated, requiring the PWS to report data that are below the analytical range of the method. EPA recommends bracketing the target sample volume (i.e., analyzing a sample volume above and below the sample volume expected to yield useable data) to account for potential variability.

4.5.1 Sample Volume and Dilution Guidance for Multiple-Well Methods

The analysis of water samples under LT2 using multiple-well methods generally requires the use of four aliquots of samples (100, 10, 1.0, and 0.1 mL). As mentioned above, sample volumes may need to be adjusted based on confounding conditions (e.g., high turbidity, heavy rainfall, etc.). Because a 100 mL volume is necessary to fill all of the wells, it will be necessary to add the aliquots of the sample to sterile reagent water blanks. The total volume after the sample aliquot is added to the reagent water blank should be approximately 100 mL (i.e., a 90 mL blank should be used for the 10 mL sample volume, a 99- mL reagent water blank should be used for the 1.0 mL aliquot). Colilert® or Colilert$_{18}$® reagent should be added to the sterile reagent water blanks prior to adding sample. If the sample is added to the reagent water before the Colilert® or Colilert$_{18}$® reagent, bacterial cells may lyse. (Reference 5.14)

4.5.2 Sample Volume and Dilution Guidance for Multiple-Tube Methods

For the analysis of water samples by a multiple-tube technique (e.g., Colilert® in a multiple-tube format, SM 9222B LTB/EC-MUG) under LT2, laboratories should consider the use of a 20-tube most probable number format (i.e., 5 tubes at each of the four dilutions, 10.0, 1.0, 0.1, and 0.01 mL). Initially, it is recommended that five sample volumes (i.e., 5 tubes for each sample volume, 10.0, 1.0, 0.1, 0.01, and 0.001 mL) are analyzed. Additional guidance on selection of dilutions for multiple-tube methods is available in *Standard Methods* 9221 (Reference 5.8).

4.5.3 Sample Volume and Dilution Guidance for Membrane Filtration

For the analysis of water samples under LT2, it is recommended that four different aliquots of sample (100, 10, 1.0, and 0.1 mL) be analyzed when using membrane filtration methods. Alternate sample volumes may be used if necessary. To ensure adequate distribution of bacteria during filtration, dispense aliquots of the sample, with the exception of the 100 mL volume, into sterile buffered water blanks (at least 30 mL, depending on the sample volume analyzed). Alternately, 10 mL of sterile buffer may be added to the filter apparatus prior to adding the sample. The buffered water minimizes clumping of the bacteria on the filter surface.

4.6 *E. coli* Data Recording and Calculations

Laboratories performing *E. coli* analyses during the LT2 Rule must follow any data recording practices required by the certification authority granting the certification to the laboratory, and should follow any recommended data recording practices outlined in the Laboratory Certification Manual, Chapter V, Section 8.

Some of the recommended information to be recorded for each *E. coli* sample is as follows. Data for each sample should be recorded on a sample collection form used in the field and maintained in the same file as the *E. coli* laboratory data or on the laboratory data form. Note that some of these data will be entered into the LT2/Stage 2 Data Collection and Tracking System (DCTS):

- PWS ID
- Facility ID
- Sample Identification (if any)
- Sample collection point ID
- Sample collection date and time
- Sample type
- Analytical method number
- Method type
- Name of sampler
- Turbidity
- Source water type

The laboratory should record all of the primary measurements associated with each analysis, as they are needed to calculate the final concentration of *E. coli* per 100 mL. Primary measurements for membrane filtration methods will include the volumes filtered and the plate counts for each volume filtered. The multiple-well and multiple-tube formats will include the volumes or dilutions of samples analyzed and the number of positive wells or tubes per each volume analyzed. Method-specific data to record for each of the individual method types are discussed in Sections 4.6.1 — 4.6.3 of this manual.

The suggested data elements shown below are important as they allow the laboratory to ensure that final sample results can be verified using primary data and to demonstrate that sample analyses were performed within method-specified holding times. Please note that not all of the data that may be recorded will be reported in the DCTS. *E. coli* data reporting under the LT2 Rule is discussed in Section 4.7 of this manual.

The final *E. coli* concentration for field samples will be reported as CFU/100 mL or MPN/100 mL depending on the method used for analysis. If no *E. coli* are detected in the sample, the detection limit based on the volume of sample analyzed may be reported (e.g., <1 CFU /100 mL or <1.8 MPN/100 mL) or a zero for purposes of the DCTS.

In addition, this section also provides standardized procedures for determining *E. coli* concentration for LT2 samples for the various analytical techniques that are approved for use under the rule. Because these analyses will be performed on source waters rather than finished drinking waters, and multiple dilutions may be necessary to assess higher *E. coli* levels, it is recommended that laboratories consistently use the same analytical procedures for determining *E. coli* sample concentrations to reduce variability.

4.6.1 Multiple-Well Data

In addition to the data elements contained in the bulleted list in Section 4.6 of this manual, laboratories using Colilert® and Colilert-18® methods for *E. coli* sample analyses in support of the LT2 Rule should record the data elements specific to multiple-well techniques. Data elements to be recorded for the 97-well format (Quanti-Tray 2000®) are noted in **Table 4-4**; data to be recorded for the 51-well format (Quanti-Tray®) are noted in **Table 4-5**. These elements include the primary measurements needed to calculate the *E. coli* concentration in the sample as well as all method-required incubation and read times needed to verify that the sample analyses were conducted under analytical control.

Calculations for determining the *E. coli* concentration using the Quanti-Tray 2000® (97-well) and Quanti-Tray® (51-well) formats are provided after each table.

4.6.1.1 Data Elements for Quanti-Tray 2000® (97-well) Analyses

The recommended data elements to record for Quanti-Tray 2000® (97-well) analyses are provided in **Table 4-4**.

Table 4-4. Recommended Minimum Record for Quanti-tray 2000® Colilert® and Colilert-18® Analyses

Primary Measurements
mL of sample added to tray (does not include reagent water volume)
Large wells positive: Total coliform positive and UV fluorescence
Small wells positive: Total coliform positive and UV fluorescence
Holding Time / Incubation Time Information
Incubation start: date/time
Incubation end: date/time
Additional incubation start: date/time
Additional incubation end: date/time

4.6.1.2 Determining E. coli Concentration Using Colilert® and Colilert-18® Quanti-Tray 2000® Data

A. **Select appropriate dilution to yield countable results.** If multiple dilutions are used, the tray exhibiting positive wells in the 40 percent and 80 percent range (39 to 78 total positive large and small wells) should be used to determine MPN value.

Note: *The analytical result can be automatically calculated using the LT2/Stage 2 Data Collection and Tracking System. See Section 4.7 of this manual for additional information.*

B. **Determine MPN.** Use the MPN tables provided by the vendor. To determine the MPN using these tables, locate the number at the intersection of large positive wells and small positive wells from the appropriate dilution, identify the corresponding MPN/100 mL in the table provided by the vendor. Large well values are located in the left column; small well values are located in the top row. For example, if a 100 mL sample was analyzed, and there were 29 large positive wells and 5 small positive wells, the corresponding MPN would be 49.6 MPN/100 mL.

C. **Adjust for dilution factor.** Because the MPN/100 mL values in the table are based on 100 mL samples, the MPN value should be adjusted if less than 100 mL of sample volume was analyzed. Use the following calculation to adjust the MPN to account for the dilution:

$$\text{Analytical result} = \text{MPN value} \times \frac{100}{\text{mL of sample analyzed}}$$

Example:

Volume analyzed = 10 mL of sample (in 90 mL of dilution water)
Large wells positive = 39
Small wells positive = 5
The MPN value calculated based on the intersection of 39 and 5 in the table.

MPN = 81.3

$$\text{Analytical result} = 81.3 \times \frac{100}{10} = 813 \textit{ E. coli} \text{ MPN/100 mL}$$

4.6.1.3 Data Elements for Quanti-Tray® (51-well) Analyses

The recommended data elements to record for Quanti-Tray® (51-well) analyses are provided in **Table 4-5**.

Table 4-5. Recommended Data to Record for Quanti-Tray® Colilert® and Colilert-18® Analyses

Primary Measurements
mL of sample added to tray (does not include reagent water volume)
Number of wells positive: Total coliform positive and UV fluorescence
Holding Time / Incubation Time Information
Incubation start: date/time
Incubation end: date/time
Additional incubation start: date/time
Additional incubation end: date/time

4.6.1.4 Determining *E. coli* Concentration Using Colilert® and Colilert-18® (51-well) Data

A. **Select appropriate dilution.** If multiple dilutions are used, the tray exhibiting 80 percent positive wells (41 positive wells) should be used to determine MPN value.

> ***Note:*** *The analytical result can be automatically calculated using the LT2/Stage 2 Data Collection and Tracking System (DCTS). See Section 4.6 of this manual for additional information.*

B. **Determine MPN.** Using the number of positive wells from the appropriate dilution, identify the corresponding MPN/100 mL using the MPN table provided by the vendor. For example, if a 100 mL sample was analyzed, and there were 41 positive wells, the corresponding MPN would be 83.1 MPN/100 mL.

C. **Adjust for dilution factor.** Because the MPN/100 mL values in the table are based on 100 mL samples, the MPN value should be adjusted if less than 100 mL of sample volume was analyzed. Use the following calculation to adjust the MPN to account for the dilution:

$$\text{MPN value} \times \frac{100}{\text{mL of sample analyzed}} = \textit{E. coli}\ \text{MPN/100 mL}$$

Example:

Volume analyzed (mL) = 10 mL (in 90 mL of dilution water)
Number of positive wells = 41
MPN = 83.1

The analytical result is calculated as follows:

$$\text{Analytical result} = 83.1 \times \frac{100}{10} = 831\ \textit{E. coli}\ \text{MPN/100 mL}$$

4.6.2 Membrane Filtration Data

In addition to the general sample data contained in the bulleted list in Section 4.6 of this manual, laboratories using membrane filtration methods for *E. coli* sample analyses in support of the LT2 Rule should record the data elements specific to this technique. These are noted in **Table 4-6**, and include the primary measurements needed to calculate the *E. coli* concentration in the sample, as well as all method-required incubation and read times needed to verify that the sample analyses were conducted under analytical control.

4.6.2.1 Data Elements for Membrane Filtration Analyses

The recommended data elements to record for membrane filtration analyses are provided in **Table 4-6**.

Table 4-6. Recommended Data Elements for Record for Membrane Filtration Analyses

Primary Measurements
Filter 1 volume (mL) (e.g., 100 mL)
CFU on Filter 1
Filter 2 volume (mL) (e.g., 10 mL)
CFU on Filter 2
Filter 3 volume (mL) (e.g., 1.0 mL)
CFU on Filter 3
Filter 4 volume (mL) (e.g., 0.1 mL)
CFU on Filter 4
Holding Time / Incubation Time Information
Primary isolation medium (e.g., mENDO, mFC) incubation start: date/time
Primary isolation medium (e.g., mENDO, mFC) incubation end: date/time
Secondary isolation medium (e.g, NA-MUG) incubation start: date/time
Secondary isolation medium (e.g, NA-MUG) incubation end: date/time

4.6.2.2 Determining *E. coli* Concentrations Using Membrane Filter Data

A. *E. coli* counts should be determined from the volume(s) filtered that yielded 20 to 80 *E. coli* colonies (20-60 for mFC-NA-MUG), and not more than 200 total colonies per plate. (Guidance for samples that do not yield countable plates is provided in Sections E and F below) (References 5.8 and 5.10-5.12)

Note: *The analytical result can be automatically calculated using the LT2/Stage 2 Data Collection and Tracking System (DCTS). See Section 4.7 of this manual for additional information.*

B. If there are greater than 200 colonies per membrane, even for the lowest dilution, the result is recorded as "too numerous to count" (TNTC). These results should not be reported for LT2 monitoring, as they cannot be used for the required data analyses. During the next sampling event, analyze an additional, lower dilution volume (the highest dilution volume may be omitted) unless conditions were unusual (e.g., heavy rains, flooding, etc.) during the sampling event yielding TNTC for all dilutions.

C. If colonies are not sufficiently distinct for accurate counting, the result is recorded as "confluent growth" (CNFG). To prevent CNFG from occurring, smaller sample aliquots should be filtered. For example, if sample volumes of 100, 10, 1 and 0.1 mL are analyzed and even the 0.1 mL plate results in CNFG, then potentially 0.01 mL should be analyzed during the next sampling event. For sample volumes less than 1 mL, serial dilutions should be used, and 1 mL volumes of the dilutions should be filtered. The 100 mL volume can be eliminated. *Note: If growth is due to high levels of total coliforms but low E. coli then another method should be chosen for analyses that does not rely on total coliform determination prior to or simultaneously with E. coli determination.*

Note: *Results that are TNTC or CNFG are not appropriate for LT2 microbial data analysis, and cannot be entered into the LT2/Stage 2 Data Collection and Tracking System (DCTS). These results should not be reported.*

D. Using the *E. coli* counts from the appropriate dilution, *E. coli* CFU/100 mL should be calculated based on the following equation:

$$\textit{E. coli}\ \text{CFU} \times \frac{100}{\text{mL sample filtered}} = \textit{E. coli}\ \text{CFU/100 mL}$$

Example 1:

Filter 1 volume = 100 mL	CFU = TNTC
Filter 2 volume = 10 mL	CFU = 40
Filter 3 volume = 1.0 mL	CFU = 9
Filter 4 volume = 0.1 mL	CFU = 0

Using the guidance on countable colonies in Step A, the count from the 10 mL plate will be used to calculate the *E. coli* concentration for the sample:

$$40\ \textit{E. coli}\ \text{CFU} \times \frac{100}{10\ \text{mL}} = 400\ \textit{E. coli}\ \text{CFU/100 mL}$$

E. If no *E. coli* colonies are present, the detection limit (i.e., 1 CFU per volume filtered) is calculated and reported per 100 mL (see example below).

Example 2:

Filter 1 volume (mL) = 100 mL	CFU = 0
Filter 2 volume (mL) = 10 mL	CFU = 0
Filter 3 volume (mL) = 1.0 mL	CFU = 0

$$\text{Detection limit} = \frac{100\ \text{mL}}{\text{Largest volume filtered}} = \textit{E. coli}\ \text{CFU/100 mL}$$

$$\frac{100\ \text{mL}}{100\ \text{mL}} = <1\ \textit{E. coli}\ /100\ \text{mL}$$

Example 3:

Filter 1 volume (mL) = 100 mL	CFU = Lab accident, no data available
Filter 2 volume (mL) = 10 mL	CFU = 0
Filter 3 volume (mL) = 1.0 mL	CFU = 0

Calculation of *E. coli*/100 mL:

$$\frac{100\text{ mL}}{10\text{ mL}} = <10\ \textit{E. coli}\ \text{CFU /100 mL}$$

F. If there are no filters with *E. coli* counts in the 20-80 colony range (20-60 for mFC-NA-MUG), sum the *E. coli* counts on all filters, divide by the volume filtered and report as number per 100 mL.

Example 4:

Filter 1 volume (mL) = 50 mL	CFU = 15
Filter 2 volume (mL) = 25 mL	CFU = 6
Filter 3 volume (mL) = 10 mL	CFU = 0

The analytical result is calculated as:

$$(15 + 6 + 0) \times \frac{100}{(50+25+10)} = 25\ \textit{E. coli}\ \text{CFU/100 mL}$$

Example 5:

Filter 1 volume (mL) = 50 mL	CFU = 105
Filter 2 volume (mL) = 25 mL	CFU = 92
Filter 3 volume (mL) = 10 mL	CFU = 85

The analytical result is calculated as:

$$(105 + 92 + 85) \times \frac{100}{(50 + 25 + 10)} = 332\ \textit{E. coli}\ \text{CFU/100 mL}$$

Example 6:

Filter 1 volume (mL) = 100 mL	CFU = 82
Filter 2 volume (mL) = 10 mL	CFU = 18
Filter 3 volume (mL) = 1.0 mL	CFU = 0

The analytical result is calculated as:

$$(82 + 18 + 0) \times \frac{100}{(100 + 10 + 1)} = 90\ \textit{E. coli}\ \text{CFU/100 mL}$$

Example 7:

Filter 1 volume (mL) = 50 mL	CFU = TNTC
Filter 2 volume (mL) = 25 mL	CFU = TNTC
Filter 3 volume (mL) = 10 mL	CFU = 83

The analytical result is calculated as:

$$83 \times \frac{100}{10} = 830 \textit{ E. coli} \text{ CFU/100 mL}$$

4.6.3 Multiple-Tube Data

In addition to the general sample data contained in the bulleted list in Section 4.6 of this manual, laboratories using multiple-tube methods for *E. coli* sample analyses in support of the LT2 Rule should record the data elements specific to these techniques. These data elements are noted in **Table 4-7** for 15-tube most probable number methods and **Table 4-8** for 15-tube multiple-tube fermentation methods. The data elements include the primary measurements needed to calculate the *E. coli* concentration in the sample, as well as all method-required incubation and read times needed to verify that the sample analyses were conducted under analytical control.

Calculations for determining the *E. coli* concentration using multiple tube formats are provided in Section 4.6.3.2.

4.6.3.1 Data Elements for Multiple-Tube Analyses

The recommended data elements to record for 15-tube most probable number methods are provided in **Table 4-7** and **Table 4-8** for 15-tube multiple-tube fermentation methods.

Table 4-7. Recommended Data Elements to Record for 15-Tube MPN Methods (Colilert®)

Primary Measurements (Note: not all dilutions listed below may be used)
Number of positive 10.0 mL tubes: Total coliform positive and UV fluorescence
Number of positive 1.0 mL tubes: Total coliform positive and UV fluorescence
Number of positive 0.1 mL tubes: Total coliform positive and UV fluorescence
Number of positive 0.01 mL tubes: Total coliform positive and UV fluorescence
Number of positive 0.001 mL tubes: Total coliform positive and UV fluorescence
Holding Time / Incubation Time Information
Incubation start: date/time
Incubation end: date/time
Additional incubation start: date/time
Additional incubation end: date/time

Table 4-8. Recommended Data Elements to Record for 15-Tube Fermentation Methods

Primary Measurements (Note: not all dilutions listed below may be used)
Number of positive tubes 10.0 mL
Number of positive tubes 1.0 mL
Number of positive tubes 0.1 mL
Number of positive tubes 0.01 mL
Number of positive tubes 0.001 mL
Number of positive tubes 0.0001 mL
Holding Time / Incubation Time Information
LTB incubation start date/time
LTB 24-hour incubation end date/time
LTB 48-hour incubation read date/time
EC-MUG incubation 24-hour read date/time (from 24-hour LTB)
EC-MUG incubation 24-hour read date/time (from 48-hour LTB)

4.6.3.2 Determination of *E. coli* Concentrations Using Multiple-Tube Methods[1]:

The guidance and examples for determining *E. coli* concentrations using multiple-tube methods are based on the revision of *Standard Methods* 9221C included in the *2001 Supplement to the 20th Edition of Standard Methods*, approved by the Standard Methods Committee in 1999.

Note: *The analytical result can be automatically calculated using the LT2/Stage 2 Data Collectionand Tracking System (DCTS). See Section 4.7 of this manual for additional information.*

A. For each sample volume (e.g., 10, 1, 0.1, and 0.01 mL or additional sample volumes as necessary), determine the number of positive tubes out of five.

B. A dilution refers to the volume of original sample that was inoculated into each series of tubes. Only three of the dilution series will be used to estimate the MPN. The three selected dilutions are called significant dilutions and are selected according to the following criteria. Examples of significant dilution selections are provided in **Table 4-9**, below.

- Choose the highest dilution (the most dilute, with the least amount of sample) giving positive results in all five tubes inoculated and the two succeeding higher (more dilute) dilutions. (**Table 4-9**, Example A).

- When the lowest dilution (least dilute) tested has less than five tubes with positive results, select it and the two next succeeding higher dilutions (**Table 4-9**, Examples B and C).

[1] Adapted from *2001 Supplement to the 20th Edition of Standard Methods* 9221 C: Explanation of Bacterial Density. This supplement is available for download at http://www.techstreet.com/cgi-bin/detail?product_id=923645.

- When a positive result occurs in a dilution higher (more dilute) than the three significant dilutions selected according to the rules above, change the selection to the lowest dilution (least dilute) that has less than five positive results and the next two higher dilutions (more dilute) (**Table 4-9**, Example D).

- When the selection rules above have left unselected any higher dilutions (more dilute) with positive results, add those higher-dilution positive results to the results for the highest selected dilution (**Table 4-9**, Example E).

- When there are not enough higher dilutions tested to select three dilutions, then select the next lower dilution (**Table 4-9**, Example F).

C. MPN values must be adjusted based on the significant dilutions series selected above. Because the MPN/100 mL values in the table are based on the analysis of 10, 1, and 0.1 mL dilutions, per method requirements, the MPN value must be adjusted if these are not the significant dilutions selected. Use the following calculation to adjust the MPN when the 10, 1, and 0.1 mL dilutions are not the significant dilutions selected:

$$\text{Analytical result} = \frac{\text{MPN value}}{\text{\# of mL in middle dilution}} = \textit{E. coli}\ \text{MPN/100 mL}$$

Table 4-9. Examples of Different Combinations of Positive Tubes (Significant Dilution Results Are in *Bold* and Underlined)

Example	Least dilute Most dilute (Lowest) (Highest)					Combination of positives	MPN Index from *Standard Methods*	*E. coli*/100 mL (after adjustment)
	10 mL	1 mL	0.1 mL	0.01 mL	0.001 mL			
A	5	***5***	***1***	***0***	0	5-1-0	33	330
B	***4***	***5***	***1***	0	0	4-5-1	48	48
C	***0***	***0***	***1***	0	0	0-0-1	1.8	1.8
D	5	***4***	***4***	***1***	0	4-4-1	40	400
E	5	***4***	***4***	***0***	***1***	4-4-1	40	400
F	5	5	***5***	***5***	***2***	5-5-2	540	54,000

Example A: The significant dilution series for the 5-1-0 combination of positives includes the 1, 0.1, and 0.01 mL dilutions. Because the 10, 1, and 0.1 mL dilutions were not selected, an adjustment is necessary to account for the dilutions selected:

$$\text{Analytical result} = \frac{33}{0.1} = 330\ \textit{E. coli}\ /\ 100\ \text{mL}$$

Example B: Because the 10, 1, and 0.1 mL dilutions are the significant dilutions, no adjustment is necessary and the result is 48 *E. coli*/100 mL.

Example C: Because the 10, 1, and 0.1 mL dilutions are the significant dilutions, no adjustment is necessary and the result is 1.8 *E. coli*/100 mL.

Examples D and E: The significant dilution series for the 4-4-1 combination of positives includes the 1, 0.1, and 0.01 mL dilutions. Because the 10, 1, and 0.1 mL dilutions were not selected, an adjustment is necessary to account for the dilutions selected:

$$\text{Analytical result} = \frac{40}{0.1} = 400 \textit{ E. coli} / 100 \text{ mL}$$

Example F: The significant dilution series for the 5-5-2 combination of positives includes the 0.1, 0.01 and 0.001 mL dilutions. Because the 10, 1, and 0.1 mL dilutions were not selected, an adjustment is necessary to account for the dilutions selected:

$$\text{Analytical result} = \frac{540}{0.01} = 54{,}000 \textit{ E. coli} / 100 \text{ mL}$$

4.7 Electronic Data Reporting

During the LT2 Rule, laboratories will report *E. coli* data electronically through EPA's LT2/Stage 2 Data Collection and Tracking System (DCTS) to the PWS staff responsible for approving and submitting monitoring results to EPA. The DCTS is a web-based application that allows laboratory users to enter or upload data, then electronically "release" the data to the appropriate PWS staff for review, approval, and submission to EPA and the State. Although ownership of the data resides with the PWS throughout this process, the DCTS increases the ease and efficiency of the data entry and transfer process from one party to another by transferring the ability to access the data from the laboratory to the PWS to EPA and the State, and ensuring that data cannot be viewed or changed by unauthorized parties. A summary of the data entry, review, and transfer process through the DCTS for both *Cryptosporidium* and *E. coli* samples is provided in **Table 3-8**, in Section 3.7.

The data reporting process is summarized below, in Sections 4.7.1 through 4.7.3, and discussed in detail in the *Users' Manual for the LT2/Stage 2 Data Collection and Tracking System (DCTS)*. The DCTS users' manual also provides detailed information on the laboratory registration process. Information on the DCTS and a downloadable users' manual are available at http://www.epa.gov/safewater/lt2/index.html.

4.7.1 Data Entry/Upload

The analyst or another laboratory staff member enters a subset of the data recorded at the bench (Section 4.6 of this manual) into the DCTS either by entering the data using web forms or by uploading data in XML format. This information includes the following:

- Sample ID (optional)
- PWS ID

- Facility ID
- Sample collection date
- Analytical method number
- Method type
- Source water type (provided by PWS on sample collection form)
- *E. coli*/100 mL (see note below)
- Turbidity result (provided by PWS on sample collection form)

Note: The laboratory may enter the final result for the *E. coli* sample or may enter the primary measurements recorded at the bench, and have the DCTS automatically calculate the final *E. coli* concentration. Because this information is specific to method type (membrane filtration, multiple-tube, 51-well, and 97-well), the system provides different entry screens for each method type. By entering *E. coli* data into the system, the laboratory acknowledges that the following QC requirements were met: all holding and incubation times and temperatures, sample condition on receipt, all method-specific QC requirements, and all QA/QC criteria and procedures specified in the Lab Certification Manual.

The laboratory should establish a contact person that is responsible for verifying the quality and accuracy of all sample results in the laboratory, and should review and approve the results before they are submitted to the PWS for review. If inaccuracies or other problems are identified, the official contact discusses the sample information with the analyst or data entry staff and resolves the issues before the data are released to the PWS for review.

If no inaccuracies or other issues are identified, the laboratory's official contact approves the data for "release" to the PWS for review (EPA does not receive the data at this point). When the data are approved by the laboratory, the rights to the data are transferred electronically by the system to the PWS, and the data can no longer be changed by the laboratory.

4.7.2 PWS Data Review

After the laboratory has released *E. coli* data electronically to the PWS using the DCTS, the PWS will review the results. The PWS user cannot edit the data, but if the PWS has an issue with the sample result, such as if the PWS believes that the sample collection point or collection date is incorrect, the PWS can release the results back to the laboratory for issue resolution. In addition to noting the reason in the DCTS for the return of the data to the laboratory, the PWS should contact the laboratory verbally to discuss the issue.

If the PWS determines that the data are accurate, the PWS releases the results to EPA (and the State, if applicable) as "approved" results. If the PWS determines that the data are accurate, but believes that the data are not valid for other reasons, the PWS can release the results as "contested."

4.7.3 EPA/State Review

After the PWS has released the results as approved or contested, they are available to EPA and State users to review through the DCTS. EPA and State users cannot edit the data. EPA or State users approve results where appropriate. Pursuant to 141.702(b)(2), resampling must occur whenever EPA or the State rejects results or indicates agreement with a PWS action to contest a result.

4.8 Data Archiving

The PWS is required to keep all original, hardcopy monitoring results associated with LT2 sample analyses (both initial and second round of monitoring) for 3 years after bin classification for filtered systems or determination of the mean *Cryptosporidium* level for unfiltered systems [40 CFR § 141.722(a)]. Although it is the PWS's responsibility to meet LT2 Rule data storage requirements for compliance monitoring samples, including MS samples, the PWS may contract this work to the laboratory.

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SECTION 5: REFERENCES

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5.2 USEPA. 2005. Method 1622: *Cryptosporidium* in Water by Filtration/IMS/FA. U.S. Environmental Protection Agency, Office of Water, Washington, D.C. EPA-815-R-05-001.

5.3 USEPA. 2005. Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA. U.S. Environmental Protection Agency, Office of Water, Washington, D.C. EPA-815-R-05-002.

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5.9 IDEXX Laboratories, Inc., Description of Colilert®, Colilert-18®, Quanti-Tray®, Quanti-Tray®/2000, and Colisure™ methods may be obtained from: IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, Maine 04092.

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5.12 USEPA. 2002. Method 1604: Total coliforms and *Escherichia coli* (*E. coli*) in Water by Membrane Filtration Using a Simultaneous Detection Technique (MI Medium). U.S. Environmental Protection Agency, Office of Water, Washington, D.C. EPA-821-R-02-024.

5.13 Hach Company, Inc. m-ColiBlue24 Method is available from: Hach Company, P.O. Box 389, Loveland, CO 80539

5.14 Noble, Rachel T., Dorsey, J., Leecaster, M., Mazur, M., McGee, C., Moore, D., Victoria, O., Reid, D., Schiff, K., Vainik P., Weisberg, S. 1999. Southern California Bight 1998 Regional Monitoring Program, Vol I: Summer shoreline microbiology. Southern California Coastal Water Research Project, Westminster, CA.

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Appendix A

Biosafety Guidelines for Laboratories Analyzing Environmental Samples for *Cryptosporidium*

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BIOSAFETY GUIDELINES FOR LABORATORIES ANALYZING ENVIRONMENTAL SAMPLES FOR *CRYPTOSPORIDIUM*

1.0 Introduction

Laboratory-related infections with *Cryptosporidium* can occur if personnel are not properly trained in biosafety techniques. In addition to waterborne, fecal-oral, person to person transmission, and animal to person, circumstantial evidence suggests that airborne transmission of oocysts may occur[1]. Adoption of a biosafety policy by laboratory management that includes commitment to technician safety, training and supervision, as well as, rigid adherence to biosafety guidelines will prevent the occurrence of *Cryptosporidium* infection (cryptosporidiosis) in laboratory personnel.

Symptoms associated with cryptosporidiosis may include: watery diarrhea, abdominal cramps, nausea, low-grade fever, dehydration, weight loss, and loss of appetite. Symptoms may develop within 2 to 10 days after infection. There are no antibiotics or drug treatments that will cure cryptosporidiosis. For additional information : www.cdc.gov/ncidod/dpd/parasites/cryptosporidiosis/default.htm

Biosafety Level 2 (BSL 2) practices and facilities are recommended for activities with infective stages of *Cryptosporidium*. A BSL 2 facility is appropriate for agents known to cause disease in humans.

Biosafety Level 2 practices and facilities include the following requirements:

- Laboratory personnel have specific training in handling pathogenic agents and are directed by competent scientists
- Access to the laboratory is limited when work is being conducted
- Certain procedures in which infectious aerosols or splashes may be created are conducted in biological safety II cabinets

2.0 Scope and Application

The biosafety guidelines described in this document are adapted from *Laboratory Safety: Principles and Practices, Second Edition* (Reference 8.1) and *Biosafety in Microbiological and Biomedical Laboratories* (BMBL) *Fourth Edition* (Reference 8.2). A readily available laboratory-specific biosafety manual may be developed, and maintained, to address the safety, handling, and laboratory practices described below. The manual can be distributed to all employees and available at all times. It can be reviewed annually, or as recommended, by the laboratory safety officer. Personnel may read and sign off on the document on a regular basis, as determined by the laboratory safety officer.

3.0 General Safety Practices

3.1 Basic Laboratory Safety Recommendations

3.1.1 Closed-toed shoes worn in the laboratory.

3.1.2 All work surfaces and floors cleaned regularly and free of clutter.

3.1.3 All emergency numbers posted in the laboratory.

---

[1]Hojlyng, N., Holten-Andersen, W., and S. Jepsen. 1987. Cryptosporidiosis: a case of airborne transmission. Lancet. 2:271-272.

Blagburn, B.L., and W.L. Current. 1983. Accidental infection of a researcher with human *Cryptosporidium*. J. Infect. Dis. 142:772-773.

3.1.4 All employees trained in the use and location of all safety/emergency equipment in each work area.

3.1.5 Biological safety II cabinets tested and certified annually.

3.1.6 All laboratory personnel trained in the proper procedures to clean up biological spills.

4.0 Recommended Microbiological Practices

4.1 Access to the laboratory is limited or restricted at the discretion of the laboratory director or laboratory safety officer when experiments are in progress.

4.2 Persons wash their hands after they handle viable materials and animals, after removing gloves, and before leaving the laboratory.

4.3 Eating, drinking, smoking, handling contact lenses, and applying cosmetics are not permitted in the work areas. Persons who wear contact lenses in laboratories should also wear goggles or a face shield. Food is stored outside the work area in cabinets or refrigerators designated for this purpose only.

4.4 Mouth pipetting recognized as poor practice.

4.5 All procedures are performed carefully to minimize the creation of splashes or aerosols. Ensure that lids are used during all centrifugation and vortexing. Any procedures with open containers are performed inside a BSL 2 hood.

4.6 Work surfaces are decontaminated before and after each use and after any spill of viable material.

4.7 All cultures, stocks, and other regulated wastes are decontaminated before disposal by an approved decontamination method such as autoclaving.

4.7.1 Materials to be decontaminated outside of the immediate laboratory are to be placed in a durable, leakproof container and closed for transport from the laboratory.

4.7.2 Materials to be decontaminated off-site from the laboratory are packaged in accordance with applicable local, state, and federal regulations before removal from the facility.

5.0 Recommended Special Practices when Processing *Cryptosporidium* Samples

5.1 In general, persons who are at increased risk of acquiring infection or for whom infection may be unusually hazardous are not allowed in the laboratory or animal rooms. For example, persons who are immunocompromised may be at risk of acquiring infections.

5.2 The laboratory safety officer has the final responsibility for assessing each circumstance and determining who may enter or work in the laboratory. The laboratory safety officer will report to the laboratory director on a regular basis on the status of safety in the laboratory, conduct training and maintain outside professional contacts to exchange safety information relevant to laboratory operations.

5.3 The laboratory director or laboratory safety officer, establishes policies and procedures, whereby only persons who have been advised of the potential hazard and meet specific entry requirements are allowed to enter the laboratory or animal rooms.

5.4 When the infectious agent(s) in use in the laboratory require special provisions or special training for entry, a hazard warning sign incorporating the universal biohazard symbol is posted on the access door to the laboratory work area. The hazard warning sign identifies the infectious agent,

lists the name and telephone number of the laboratory director or other responsible person(s), and indicates the special requirement(s) for entering the laboratory.

5.5 Laboratory personnel receive appropriate training on the potential hazards associated with the work involved, the necessary precautions to prevent exposures, and the exposure evaluation procedures. Personnel receive annual updates, or additional training as necessary for procedural or policy changes.

5.6 Materials containing *Cryptosporidium* are placed in a container that prevents leakage during collection, handling, processing, storage, transport, or shipping.

5.7 Laboratory equipment is decontaminated with an appropriate disinfectant before and after the equipment is used, and especially after overt spills, splashes, or other contamination by infectious materials. Contaminated equipment is decontaminated according to any local, state, or federal regulations before it is sent for repair or maintenance or packaged for transport in accordance with applicable local, state, or federal regulations before removal from the facility.

5.8 Spills and accidents which result in overt exposures to infectious materials are immediately reported to the laboratory director or laboratory safety officer. Medical evaluation, surveillance, and treatment are provided as appropriate and written records are maintained.

6.0 Recommended Safety Equipment (Primary Barriers)

6.1 Properly maintained biological safety II cabinets, and other appropriate personal protective equipment or physical containment devices are used whenever:

6.1.1 Procedures with potential for creating infectious aerosols or splashes are conducted. These may include centrifuging, vortexing, grinding, blending, vigorous shaking or mixing, sonic disruption, or opening containers of infectious materials whose internal pressures may be different from ambient pressures.

6.1.2 High concentrations or large volumes of infectious agents are used. Such materials may be centrifuged in the open laboratory if sealed rotor heads or centrifuge safety cups are used, and if these rotors or safety cups are opened only in a biological safety cabinet.

6.2 Face protection (goggles, mask, faceshield, or other splatter guards) is used for anticipated splashes or sprays of infectious or other hazardous materials to the face, when the microorganisms are manipulated outside the biological safety cabinet.

6.3 Protective laboratory coats, gowns, smocks, or uniforms designated for lab use are worn while in the laboratory. This protective clothing is removed and left in the laboratory before leaving for non-laboratory areas (e.g., cafeteria, library, administrative offices). All protective clothing is either disposed of in the laboratory or laundered by the institution; and is not taken home by personnel.

6.4 Gloves are worn when handling infected animals and when hands may contact infectious materials, contaminated surfaces, or equipment.

6.4.1 Wearing two pairs of gloves may be appropriate; if a spill or splatter occurs, the hand will be protected after the contaminated glove is removed.

6.4.2 Gloves are disposed of when contaminated, removed when work with infectious materials is complete, and are not worn outside the laboratory.

6.4.3 Disposable gloves are not washed or reused.

7.0 Recommendations for Laboratory Facilities (Secondary Barriers)

7.1 Each laboratory contains a sink for handwashing.

7.2 The laboratory is designed so that it can be easily cleaned. Rugs in laboratories are not appropriate, because proper decontamination following a spill is extremely difficult to achieve.

7.3 Bench tops are impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat.

7.4 Laboratory furniture is sturdy, and spaces between benches, cabinets, and equipment are accessible for cleaning.

7.5 If the laboratory has windows that open, they are fitted with fly screens.

7.6 A method for decontamination of infectious or regulated laboratory wastes is available (e.g., autoclave, chemical disinfection, incinerator, or other approved decontamination system).

7.7 An eyewash facility is readily available.

7.8 The laboratory facilities are clean, temperature and humidity controlled, and have adequate lighting at bench tops.

8.0 References

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8.2 US Department of Health and Human Services. *Biosafety in Microbiological and Biomedical Laboratories*. 1999. Fourth Edition. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, and National Institutes of Health, US Government Printing Office, Washington, D.C., publisher.

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Appendix B

Method 1622: *Cryptosporidium* in Water by Filtration/IMS/FA

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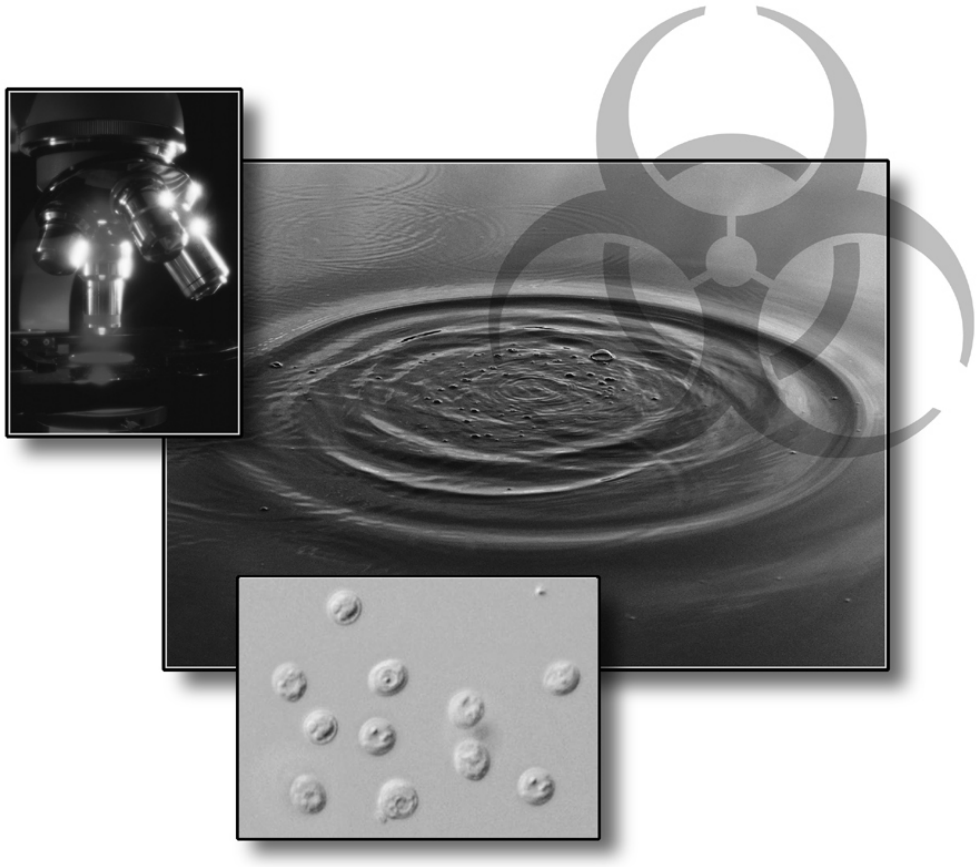
EPA
United States
Environmental Protection
Agency

Method 1622: *Cryptosporidium* in Water by Filtration/IMS/FA

December 2005



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December 2005

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Cryptosporidium cover photo courtesy of the U.S. Centers for Disease Control

Disclaimer

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

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Introduction

To support future regulation of protozoa in drinking water, the Safe Drinking Water Act Amendments of 1996 require the U.S. Environmental Protection Agency (EPA) to evaluate the risk to public health posed by drinking water contaminants, including waterborne parasites, such as *Cryptosporidium*. To implement these requirements, EPA must assess *Cryptosporidium* occurrence in raw surface waters used as source waters for drinking water treatment plants. EPA Method 1622 was developed to support this assessment.

Method Development and Validation

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

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

Changes in the April 2001 Version of the Method

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

- Nationwide approval of modified versions of the methods using the following components:
  (a) Whatman Nuclepore CrypTest™ filter
  (b) IDEXX Filta-Max® filter
  (c) Waterborne Aqua-Glo™ G/C Direct FL antibody stain
  (d) Waterborne Crypt-a-Glo™ and Giardi-a-Glo™ antibody stains
- Clarified sample acceptance criteria
- Modified capsule filter elution procedure
- Modified concentrate aspiration procedure
- Modified IMS acid dissociation procedure
- Updated QC acceptance criteria for IPR and OPR tests
- Addition of a troubleshooting section for QC failures
- Modified holding times
- Inclusion of flow cytometry–sorted spiking suspensions

Changes in the June 2003 Version of the Method

The method was revised again in June 2003 to support proposal of EPA's Long Term 2 Enhanced Surface Water Treatment Rule. Changes incorporated into the December 2002 version include:

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

Changes in the December 2005 Version of the Method

The method was revised again in 2005 to support promulgation of EPA's Long Term 2 Enhanced Surface Water Treatment Rule. Changes incorporated into the June 2003 version include:

- Nationwide approval of the use of portable continuous-flow centrifugation as a modified version of the method. The product met all method acceptance criteria for *Cryptosporidium* using 50-L source water samples.
- Addition of BTF EasyStain™ monoclonal antibody stain as an acceptable reagent for staining in Methods 1622. The product was validated through an interlaboratory validation study using the Pall Envirochek™ HV filter.
- Clarification of the analyst verification procedure
- Clarification of sample condition criteria upon receipt

Performance-Based Method Concept and Modifications Approved for Nationwide Use

EPA Method 1622 is a performance-based method applicable to the determination of *Cryptosporidium* in aqueous matrices. EPA Method 1622 requires filtration, immunomagnetic separation of the oocysts from the material captured, and enumeration of the target organisms based on the results of immunofluorescence assay, 4',6-diamidino-2-phenylindole (DAPI) staining results, and differential interference contrast microscopy.

The interlaboratory validation of EPA Method 1622 conducted by EPA used the Pall Gelman capsule filtration procedure, Dynal immunomagnetic separation (IMS) procedure, and Meridian sample staining procedure described in this document. Alternate procedures are allowed, provided that required quality control tests are performed and all quality control acceptance criteria in this method are met.

Since the interlaboratory validation of EPA Method 1622, interlaboratory validation studies have been performed to demonstrate the equivalency of modified versions of the method using the following components:

- Whatman Nuclepore CryptTest™ filter (no longer available)
- IDEXX Filta-Max® filter
- Pall Gelman Envirochek™ HV filter
- Portable Continuous-Flow Centrifugation (PCFC)
- Waterborne Aqua-Glo™ G/C Direct FL antibody stain
- Waterborne Crypt-a-Glo™ and Giardi-a-Glo™ antibody stains
- BTF EasyStain™ antibody stain
- BTF EasySeed™ irradiated oocysts for use in routine QC samples

The validation studies for the modified versions of the method met EPA's performance-based measurement system Tier 2 validation for nationwide use (see Section 9.1.2 for details), and have been accepted by EPA as equivalent in performance to the original version of the method validated by EPA. The equipment and reagents used in these modified versions of the method are noted in Sections 6 and 7 of the method.

Because this is a performance-based method, other alternative components not listed in the method may be available for evaluation and use by the laboratory. Confirming the acceptable performance of a modified version of the method using alternate components in a single laboratory does not require that an interlaboratory validation study be conducted. However, method modifications validated only in a single laboratory have not undergone sufficient testing to merit inclusion in the method. Only those modified versions of the method that have been demonstrated as equivalent at multiple laboratories on multiple water sources through a Tier 2 interlaboratory study will be cited in the method.

Table of Contents

Method 1622: *Cryptosporidium* in Water by Filtration/IMS/FA

1.0 Scope and Application

1.1 This method is for the detection of *Cryptosporidium* (CAS Registry number 137259-50-8) and in water by concentration, immunomagnetic separation (IMS), and immunofluorescence assay (FA) microscopy. *Cryptosporidium* may be verified using 4',6-diamidino-2-phenylindole (DAPI) staining and differential interference contrast (DIC) microscopy. The method has been validated in surface water, but may be used in other waters, provided the laboratory demonstrates that the method's performance acceptance criteria are met.

1.2 This method is designed to meet the survey and monitoring requirements of the U.S. Environmental Protection Agency (EPA). It is based on laboratory testing of recommendations by a panel of experts convened by EPA. The panel was charged with recommending an improved protocol for recovery and detection of protozoa that could be tested and implemented with minimal additional research.

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

1.4 This method is for use only by persons experienced in the determination of *Cryptosporidium* by filtration, IMS, and FA. Experienced persons are defined in Section 22.2 as analysts or principal analysts. Laboratories unfamiliar with analyses of environmental samples by the techniques in this method should gain experience using water filtration techniques, IMS, fluorescent antibody staining with monoclonal antibodies, and microscopic examination of biological particulates using bright-field and DIC microscopy.

1.5 Any modification of the method beyond those expressly permitted is subject to the application and approval of alternative test procedures under 40 *CFR* Part 141.27.

2.0 Summary of Method

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

2.2 Elution and separation

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

2.2.2 The oocysts are magnetized by attachment of magnetic beads conjugated to anti-*Cryptosporidium* antibodies. The magnetized oocysts are separated from the extraneous materials using a magnet, and the extraneous materials are discarded. The magnetic bead complex is then detached from the oocysts.

2.3 Enumeration

2.3.1 The oocysts are stained on well slides with fluorescently labeled monoclonal antibodies and 4',6-diamidino-2-phenylindole (DAPI). The stained sample is examined using fluorescence and differential interference contrast (DIC) microscopy.

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

2.3.3 Quantitative analysis is performed by counting the total number of objects on the slide confirmed as oocysts.

2.4 Quality is assured through reproducible calibration and testing of the filtration, immunomagnetic separation (IMS), staining, and microscopy systems. Detailed information on these tests is provided in Section 9.0.

3.0 Definitions

3.1 *Cryptosporidium* is a genus of protozoan parasites potentially found in water and other media. The recent taxonomy of the genus *Cryptosporidium* includes the following species and their potential hosts: *C. hominis* (humans; formerly *C. parvum* genotype I; Reference 20.1); *C. parvum* (bovine and other mammals including humans; formerly genotype II;); *C. baileyi* and *C. meleagridis* (birds); *C. muris* (rodents); *C. canis* (dogs); *C. felis* (cats); *C. serpentis* (reptiles); and *C. nasorum* (fish). *Cryptosporidium* oocysts are defined in this method as objects exhibiting brilliant apple green fluorescence under UV light (FA-positive), typical size (4 to 6 µm) and shape (round to oval), and no atypical characteristics by FA, DAPI fluorescence, or DIC microscopy. Examination and characterization using fluorescence (FITC and DAPI stain) and DIC microscopy are required for exclusion of atypical organisms (e.g., those possessing spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.).

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

4.0 Contamination, Interferences, and Organism Degradation

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

4.2 Organisms and debris that autofluoresce or demonstrate non-specific immunofluorescence, such as algal and yeast cells, when examined by epifluorescent microscopy, may interfere with the detection of oocysts and contribute to false positives by immunofluorescence assay (FA) (Reference 20.3).

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

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

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

5.0 Safety

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

5.2 The toxicity or carcinogenicity of each compound or reagent used in this method has not been precisely determined; however, each chemical compound should be treated as a potential health hazard. Exposure to these compounds should be reduced to the lowest possible level. The laboratory is responsible for maintaining current knowledge of Occupational Safety and Health Administration regulations regarding the safe handling of the chemicals specified in this method. A reference file of material safety data sheets should be made available to all personnel involved in these analyses. Additional information on laboratory safety can be found in References 20.4 through 20.7.

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

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

5.5 Centers for Disease Control (CDC) regulations (42 CFR 72) prohibit interstate shipment of more than 4 L of solution known to contain infectious materials (see http://www.cdc.gov/od/ohs/biosfty/shipregs.htm for details). State regulations may contain similar regulations for intrastate commerce. Unless the sample is known or suspected to contain *Cryptosporidium* or other infectious agents (e.g., during an outbreak), samples should be shipped as noninfectious and should not be marked as infectious. If a sample is known or suspected to be infectious, and the sample must be shipped to a laboratory by a transportation means affected by CDC or state regulations, the sample should be shipped in accordance with these regulations.

6.0 Equipment and Supplies

NOTE: Brand names, suppliers, and part numbers are for illustrative purposes only. No endorsement is implied. Equivalent performance may be achieved using apparatus and materials other than those specified here, but demonstration of equivalent performance that meets the requirements of this method is the responsibility of the laboratory.

6.1 Sample collection equipment for shipment of bulk water samples for laboratory filtration.

Collapsible LDPE cubitainer for collection of 10-L bulk sample(s)—Cole Parmer cat. no. U-06100-30 or equivalent. Fill completely to ensure collection of a full 10-L sample. Discard after one use.

6.2 Equipment for sample filtration. Four options have been demonstrated to be acceptable for use with Method 1622. Other options may be used if their acceptability is demonstrated according to the procedures outlined in Section 9.1.2.

6.2.1 Cubitainer spigot to facilitate laboratory filtration of sample (for use with any filtration option)—Cole Parmer cat. no. U-06061-01, or equivalent.

6.2.2 Original Envirochek™ sampling capsule or Envirochek™ HV sampling capsule equipment requirements (for use with the procedure described in Section 12.2). The versions of the method using these filters were validated using 10-L and 50-L sample volumes, respectively. Alternate sample volumes may be used, provided the laboratory demonstrates acceptable performance on initial and ongoing spiked reagent water and source water samples (Section 9.1.2).

6.2.2.1 Sampling capsule

6.2.2.1.1 Envirochek™, Pall Corporation, Ann Arbor, MI, part no. 12110 (individual filter) and or part no.12107 (box of 25 filters) (www.pall.com or (800) 521-1520 ext. 2)

6.2.2.1.2 Envirochek™ HV, Pall Corporation, Ann Arbor, MI, part no. 12099 (individual filter) or part no.12098 (box of 25 filters) (www.pall.com or (800) 521-1520 ext. 2)

6.2.2.2 Laboratory shaker with arms for agitation of sampling capsules

6.2.2.2.1 Laboratory shaker—Lab-Line model 3589 (available through VWR Scientific cat. no. 57039-055), Pall Corporation part no. 4821, Fisher cat. no. 14260-11, or equivalent

6.2.2.2.2 Side arms for laboratory shaker—Lab-Line Model 3587-4 (available through VWR Scientific cat. no. 57039-045), Fisher cat. no. 14260-13, or equivalent

6.2.3 Filta-Max® foam filter equipment requirements (for use with the procedure described in Section 12.3). The version of the method using this filter was validated using 50-L sample volumes; alternate sample volumes may be used, provided the laboratory demonstrates acceptable performance on initial and ongoing spiked reagent water and matrix samples (Section 9.1.2).

6.2.3.1 Foam filter—Filta-Max®, IDEXX, Westbrook, ME. Filter module cat. no. FMC 10603

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

6.2.3.2 Filter processing equipment—Filta-Max® starter kit, IDEXX, Westbrook, ME, cat. no. FMC 11002. Starter kit includes manual wash station with clamp set (FMC 10101 or 10106) including plunger head (FMC 12001), tubing set (FMC 10307), vacuum set (FMC 10401), MKII filter housing with hose-tail fittings (FMC 10504) and green housing tools (FMC 10506). In addition, processing requires magnetic stirrer (FMC 10901) and filter membranes, 100 pk, (FMC 10800).

6.2.4 Portable Continuous-Flow Centrifuge (PCFC) requirements (for use with procedures described in Section 12.4). The version of the method using this technique was validated for *Cryptosporidium* in 50-L sample volumes; alternate sample volumes may be used,

provided the laboratory demonstrates acceptable performance on initial and ongoing spiked reagent water and matrix samples (Section 9.1.2). The technique is based on technology from Haemonetics Corporation, Braintree, MA.

6.3 Ancillary sampling equipment

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

6.3.2 Flow control valve—0.5 gpm (0.03 L/s), Bertram Controls, Plast-O-Matic cat. no. FC050B½-PV, or equivalent; or 0.4- to 4-Lpm flow meter with valve, Alamo Water Treatment, San Antonio, TX, cat. no. R5310, or equivalent

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

6.3.4 Flow meter—SaMeCo cold water totalizer, E. Clark and Associates, Northboro, MA, product no. WFU 10.110; Omega flow meter, Stamford, CT, model FTB4105; or equivalent. Alternatively, use a graduated carboy(s) (See Section 6.18)

6.4 Equipment for spiking samples in the laboratory

6.4.1 Collapsible 10-L LDPE cubitainer with cubitainer spigot—Cole Parmer cat. no. U-06100-30 or equivalent and Cole Parmer cat. no. U-06061-01, or equivalent. Discard after one use to eliminate possible contamination. Alternatively, use clean, 10-L carboy with bottom delivery port (½"), Cole-Palmer cat. no. 06080-42, or equivalent; calibrate to 10.0 L and mark level with waterproof marker

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

6.4.3 Stir plate—Fisher cat. no. 11-510-49S, S50461HP, or equivalent

6.4.4 Hemacytometer—Neubauer type, Hausser Scientific, Horsham, PA, product no. 3200 or 1475, or equivalent

6.4.5 Hemacytometer coverslip—Hausser Scientific, product no. 5000 (for hemacytometer cat. no. 3200) or 1461 (for hemacytometer cat. no 1475), or equivalent

6.4.6 Lens paper without silicone—Fisher cat. no. 11-995, or equivalent

6.4.7 Polystyrene or polypropylene conical tubes with screw caps—15- and 50-mL

6.4.8 Equipment required for enumeration of spiking suspensions using membrane filters

6.4.8.1 Glass microanalysis filter holder—25-mm-diameter, with fritted glass support, Fisher cat. no. 09-753E, or equivalent. Replace stopper with size 8, one-hole rubber stopper, Fisher Cat. No. 14-135M, or equivalent.

6.4.8.2 Three-port vacuum filtration manifold and vacuum source—Fisher Cat. No. 09-753-39A, or equivalent

6.4.8.3 Cellulose acetate support membrane—1.2-µm-pore-size, 25-mm-diameter, Fisher cat. no. A12SP02500, or equivalent

6.4.8.4 Polycarbonate track-etch hydrophilic membrane filter—1-µm-pore-size, 25-mm-diameter, Fisher cat. no. K10CP02500, or equivalent

6.4.8.5 100 × 15 mm polystyrene petri dishes (bottoms only)

6.4.8.6 60 × 15 mm polystyrene petri dishes

6.4.8.7 Glass microscope slides—1 in. × 3 in or 2 in. × 3 in.

6.4.8.8 Coverslips—25 mm^2

6.5 Immunomagnetic separation (IMS) apparatus

6.5.1 Sample mixer—Dynal Inc., Lake Success, NY, cat. no. 947.01, or equivalent

6.5.2 Magnetic particle concentrator for 10-mL test tubes—Dynal MPC®-1 , cat. no. 120.01 or MPC®-6, cat. No 120.02, or equivalent

6.5.3 Magnetic particle concentrator for microcentrifuge tubes—Dynal MPC®-M, cat. no. 120.09 (no longer available); Dynal MPC®-S, cat. no. 120.20, or equivalent

6.5.4 Flat-sided sample tubes—16 × 125 mm Leighton-type tubes with 60 × 10 mm flat-sided magnetic capture area, Dynal L10, cat. no. 740.03, or equivalent

6.6 Powder-free latex gloves—Fisher cat no. 113945B, or equivalent

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

6.8 Centrifuges

6.8.1 Centrifuge capable of accepting 15- to 250-mL conical centrifuge tubes and achieving 1500 × G—International Equipment Company, Needham Heights, MA, Centrifuge Size 2, Model K with swinging bucket, or equivalent

6.8.2 Centrifuge tubes—Conical, graduated, 1.5-, 50-, and 250-mL

6.9 Microscope

6.9.1 Epifluorescence/differential interference contrast (DIC) with stage and ocular micrometers and 20X (N.A.=0.4) to 100X (N.A.=1.3) objectives—Zeiss™ Axioskop, Olympus™ BH, or equivalent. Hoffman Modulation Contrast optics may be equivalent.

6.9.2 Excitation/band-pass filters for immunofluorescence assay (FA)—Zeiss™ 487909 or equivalent, including, 450- to 490-nm exciter filter, 510-nm dicroic beam-splitting mirror, and 515- to 520-nm barrier or suppression filter

6.9.3 Excitation/band-pass filters for DAPI—Filters cited below (Chroma Technology, Brattleboro, VT), or equivalent

Microscope model	Fluoro-chrome	Excitation filter (nm)	Dichroic beam- splitting mirror (nm)	Barrier or suppression filter (nm)	Chroma catalog number
Zeiss™ - Axioskop	DAPI (UV)	340-380	400	420	CZ902
Zeiss™ -IM35	DAPI (UV)	340-380	400	420	CZ702
Olympus™ BH	DAPI (UV)	340-380	400	420	11000
	Filter holder				91002
Olympus™ BX	DAPI (UV)	340-380	400	420	11000
	Filter holder				91008
Olympus™ IMT2	DAPI (UV)	340-380	400	420	11000
	Filter holder				91003

6.10 Ancillary equipment for microscopy

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

6.10.2 Glass coverslips—22 × 50 mm

6.10.3 Nonfluorescing immersion oil—Type FF, Cargille cat. no. 16212, or equivalent

6.10.4 Micropipette, adjustable: 0- to 10-µL with 0- to 10-µL tips
10- to 100-µL, with 10- to 200-µL tips
100- to 1000-µL with 100- to 1000-µL tips

6.10.5 Forceps—Splinter, fine tip

6.10.6 Forceps—Blunt-end

6.10.7 Desiccant—Drierite™ Absorbent, Fisher cat. no. 07-577-1A, or equivalent

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

6.11 Pipettes—Glass or plastic

6.11.1 5-, 10-, and 25-mL

6.11.2 Pasteur, disposable

6.12 Balances

6.12.1 Analytical—Capable of weighing 0.1 mg

6.12.2 Top loading—Capable of weighing 10 mg

6.13 pH meter

6.14 Incubator—Fisher Scientific Isotemp™, or equivalent

6.15 Vortex mixer—Fisons Whirlmixer, or equivalent

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

6.17 Miscellaneous labware and supplies

6.17.1 Test tubes and rack

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

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

6.17.4 Lint-free tissues

6.18 10- to 15-L graduated container—Fisher cat. no. 02-961-50B, or equivalent; calibrate to 9.0, 9.5, 10.0, 10.5, and 11.0 L and mark levels with waterproof marker

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

7.0 Reagents and Standards

7.1 Reagents for adjusting pH

7.1.1 Sodium hydroxide (NaOH)—ACS reagent grade, 6.0 N and 1.0 N in reagent water

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

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

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

7.3 Reagent water—Water in which oocysts and interfering materials and substances, including magnetic minerals, are not detected by this method. See Reference 20.8 (Section 9020) for reagent water requirements.

7.4 Reagents for eluting filters

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

7.4.1 Reagents for eluting Envirochek™ and Envirochek™ HV sampling capsules (Section 6.2.2)

7.4.1.1 Laureth-12—PPG Industries, Gurnee, IL, cat. no. 06194, or equivalent. Store Laureth-12 as a 10% solution in reagent water. Weigh 10 g of Laureth-12 and dissolve using a microwave or hot plate in 90 mL of reagent water. Dispense 10-mL aliquots into sterile vials and store at room temperature for up to 2 months, or in the freezer for up to a year.

7.4.1.2 1 M Tris, pH 7.4—Dissolve 121.1 g Tris (Fisher cat. no. BP152) in 700 mL of reagent water and adjust pH to 7.4 with 1 N HCl or NaOH. Dilute to a final 1000 mL with reagent water and adjust the final pH. Filter-sterilize through a 0.2-µm membrane into a sterile plastic container and store at room temperature. Alternatively, use prepared TRIS, Sigma T6066 or equivalent.

7.4.1.3 0.5 M EDTA, 2 Na, pH 8.0—Dissolve 186.1 g ethylenediamine tetraacetic acid, disodium salt dihydrate (Fisher cat. no. S311) in 800 mL of reagent water and adjust pH to 8.0 with 6.0 N HCl or NaOH. Dilute to a final volume of 1000 mL with reagent water and adjust to pH 8.0 with 1.0 N HCl or NaOH. Alternatively, use prepared EDTA, Sigma E5134 or equivalent.

7.4.1.4 Antifoam A—Sigma Chemical Co. cat. no. A5758, or equivalent

7.4.1.5 Preparation of elution buffer solution—Add the contents of a pre-prepared Laureth-12 vial (Section 7.4.1.1) to a 1000-mL graduated cylinder. Rinse the vial several times to ensure the transfer of the detergent to the cylinder. Add 10 mL of Tris solution (Section 7.4.1.2), 2 mL of EDTA solution (Section 7.4.1.3), and 150 µL Antifoam A (Section 7.4.1.4). Dilute to 1000 mL with reagent water.

7.4.2 Reagents for eluting Filta-Max® foam filters (Section 6.2.3)

7.4.2.1 Phosphate buffered saline (PBS), pH 7.4—Sigma Chemical Co. cat. no. P-3813, or equivalent. Alternately, prepare PBS by adding the following to 1 L of reagent water: 8 g NaCl; 0.2 g KCl; 1.15 g Na_2HPO_4, anhydrous; and 0.2 g KH_2PO_4.

7.4.2.2 Tween® 20 —Sigma Chemical Co. cat. no. P-7949, or equivalent

7.4.2.3 High-vacuum grease—BDH/Merck. cat. no. 636082B, or equivalent

7.4.2.4 Preparation of PBST elution buffer. Add 100 µL of Tween® 20 to prepared PBS (Section 7.4.2.1). Alternatively, add the contents of one packet of PBS to 1.0 L of reagent water. Dissolve by stirring for 30 minutes. Add 100 µL of Tween® 20 . Mix by stirring for 5 minutes.

7.4.3 Reagents for Portable Continuous-Flow Centrifuge (Section 6.2.4)

7.4.3.1 Sodium dodecyl sulfate—Sigma Chemical Co. cat. no. 71730 or equivalent

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

7.4.3.3 Antifoam A—Sigma Chemical Co. cat. no. A5758, or equivalent

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

7.5 Reagents for immunomagnetic separation (IMS)—Dynabeads® anti-Cryptosporidium beads, Dynal cat. nos. 730.01/730.11, or equivalent

7.6 Direct antibody labeling reagents for detection of oocysts. Store reagents between 1°C and 10°C and return promptly to this temperature after each use. Do not allow any of the reagents to freeze. The reagents should be protected from exposure to light. Diluted, unused working reagents should be discarded after 48 hours. Discard reagents after the expiration date is reached. The labeling reagents in Sections 7.6.1-7.6.3 have been approved for use with this method.

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

7.6.2 Aqua-Glo™ G/C Direct FL, Waterborne cat. no. A100FLR, New Orleans, LA, or equivalent

7.6.3 Crypt-a-Glo™, Waterborne cat. no. A400FLR, New Orleans, LA, or equivalent

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

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NOTE: If a laboratory will use multiple types of labeling reagents, the laboratory must demonstrate acceptable performance through an initial precision and recovery test (Section 9.4) for each type, and must perform positive and negative staining controls for each batch of slides stained using each product. However, the laboratory is not required to analyze additional ongoing precision and recovery samples or method blank samples for each type. The performance of each labeling reagent used also should be monitored in each source water type.

7.6.5 Diluent for labeling reagents—Phosphate buffered saline (PBS) (Section 7.4.2).

7.7 4',6-diamidino-2-phenylindole (DAPI) stain—Sigma Chemical Co. cat. no. D9542, or equivalent

7.7.1 Stock solution—Dissolve 2 mg/mL DAPI in absolute methanol. Prepare volume consistent with minimum use. Store between 1°C and 10°C in the dark. Do not allow to freeze. Discard unused solution when positive staining control fails or after specified time determined by laboratory.

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

7.8 Mounting medium

7.8.1 DABCO/glycerol mounting medium (2%)—Dissolve 2 g of DABCO (Sigma Chemical Co. cat no. D-2522, or equivalent) in 95 mL of warm glycerol/PBS (60% glycerol, 40% PBS). After the DABCO has dissolved completely, adjust the solution volume to 100 mL by adding an appropriate volume of glycerol/PBS solution. Alternately, dissolve the DABCO in 40 mL of PBS, then add azide (1 mL of 100X, or 10% solution), then 60 mL of glycerol.

7.8.2 Mounting medium supplied with MeriFluor® *Cryptosporidium/Giardia*, Meridian Diagnostics cat. no. 250050, or equivalent (Section 7.6.1)

7.8.3 Mounting medium supplied with Aqua-Glo™ G/C Direct FL kit, Waterborne cat. no. A100FLR, cat. no. M101, or equivalent (Section 7.6.2)

7.8.4 Mounting medium supplied with EasyStain™C&G, BTF Pty Limited or equivalent (Section 7.6.4)

7.8.5 Elvanol or equivalent permanent, non-fade archiving mounting medium

7.9 Clear fingernail polish or clear fixative, PGC Scientifics, Gaithersburg, MD, cat. no. 60-4890-00, or equivalent

7.10 Oocyst suspensions for spiking

7.10.1 Enumerated spiking suspensions prepared by flow cytometer—not formalin fixed.

7.10.1.1 Live, flow cytometer–sorted oocysts —Wisconsin State Laboratory of Hygiene Flow Cytometry Unit ([608] 224-6260), or equivalent

7.10.1.2 Irradiated, flow cytometer–sorted oocysts —flow cytometer–sorted oocysts —BTF EasySeed™ (contact@btfbio.com), or equivalent

7.10.2 Materials for manual enumeration of spiking suspensions

7.10.2.1 Purified *Cryptosporidium* oocyst stock suspension for manual enumeration—not formalin-fixed: Sterling Parasitology Laboratory, University of Arizona, Tucson, or equivalent

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

7.10.3 Storage procedure—Store oocyst suspensions between 1°C and 10°C, until ready to use; do not allow to freeze

7.11 Additional reagents for enumeration of spiking suspensions using membrane filtration (Section 11.3.6)—Sigmacote® Sigma Company Product No. SL-2, or equivalent

8.0 Sample Collection and Storage

8.1 Sample collection, shipment, and receipt

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

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

NOTE: *See transportation precautions in Section 5.5.*

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

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

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

8.1.2.4 Public water systems shipping samples to off-site laboratories for analysis should include in the shipping container a means for monitoring the temperature of the sample during shipping to verify that the sample did not freeze or exceed 20°C. Suggested approaches for monitoring sample temperature during shipping are discussed in Section 8.1.4.

8.1.3 Sample receipt. Upon receipt, the laboratory must record the sample temperature.

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

8.1.4 Suggestions on measuring sample temperature. Given the importance of maintaining sample temperatures for *Cryptosporidium* determination, laboratories performing analyses using this method must establish acceptance criteria for receipt of samples transported to their laboratory. Several options are available to measure sample temperature upon receipt at the laboratory and, in some cases, during shipment:

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

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

8.1.4.3 **iButton.** Measures the sample temperature during shipment and upon receipt. An iButton is a small, waterproof device that contains a computer chip that can be programmed to record temperature at different time intervals. The information is then downloaded from the iButton onto a computer. The iButton should be placed in a temperature sample, rather than placed loose in the cooler, or attached to the sample container. This option is appropriate for use with both filtered and bulk samples. Information on Thermocron® iButtons is available from http://www.ibutton.com/. Distributors include http://www.pointsix.com/, http://www.rdsdistributing.com, and http://www.scigiene.com/.

8.1.4.4 **Stick-on temperature strips.** Another option is for the laboratory to apply a stick-on temperature strip to the outside of the sample container upon receipt at the laboratory. This option does not measure temperature as precisely as the other options, but provides an indication of sample temperature to verify that the sample temperature is acceptable. This option is appropriate for use with both filtered and bulk samples. *Example product:* Cole Parmer cat. no. U-90316-00.

8.1.4.5 **Infrared thermometers.** A final option is to measure the temperature of the surface of the sample container or filter using an infrared thermometer. The thermometer is pointed at the sample, and measures the temperature without coming in contact with the sample volume. This option is appropriate for use with both filtered and bulk samples. *Example product*: Cole Parmer cat. no. EW-39641-00.

As with other laboratory equipment, all temperature measurement devices must be calibrated routinely to ensure accurate measurements. See the EPA *Manual for the Certification of Laboratories Analyzing Drinking Water* (Reference 20.9) for more information.

8.2 **Sample holding times.** Samples must be processed or examined within each of the holding times specified in Sections 8.2.1 through 8.2.4. Sample processing should be completed as soon as possible by the laboratory. The laboratory should complete sample filtration, elution, concentration, purification, and staining the day the sample is received wherever possible. However, the laboratory is permitted to split up the sample processing steps if processing a sample completely in one day is not possible. If this is necessary, sample processing can be halted after filtration, application of the purified sample onto the slide, or staining. **Table 1**, in Section 21.0 provides a breakdown of the holding times for each set of steps. Sections 8.2.1 through 8.2.4 provide descriptions of these holding times.

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

8.2.2 **Sample elution, concentration, and purification.** The laboratory must complete elution, concentration, and purification (Sections 12.2.6 through 13.3.3.11) in one work day. It is critical that these steps be completed in one work day to minimize the time that any target organisms present in the sample sit in eluate or concentrated matrix. This process ends with the application of the purified sample on the slide for drying.

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

8.2.4 **Examination.** Although immunofluorescence assay (FA) and 4',6-diamidino-2-phenylindole (DAPI) and differential interference contrast (DIC) microscopy examination and characterization should be performed immediately after staining is complete, laboratories have up to 168 hours (7 days) from the completion of sample staining to perform the examination and verification of samples. However, if fading/diffusion of FITC or DAPI staining is noticed, the laboratory must reduce this holding time. In addition the laboratory may adjust the concentration of the DAPI staining solution (Sections 7.7.2) so that fading/diffusion does not occur.

8.3 **Spiking suspension enumeration holding times.** Flow-cytometer-sorted spiking suspensions (Sections 7.10.1 and 11.2) used for spiked quality control (QC) samples (Section 9) must be used within the expiration date noted on the suspension. Manually enumerated spiking suspensions must be used within 24 hours of enumeration of the spiking suspension if the hemacytometer chamber technique is used (Section 11.3.4); or within 24 hours of application of the spiking suspension to the slides if the well slide or membrane filter enumeration technique is used (Sections 11.3.5 and 11.3.6). Oocyst suspensions must be stored between 1°C and 10°C, until ready to use; do not allow to freeze.

9.0 Quality Control

9.1 Each laboratory that uses this method is required to operate a formal quality assurance (QA) program that addresses and documents data quality, instrument and equipment maintenance and performance, reagent quality and performance, analyst training and certification, and records storage and retrieval. General requirements and recommendations for QA and quality control (QC) procedures for microbiology laboratories are provided in References 20.8, 20.9, 20.10. The minimum analytical requirements of this program consist of an initial demonstration of laboratory capability (IDC) through performance of the initial precision and recovery (IPR) test (Section 9.4), and ongoing demonstration of laboratory capability and method performance through the matrix spike (MS) test (Section 9.5.1), the method blank test (Section 9.6), the ongoing precision and recovery (OPR) test (Section 9.7), staining controls (Section 14.1 and 15.2.1), and analyst verification tests (Section 10.6). Laboratory performance is compared to established performance criteria to determine if the results of analyses meet the performance characteristics of the method.

9.1.1 A test of the microscope used for detection of oocysts is performed prior to examination of slides. This test is described in Section 10.0.

9.1.2 In recognition of advances that are occurring in analytical technology, the laboratory is permitted to modify certain method procedures to improve recovery or lower the costs of measurements, provided that all required quality control (QC) tests are performed and all QC acceptance criteria are met. Method procedures that can be modified include front-end techniques, such as filtration or immunomagnetic separation (IMS). The laboratory is not permitted to use an alternate determinative technique to replace immunofluorescence assay in this method (the use of different determinative techniques are considered to be different methods, rather than modified version of this method). However, the laboratory is permitted to modify the immunofluorescence assay procedure, provided that all required QC tests are performed (Section 9.1.2.1) and all QC acceptance criteria are met (see guidance on the use of multiple labeling reagents in Section 7.6).

NOTE: Method modifications should be considered only to improve method performance, reduce cost, or reduce sample processing time. Method modifications that reduce cost or sample processing time, but that result in poorer method performance should not be used.

9.1.2.1 Method modification validation/equivalency demonstration requirements

9.1.2.1.1 **Method modifications at a single laboratory.** Each time a modification is made to this method for use in a single laboratory, the laboratory must, at a minimum, validate the modification according to Tier 1 of EPA's performance-based measurement system (PBMS) (Table 2) to demonstrate that the modification produces results equivalent or superior to results produced by this method as written. Briefly, each time a modification is made to this method, the laboratory is required to demonstrate acceptable modified method performance through the IPR test (Section 9.4). IPR results must meet the QC acceptance criteria in Tables 3 and 4 in Section 21.0, and should be comparable to previous results using the unmodified procedure. Although not required, the laboratory also should perform a matrix spike/matrix spike duplicate (MS/MSD) test to demonstrate the performance of the modified method in at least one real-world matrix before analyzing field samples using the modified method. The laboratory is required to perform MS samples using the modified method at the frequency noted in Section 9.1.8. If the modified method involves changes that cannot be adequately evaluated through these tests, additional tests may be required to demonstrate acceptability.

9.1.2.1.2 **Method modifications for nationwide approval.** If the laboratory or a manufacturer seeks EPA approval of a method modification for nationwide use, the laboratory or manufacturer must, at a minimum, validate the modification according to Tier 2 of EPA's PBMS (Table 2). Briefly, at least three laboratories must perform IPR tests (Section 9.4) and MS/MSD (Section 9.5) tests using the modified method, and all tests must meet the QC acceptance criteria specified in Tables 3 and 4 in Section 21.0. Upon nationwide approval, laboratories electing to use the modified method still must demonstrate acceptable performance in their own laboratory according to the requirements in Section 9.1.2.1.1. If the modified method involves changes that cannot be adequately evaluated through these tests, additional tests may be required to demonstrate acceptability.

9.1.2.2 The laboratory is required to maintain records of modifications made to this method. These records include the following, at a minimum:

9.1.2.2.1 The names, titles, addresses, and telephone numbers of the analyst(s) who performed the analyses and

modification, and of the quality control officer who witnessed and will verify the analyses and modification.

9.1.2.2.2 A listing of the analyte(s) measured (*Cryptosporidium*).

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

9.1.2.2.4 Results from all QC tests comparing the modified method to this method, including:

(a) IPR (Section 9.4)
(b) MS/MSD (Section 9.5)
(c) Analysis of method blanks (Section 9.6)

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

(a) Sample numbers and other identifiers
(b) Source of spiking suspensions, as well as lot number and date received (Section 7.10)
(c) Spike enumeration date and time
(d) All spiking suspension enumeration counts and calculations (Section 11.0)
(e) Sample spiking dates and times
(f) Volume filtered (Section 12.2.5.2)
(g) Filtration and elution dates and times
(h) Pellet volume, resuspended concentrate volume, resuspended concentrate volume transferred to IMS, and all calculations required to verify the percent of concentrate examined (Section 13.2)
(i) Purification completion dates and times (Section 13.3.3.11)
(j) Staining completion dates and times (Section 14.10)
(k) Staining control results (Section 15.2.1)
(l) All required examination information (Section 15.2.2)
(m) Examination completion dates and times (Section 15.2.4)
(n) Analysis sequence/run chronology
(o) Lot numbers of elution, IMS, and staining reagents
(p) Copies of bench sheets, logbooks, and other recordings of raw data
(q) Data system outputs, and other data to link the raw data to the results reported

9.1.3 The laboratory shall spike a separate sample aliquot from the same source to monitor method performance. The frequency of the MS test is described in Section 9.1.8 and the procedures are described in Section 9.5.1.

9.1.4 Analysis of method blanks is required to demonstrate freedom from contamination. The frequency of the analysis of method blanks is described in Section 9.1.7 and the procedures and criteria for analysis of a method blank are described in Section 9.6.

9.1.5 The laboratory shall, on an ongoing basis, demonstrate through analysis of the ongoing precision and recovery (OPR) sample that the analysis system is in control. Frequency of OPR samples is described in Section 9.1.7 and the procedures are described in Section 9.7.

9.1.6 The laboratory shall maintain records to define the quality of data that are generated. Development of accuracy statements is described in Sections 9.5.1.4 and 9.7.6.

9.1.7 The laboratory shall analyze one method blank (Section 9.6) and one OPR sample (Section 9.7) each week (7 day or 168 hours time period which begins with processing the OPR) in which samples are analyzed if 20 or fewer field samples are analyzed during this period. The laboratory shall analyze one laboratory blank and one OPR sample for every 20 samples if more than 20 samples are analyzed in a one week (7 day or 168 hours) period.

9.1.8 The laboratory shall analyze MS samples (Section 9.5.1) at a minimum frequency of 1 MS sample per 20 field samples from each source analyzed. The laboratory should analyze an MS sample when samples are first received from a PWS for which the laboratory has never before analyzed samples to identify potential method performance issues with the matrix (Section 9.5.1; Tables 3 and 4). If an MS sample cannot be analyzed on the first sampling event, the first MS sample should be analyzed as soon as possible to identify potential method performance issues with the matrix.

9.2 Micropipette calibration

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

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

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

9.2.4 If the weight of the reagent water is outside the acceptable limits, consult the manufacturer's instruction manual troubleshooting section and repeat steps described in Section 9.2.3. If problems with the pipette persist, the laboratory must send the pipette to the manufacturer for recalibration.

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

9.4 Initial precision and recovery (IPR)—To establish the ability to demonstrate control over the analytical system and to generate acceptable precision and recovery, the laboratory shall perform the following operations:

9.4.1 Using the spiking procedure in Section 11.4 and enumerated spiking suspensions (Section 7.10.1 or Section 11.3), spike, filter, elute, concentrate, separate (purify), stain, and examine the four reagent water samples spiked with ~100-500 oocysts.

9.4.1.1 The laboratory is permitted to analyze the four spiked reagent samples on the same day or on as many as four different days (provided that the spiked reagent samples are analyzed consecutively), and also may use

different analysts and/or reagent lots for each sample (however, the procedures used for all analyses must be identical). Laboratories should note that the variability of four measurements performed on multiple days or using multiple analysts or reagent lots may be greater than the variability of measurements performed on the same day with the same analysts and reagent lots. As a result, the laboratory is at a greater risk of generating unacceptable IPR results if the test is performed across multiple days, analysts, and /or reagent lots.

9.4.1.2 If more than one modification will be used for filtration and/or separation of samples, a separate set of IPR samples must be prepared for each modification.

9.4.1.3 The set of four IPR samples must be accompanied by analysis of an acceptable method blank (Section 9.6).

9.4.2 Calculate the percent recovery (R) using the following equation:

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

where:

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

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

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

9.4.4 Compare the mean and RSD to the corresponding method performance acceptance criteria for initial precision and recovery in Table 3 in Section 21.0. If the mean and RSD for recovery meet the acceptance criteria, system performance is acceptable and analysis of blanks and samples may begin. If the mean or RSD falls outside the range for recovery, system performance is unacceptable. In this event, trouble-shoot the problem by starting at the end of the method (see guidance in Section 9.7.5), correct the problem and repeat the IPR test (Section 9.4.1).

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

9.4.6 Using 200X to 400X magnification, more than 50% of the oocysts must appear undamaged and morphologically intact; otherwise, the organisms in the spiking suspension may be of unacceptable quality or the analytical process may be damaging the organisms. If the quality of the organisms on the IPR test slides is unacceptable, examine the spiking suspension organisms directly (by centrifuging, if possible, to concentrate the organisms in a volume that can be applied directly to a slide). If the unprocessed organisms appear undamaged and morphologically intact under DIC, determine the step or reagent that is causing damage to the organisms. Correct the problem (see Section 9.7.5) and repeat the IPR test.

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

9.5.1 Matrix spike— The laboratory shall spike and analyze a separate field sample aliquot to determine the effect of the matrix on the method's oocyst recovery. The MS and field sample must be that was collected from the same sampling location as split samples or as samples sequentially collected immediately after one another. The MS sample volume analyzed must be within 10% of the field sample volume. The MS shall be analyzed according to the frequency in Section 9.1.8.

9.5.1.1 Analyze an unspiked field sample according to the procedures in Sections 12.0 to 15.0. Using the spiking procedure in Section 11.4 and enumerated spiking suspensions (Section 7.10.1 or Section 11.3), spike, filter, elute, concentrate, separate (purify), stain, and examine a second field sample aliquot with a similar number of organisms as that used in the IPR or OPR tests (Sections 9.4 and 9.7).

9.5.1.2 Calculate the percent recovery (R) using the following equation.

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

where

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

9.5.1.3 Compare the recovery with the acceptance criteria in Table 3 in Section 21.0.

NOTE: Some sample matrices may prevent the acceptance criteria in Tables 3 from being met. An assessment of the distribution of MS recoveries across 430 MS samples from 87 sites during the ICR Supplemental Surveys is provided in Table 5.

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

9.5.2 Matrix spike duplicate—MSD analysis is required as part of Tier 2 or nationwide approval of a modified version of this method to demonstrate that the modified version of this method produces results equal or superior to results produced by the method as written (Section 9.1.2.1.2). At the same time the laboratory spikes and analyzes the second field sample aliquot in Section 9.5.1.1, the laboratory shall spike and analyze a third, identical field sample aliquot.

NOTE: Matrix spike duplicate samples are only required for Tier 2 validation studies. They are recommended for Tier 1 validation, but not required.

9.5.2.1 Calculate the percent recovery (R) using the equation in Section 9.5.1.2.

9.5.2.2 Calculate the mean of the number of oocysts in the MS and MSD (X_{mean}) (= [MS+MSD]/2).

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

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

where

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

9.5.2.4 Compare the mean MS/MSD recovery and RPD with the acceptance criteria in Table 3 in Section 21.0.

9.6 Method blank (negative control sample, laboratory blank)—Reagent water blanks are routinely analyzed to demonstrate freedom from contamination. Analyze the blank immediately after analysis of the IPR test (Section 9.4) and OPR test (Section 9.7) and prior to analysis of samples for the week to demonstrate freedom from contamination.

9.6.1 Filter, elute, concentrate, separate (purify), stain, and examine at least one reagent water method blank per week (Section 9.1.7) according to the procedures in Sections 12.0 to 15.0. A method blank must be analyzed each week (7 day or 168 hours time period that begins with processing the OPR) in which samples are analyzed if 20 or fewer field samples are analyzed during this period. If more than 20 samples are analyzed in a week (7 days or 168 hours), process and analyze one reagent water method blank for every 20 samples.

9.6.2 Actions

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

9.6.2.2 If *Cryptosporidium* oocysts (as defined in Section 3) or any potentially interfering organism or materials that may be misidentified as oocysts are found in the method blank, the method blank test is unacceptable. Any field sample in a batch associated with an unacceptable method blank is assumed to be contaminated and should be recollected. Analysis of additional samples is halted until the source of contamination is eliminated, the method blank test is performed again, and no evidence of contamination is detected.

9.7 Ongoing precision and recovery (OPR; positive control sample; laboratory control sample)—Using the spiking procedure in Section 11.4 and enumerated spiking suspensions (Section 7.10.1 or Section 11.3), filter, elute, concentrate, separate (purify), stain, and examine at least one reagent water sample spiked with ~100 to 500 oocysts each week to verify all performance criteria. The laboratory must analyze one OPR sample for every 20 samples if more than 20 samples are analyzed in a week. If multiple method variations are used, separate OPR samples must be prepared for each method variation. Adjustment and/or recalibration of the analytical system shall be performed until all performance criteria are met. Only after all performance criteria are met should samples be analyzed.

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

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

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

9.7.2 Calculate the percent recovery (R) using the following equation:

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

where:

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

9.7.3 Compare the recovery with the acceptance criteria for ongoing precision and recovery in Table 3 in Section 21.0.

9.7.4 Actions

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

9.7.4.2 If the recovery for *Cryptosporidium* falls outside of the criteria, system performance is unacceptable. Any sample in a batch associated with an unacceptable OPR sample is unacceptable. Analysis of additional samples is halted until the analytical system is brought under control. Troubleshoot the problem using the procedures at Section 9.7.5 as a guide. After assessing the issue, perform another OPR test and verify that *Cryptosporidium* recoveries meet the acceptance criteria.

9.7.5 **Troubleshooting.** If an OPR sample has failed, and the cause of the failure is not known, the laboratory generally should identify the problem working backward in the analytical process from the microscopic examination to filtration.

9.7.5.1 **Quality of spiked organisms.** Examine the spiking suspension organisms directly (by centrifuging, if possible, to concentrate the organisms in a volume that can be applied directly to a slide). If the organisms appear damaged under DIC, obtain fresh spiking materials. If the organisms appear undamaged and morphologically intact, determined whether the problem is associated with the microscope system or antibody stain (Section 9.7.5.2).

9.7.5.2 **Microscope system and antibody stain:** To determine if the failure of the OPR test is due to changes in the microscope or problems with the antibody stain, re-examine the positive staining control (Section 15.2.1),

check Köhler illumination, and check the fluorescence of the fluorescein-labeled monoclonal antibodies (Mabs) and 4',6-diamidino-2-phenylindole (DAPI). If results are unacceptable, re-examine a previously-prepared positive staining control to determine whether the problem is associated with the microscope or the antibody stain.

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

9.7.5.4 **Filtration/elution/concentration system:** If the failure of the OPR test is attributable to the filtration/elution/concentration system, check system performance by processing spiked reagent water according to the procedures in Section 12.2 through 13.2.2, and filter, stain, and examine the sample concentrate according to Section 11.3.6.

9.7.6 The laboratory should add results that pass the specifications in Section 9.7.3 to initial and previous ongoing data and update the QC chart to form a graphic representation of continued laboratory performance. The laboratory should develop a statement of laboratory accuracy (reagent water, raw surface water) by calculating the mean percent recovery (R) and the standard deviation of percent recovery (s_r). Express the accuracy as a recovery interval from R – 2 s_r to R + 2 s_r. For example, if R = 95% and s_r = 25%, the accuracy is 45% to 145%.

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

9.9 The specifications contained in this method can be met if the analytical system is under control. The standards used for initial (Section 9.4) and ongoing (Section 9.7) precision and recovery should be identical, so that the most precise results will be obtained. The microscope in particular will provide the most reproducible results if dedicated to the settings and conditions required for the determination of *Cryptosporidium* by this method.

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

10.0 Microscope Calibration and Analyst Verification

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

10.2 Using the manuals provided with the microscope, all analysts must familiarize themselves with operation of the microscope.

10.3 Microscope adjustment and calibration (adapted from Reference 20.10)

10.3.1 Preparations for adjustment

10.3.1.1 The microscopy portion of this procedure depends upon proper alignment and adjustment of very sophisticated optics. Without proper alignment and adjustment, the microscope will not function at maximal efficiency, and reliable identification and enumeration of oocysts will not be

possible. Consequently, it is imperative that all portions of the microscope from the light sources to the oculars are properly adjusted.

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

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

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

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

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

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

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

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

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

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

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

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

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

10.3.2.8 Reattach the objective to the nosepiece.

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

10.3.2.10 Turn off the transmitted light and replace the card with a slide of fluorescent material. Check the field for even fluorescent illumination. Adjustment of the diffuser lens probably will be required. Additional slight adjustments as in Section 10.3.2.7 above may be required.

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

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

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

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

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

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

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

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

10.3.4 Adjustment of the interpupillary distance and oculars for each eye: These adjustments are necessary so that eye strain is reduced to a minimum, and must be made for each individual using the microscope. Section 10.3.4.2 assumes use of a microscope with both oculars adjustable; Section 10.3.4.3 assumes use of a microscope with a single adjustable ocular. The procedure must be followed each time an analyst uses the microscope.

10.3.4.1 Interpupillary distance

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

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

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

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

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

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

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

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

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

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

10.3.5.2 Adjust the stage and ocular with the micrometer so the “0" line on the ocular micrometer is exactly superimposed on the “0" line on the stage micrometer.

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

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

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

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

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

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

10.3.5.7 Follow the procedure below for each objective. Record the information as shown in the example below and keep the information available at the microscope.

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

[1]1000 µm/mm
[2](Stage micrometer length in mm × (1000 µm/mm)) ÷ no. ocular micrometer spaces
[3]N.A. refers to numerical aperature. The numerical aperature value is engraved on the barrel of the objective.

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

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

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

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

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

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

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

10.4 Microscope cleaning procedure

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

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

10.4.3 Protocol for cleaning oculars and condenser

10.4.3.1 Use a new, clean Q-tip dampened with MCS to clean each lense. Start at the center of the lens and spiral the Q-tip outward using little to no pressure. Rotate the Q-tip head while spiraling to ensure a clean surface is always contacting the lens.

10.4.3.2 Repeat the procedure using a new, dry Q-tip.

10.4.3.3 Repeat Sections 10.4.3.1 and 10.4.3.2.

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

10.4.4 Protocol for cleaning objective lenses

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

10.4.4.2 Hold a new Q-tip dampened with MCS at a 45° angle on the objective and twirl.

10.4.4.3 Repeat Sections 10.4.4.2 with a new, dry Q-tip.

10.4.4.4 Repeat Sections 10.4.4.2 and 10.4.4.3.

10.4.4.5 Clean all objectives whether they are used or not.

10.4.5 Protocol for cleaning light source lens and filters

10.4.5.1 Using a Kimwipe dampened with microscope cleaning solution, wipe off the surface of each lens and filter.

10.4.5.2 Repeat the procedure using a dry Kimwipe.

10.4.5.3 Repeat Sections 10.4.5.1 and 10.4.5.2.

10.4.6 Protocol for cleaning microscope stage

10.4.6.1 Using a Kimwipe dampened with microscope cleaning solution, wipe off the stage and stage clip. Be sure to clean off any residual immersion oil or fingernail polish. Remove the stage clip if necessary to ensure that it is thoroughly cleaned.

10.4.7 Use 409 and a paper towel to clean the bench top surrounding the microscope.

10.4.8 Frequency

10.4.8.1 Perform Sections 10.4.2, 10.4.3, 10.4.4, 10.4.5 and 10.4.7 after each microscope session.

10.4.8.2 Perform complete cleaning each week.

10.5 Protozoa libraries: Each laboratory is encouraged to develop libraries of photographs and drawings for identification of protozoa.

10.5.1 Take color photographs of *Cryptosporidium* oocysts by FA, 4',6-diamidino-2-phenylindole (DAPI), and DIC that the analysts (Section 22.2) determine are accurate (Section 15.2).

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

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

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

10.6.2 Each analyst shall determine the total number of oocysts detected by FITC on the entire slide meeting the criteria in 10.6.1. For the same 10 oocysts, each analyst shall determine the DAPI category (DAPI negative, DAPI positive internal intense blue and DAPI positive number of nuclei) and the DIC category (empty, containing amorphous structures, or containing identifiable internal structures) of each. The DAPI/DIC comparisons may be performed on the slide prepared in 10.6.1, OPR slide, MS slide, or a positive staining control slide.

10.6.3 Requirements for laboratories with multiple analysts

10.6.3.1 The total number of oocysts determined by each analyst (Section 10.6.2.) must be within ±10% of each other. If the number is not within this range, the analysts must identify the source of any variability between analysts' examination criteria, prepare a new slide, and repeat the performance verification (Sections 10.6.1 to 10.6.2). It is recommended that the DAPI and DIC categorization of the same 10 oocysts occur with all analysts at the same time, i.e. each analyst determines the categorizations independently, then the differences in the DAPI and DIC categorizations among analysts are discussed and resolved, and these resolutions documented. Alternatively, organism coordinates may be recorded for each analyst to locate and categorize the organisms at different times. Differences among analysts must be discussed and resolved.

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

10.6.3.3 Only after an analyst has passed the criteria in Section 10.6.3, may oocysts in QC samples and field samples be identified and enumerated.

10.6.4 Laboratories with only one analyst should maintain a protozoa library (Section 10.5) and compare the results of the examinations performed in Sections 10.6.1 and 10.6.2 to photographs of oocysts and interfering organisms to verify that examination results are consistent with these references. These laboratories also should perform repetitive counts of a single verification slide for FITC. These laboratories should also coordinate with other laboratories to share slides and compare counts.

11.0 Oocyst Suspension Enumeration and Sample Spiking

11.1 This method requires routine analysis of spiked QC samples to demonstrate acceptable initial and ongoing laboratory and method performance (initial precision and recovery samples [Section 9.4], matrix spike and matrix spike duplicate samples [Section 9.5], and ongoing precision and recovery samples [Section 9.7]). The organisms used for these samples must be enumerated to calculate recoveries (and precision) and monitor method performance. EPA recommends that flow cytometry be used for this enumeration, rather than manual techniques. Flow cytometer–sorted spikes generally are characterized by a relative standard deviation of ≤2.5%, versus greater variability for manual enumeration techniques (Reference 20.11). Guidance on preparing spiking suspensions using a flow cytometer is provided in Section 11.2. Manual enumeration procedures are provided in Section 11.3. The procedure for spiking bulk samples in the laboratory is provided in Section 11.4.

11.2 **Flow cytometry enumeration guidelines.** Although it is unlikely that many laboratories performing Method 1622 will have direct access to a flow cytometer for preparing spiking suspensions, flow-sorted suspensions are available from commercial vendors and other sources (Section 7.10.1). The information provided in Sections 11.2.1 through 11.2.4 is simply meant as a guideline for preparing spiking suspensions using a flow cytometer. Laboratories performing flow cytometry must develop and implement detailed standardized protocols for calibration and operation of the flow cytometer.

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

11.2.2 Spiking suspensions should be prepared using *Cryptosporidium parvum* oocysts <3 months old.

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

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

11.2.5 **Method blanks.** Depending on the operation of the flow cytometer, method blanks should be prepared and examined at the same frequency as the ongoing calibration samples (Section 11.2.4).

11.2.6 **Holding time criteria.** Flow-cytometer-sorted spiking suspensions (Sections 7.10.1 and 11.2) used for spiked quality control (QC) samples (Section 9) must be used within the expiration date noted on the suspension. The holding time specified by the flow cytometry laboratory should be determined based on a holding time study.

11.3 **Manual enumeration procedures.** Two sets of manual enumerations are required per organism before purified *Cryptosporidium* oocyst (Section 7.10.2) received from suppliers can be used to

spike samples in the laboratory. First, the stock suspension must be diluted and enumerated (Section 11.3.3) to yield a suspension at the appropriate oocyst concentration for spiking (spiking suspension). Then, 10 aliquots of spiking suspension must be enumerated to calculate a mean spike dose. Spiking suspensions can be enumerated using hemacytometer chamber counting (Section 11.3.4), well slide counting (Section 11.3.5), or membrane filter counting (Section 11.3.6).

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

11.3.2 Holding time criteria. Manually enumerated spiking suspensions must be used within 24 hours of enumeration of the spiking suspension if the hemacytometer chamber technique is used (Section 11.3.4); or within 24 hours of application of the spiking suspension or membrane filter to the slides if the well slide or membrane filter enumeration technique is used (Sections 11.3.5 and 11.3.6).

11.3.3 Enumerating and diluting stock suspensions

11.3.3.1 Purified, concentrated stock suspensions (Sections 7.10.2.1 and 7.10.2.2) must be diluted and enumerated before the diluted suspensions are used to spike samples in the laboratory. Stock suspensions should be diluted with reagent water/Tween® 20 , 0.01% (Section 7.10.2.3), to a concentration of 20 to 50 organisms per large hemacytometer square before proceeding to Section 11.3.3.2.

11.3.3.2 Apply a clean hemacytometer coverslip (Section 6.4.5) to the hemacytometer and load the hemacytometer chamber with 10 µL of vortexed suspension per chamber. If this operation has been properly executed, the liquid should amply fill the entire chamber without bubbles or overflowing into the surrounding moats. Repeat this step with a clean, dry hemacytometer and coverslip if loading has been incorrectly performed. See Section 11.3.3.13, below, for the hemacytometer cleaning procedure.

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

11.3.3.4 Use 200X magnification.

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

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

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

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

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

$$\frac{\text{number of organisms counted}}{\text{number of mm}^2\text{ counted}} \times \frac{10}{1\text{ mm}} \times \frac{\text{dilution factor}}{1} \times \frac{1\text{ mm}^3}{1\text{ µL}} = \frac{\text{number of organisms}}{\text{µL}}$$

11.3.3.10 Record the result on a hemacytometer data sheet.

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

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

NOTE: If the diluted stock suspensions (the spiking suspensions) will be enumerated using hemacytometer chamber counts (Section 11.3.4) or membrane filter counts (Section 11.3.6), then the stock suspensions should be diluted with 0.01% Tween® 20 . If the spiking suspensions will be enumerated using well slide counts (Section 11.3.5), then the stock suspensions should be diluted in reagent water.

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

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

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

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

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

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

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

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

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

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

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

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

11.3.4 Enumerating spiking suspensions using a hemacytometer chamber

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

11.3.4.1 Vortex the tube containing the spiking suspension (diluted stock suspension; Section 11.3.3) for a minimum of 2 minutes. Gently invert the tube three times.

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

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

11.3.4.4 While the stir bar is still spinning, remove a 10-µL aliquot and carefully load one side of the hemacytometer. Count all organisms on the platform, at 200X magnification using phase-contrast or darkfield microscopy. The count must include the entire area under the hemacytometer, not just the four outer 1-mm^2 squares. Repeat this procedure nine times. This step allows confirmation of the number of organisms per 10 µL (Section 11.3.3.12). Based on the 10 counts, calculate the mean, standard deviation, and RSD of the counts. Record the counts and the calculations on a spiking suspension enumeration form. The relative standard deviation (RSD) of the calculated mean spike dose must be ≤16% for *Cryptosporidium* before proceeding. If the RSD is unacceptable, or the mean number is outside the expected range, add additional oocysts from stock suspension or dilute the contents of the beaker appropriately with reagent water. Repeat the process to confirm counts. Refer to Section 11.3.3.14 for factors that may introduce errors.

11.3.5 Enumerating spiking suspensions using well slides

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

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

11.3.5.2 Vortex the tube containing the spiking suspension (diluted stock suspension; Section 11.3.3) for a minimum of 2 minutes. Gently invert the tube three times.

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

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

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

11.3.5.6 Positive and negative controls must be prepared.

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

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

11.3.5.6.3 Air-dry the control slides.

11.3.5.7 Follow the manufacturer's instructions (Section 7.6) in applying the stain to the slide.

11.3.5.8 Place the slides in a humid chamber in the dark and incubate according to manufacturer's directions. The humid chamber consists of a tightly sealed plastic container containing damp paper towels on top of which the slides are placed.

11.3.5.9 Apply one drop of wash buffer (prepared according to the manufacturer's instructions [Section 7.6]) to each well. Tilt each slide on a clean paper towel, long edge down. Gently aspirate the excess detection reagent from below the well using a clean Pasteur pipette or absorb with a paper towel or other absorbent material. Avoid disturbing the sample.

NOTE: *If using the MeriFluor® Cryptosporidium/Giardia stain (Section 7.6.1), do not allow slides to dry completely.*

11.3.5.10 Add mounting medium (Section 7.8) to each well.

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

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

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

11.3.6 Enumeration of spiking suspensions using membrane filters

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

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

11.3.6.2 Place foil around the bottoms of the 100 × 15 mm petri dishes.

11.3.6.3 Filter-sterilize (Section 6.19) approximately 10 mL of PBS (Section 7.4.2.1). Dilute detection reagent (Section 7.6) as per manufacturer's instructions using sterile PBS. Multiply the anticipated number of filters to be stained by 100 mL to calculate total volume of stain required. Divide the total volume required by 5 to obtain the microliters of antibody necessary. Subtract the volume of antibody from the total stain volume to obtain the required microliters of sterile PBS to add to the antibody.

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

11.3.6.5 Place a decontaminated and cleaned filter holder base (Section 6.4.8.1) into each of the three ports of the vacuum manifold (Section 6.4.8.2).

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

11.3.6.7 Using forceps, moisten a 1.2-µm cellulose-acetate support membrane (Section 6.4.8.3) in the 0.01% Tween® 20 and place it on the fritted glass support of one of the filter bases. Moisten a polycarbonate filter (Section 6.4.8.4) the same way and position it on top of the cellulose-acetate support membrane. Carefully clamp the glass funnel to the loaded filter support. Repeat for the other two filters.

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

11.3.6.9 Vortex the tube containing the spiking suspension (diluted stock suspension; Section 11.3.3) for a minimum of 2 minutes. Gently invert the tube three times.

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

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

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

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

11.3.6.14 Repeat Sections 11.3.6.4 through 11.3.6.10 for the next three samples (if that the diluted spiking suspension has sat less than 15 minutes, reduce the suspension vortex time to 60 seconds). Ten, 10-µL spiking suspension aliquots must be prepared and counted, and the counts averaged, to sufficiently enumerate the spike dose. Include a filter blank sample at a frequency of every five samples; rotate the position of filter blank to eventually include all three filter placements.

11.3.6.15 Repeat Sections 11.3.6.4 through 11.3.6.10 until the 10-µL spiking suspensions have been filtered. The last batch should include a 10-µL 0.01 Tween® 20 blank control and 20 µL of positive control antigen as a positive staining control.

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

11.3.6.17 To each slide, add 20 µL of mounting medium (Section 7.8).

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

11.3.6.19 Record the date and time that staining was completed. If slides will not be read immediately, store sealed slides in a closed container in the dark between 1°C and 10°C until ready for examination.

11.3.6.20 After examination of the 10 slides, calculate the mean, standard deviation, and RSD of the 10 replicates. Record the counts and the calculations on a spiking suspension enumeration form. The relative

standard deviation (RSD) of the calculated mean spike dose must be ≤16% for *Cryptosporidium* before proceeding. If the RSD is unacceptable, or the mean number is outside the expected range, add additional oocysts from stock suspension or dilute the contents of the beaker appropriately with reagent water. Repeat the process to confirm counts.

11.3.6.21 If oocysts are detected on the filter blanks, modify the rinse procedure to ensure that no carryover occurs and repeat enumeration.

11.4 Procedure for spiking samples in the laboratory with enumerated spiking suspensions.

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

11.4.2 For initial precision and recovery (Section 9.4) and ongoing precision and recovery (Section 9.7) samples, fill the container with 10 L of reagent water or a volume of reagent water equal to the volume of the field samples analyzed in the analytical batch. For matrix spike samples (Section 9.5), fill the container with the field sample to be spiked. Continuously mix the sample (using a stir bar and stir plate for smaller-volume samples and alternate means for larger-volume samples).

11.4.3 Follow the procedures in Section 11.4.3.1 or manufacturer's instructions for flow cytometer–enumerated suspensions and the procedures in Section 11.4.3.2 for manually enumerated suspensions.

11.4.3.1 For flow cytometer–enumerated suspensions (where the entire volume of a spiking suspension tube will be used):

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

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

11.4.3.1.3 Pour the suspension into the sample container.

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

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

11.4.3.1.6 Record the estimated number of organisms spiked, the date and time the sample was spiked, and the sample volume spiked on a bench sheet. Proceed to Section 11.4.4.

11.4.3.2 For manually enumerated spiking suspensions:

11.4.3.2.1 Vortex the spiking suspension(s) (Section 11.2 or Section 11.3) for a minimum of 30 seconds.

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

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

11.4.3.2.4 Record the estimated number of organisms spiked, the date and time the sample was spiked, and the sample

volume spiked on a bench sheet. Proceed to Section 11.4.4

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

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

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

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

11.4.8 Proceed to filter disassembly.

12.0 Sample Filtration and Elution

12.1 A water sample is filtered according to the procedures in Section 12.2, 12.3, or 12.4. Alternate procedures may be used if the laboratory first demonstrates that the alternate procedure provides equivalent or superior performance per Section 9.1.2.

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

12.2 Capsule filtration (adapted from Reference 20.12). This procedure was validated using 10-L sample volumes (for the original Envirochek ™ filter) and 50-L sample volumes (for the Envirochek™ HV filter). Alternate sample volumes may be used, provided the laboratory demonstrates acceptable performance on initial and ongoing spiked reagent water and source water samples (Section 9.1.2).

12.2.1 Flow rate adjustment

12.2.1.1 Connect the sampling system, minus the capsule, to a carboy filled with reagent water (Figure 3).

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

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

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

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

12.2.4 Filtration

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

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

12.2.4.2 Place the drain end of the sampling system tubing into an empty graduated container with a capacity of 10 to 15 L, calibrated at 9.0, 9.5, 10.0, 10.5, and 11.0 L (Section 6.18). This container will be used to determine the sample volume filtered. Alternately, connect a flow meter (Section 6.3.4) downstream of the filter, and record the initial meter reading.

12.2.4.3 Allow the carboy discharge tube and capsule to fill with sample water by gravity. Vent residual air using the bleed valve/vent port, gently shaking or tapping the capsule, if necessary. Turn on the pump to start water flowing through the filter. Verify that the flow rate is 2 L/min.

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

12.2.4.5 Turn off stir plate; add 1 L PBST or reagent water rinse (to 10-L carboy) or 5 L PBST or reagent water rinse (to 50-L carboy). Swirl or shake the carboy to rinse down the side walls.

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

12.2.5 Disassembly

12.2.5.1 Disconnect the inlet end of the capsule filter assembly while maintaining the level of the inlet fitting above the level of the outlet fitting to prevent backwashing and the loss of oocysts from the filter. Restart the pump and allow as much water to drain as possible. Turn off the pump.

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

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

12.2.6 Elution

NOTE: The laboratory must complete the elution, concentration, and purification (Sections 12.2.6 through 13.3.3.11) in one work day. It is critical that these steps be completed in one work day to minimize the time that any target organisms present in the sample sit in eluate or concentrated matrix. This process ends with the application of the purified sample on the slide for drying.

12.2.6.1 Setup

12.2.6.1.1 Assemble the laboratory shaker with the clamps aligned vertically so that the filters will be aligned horizontally. Extend the clamp arms to their maximum distance from the horizontal shaker rods to maximize the shaking action.

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

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

12.2.6.2 Elution

12.2.6.2.1 Record the elution date and time on the bench sheet.

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

12.2.6.2.2 Remove the inlet cap, pour elution buffer through the inlet fitting, and allow the liquid level to stabilize. Sufficient elution buffer must be added to cover the pleated white membrane with buffer solution or elution buffer may be measured to ensure the use of one 250-mL centrifuge tube. Replace the inlet cap.

12.2.6.2.3 Securely clamp the capsule in one of the clamps on the laboratory shaker with the bleed valve positioned at the top on a vertical axis (in the 12 o'clock position). Turn on the shaker and set the speed to maximum (approximately 900 rpm or per manufacturer's instructions). Agitate the capsule for approximately 5 minutes. Time the agitation using a lab timer, rather than the timer on the shaker to ensure accurate time measurement.

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

12.2.6.2.5 Clamp the capsule vertically with the inlet end up and add sufficient volume of elution buffer through the inlet fitting to cover the pleated membrane. Replace the inlet cap.

12.2.6.2.6 Return the capsule to the shaker with the bleed valve positioned at the 4 o'clock position. Turn on the shaker and agitate the capsule for approximately 5 minutes.

12.2.6.2.7 Remove the filter from the shaker, but leave the elution buffer in the capsule. Re-clamp the capsule to the shaker at the 8 o'clock position. Turn on the shaker and agitate the capsule for a final 5 minutes.

12.2.6.2.8 Remove the filter from the shaker and pour the contents into the 250-mL centrifuge tube. Rinse down the inside of the capsule filter walls with reagent water or elution buffer using a squirt bottle inserted in the inlet end of the capsule. Invert the capsule filter over the centrifuge tube and ensure that as much of the eluate as possible has been transferred.

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

12.3 Sample filtration using the Filta-Max® foam filter. This procedure was validated using 50-L sample volumes. Alternate sample volumes may be used, provided the laboratory demonstrates acceptable performance on initial and ongoing spiked reagent water and source water samples (Section 9.1.2).

NOTE: The filtration procedures specified in Sections 12.3.1.2 - 12.3.1.6.3 are specific to laboratory filtration of a bulk sample. These procedures may require modification if samples will be filtered in the field.

12.3.1 Filtration

12.3.1.1 Flow rate adjustment

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

12.3.1.1.2 Place the peristaltic pump upstream of the filter housing.

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

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

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

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

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

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

12.3.1.5 Filtration

12.3.1.5.1 Connect the sampling system to the field carboy of sample water, or transfer the sample water to the laboratory carboy used in Section 12.3.1.1.1. If the sample will be filtered from a field carboy, a spigot can be used with the carboy to facilitate sample filtration.

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

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

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

12.3.1.5.4 After all of the sample has passed through the filter, turn off the pump. Allow the pressure to decrease until flow stops.

12.3.1.6 Disassembly

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

12.3.1.6.2 Based on the water level in the graduated container or the meter reading, record the volume filtered on a bench sheet to the nearest quarter liter.

12.3.1.6.3 Loosen the outlet fitting, the filter housing should be sealed with rubber plugs.

NOTE: Filters should be prevented from drying out, as this can impair their ability to expand when decompressed.

12.3.2 Elution

12.3.2.1 The filter is eluted to wash the oocysts from the filter. This can be accomplished using the Filta-Max® wash station, which moves a plunger up and down a tube containing the filter and eluting solution (Section 12.3.2.2), or a stomacher, which uses paddles to agitate the stomacher bag containing the foam filter in the eluting solution (Section 12.3.2.3). If the Filta-Max® automatic wash station is used please see the manufacturer's operator's guide for instructions on its use. If Filta-Max® Quick Connect kit is used please follow manufacturer's instructions.

12.3.2.2 Filta-Max® wash station elution procedure

12.3.2.2.1 First wash

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

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

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

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

the jaws of the wash station and screw the elution tube (the shorter of the two tubes) firmly in place.

(e) Pull the plunger down until the filter module sits at the bottom of the elution tube; the locking pin (at the top left of the wash station) should "click" to lock the plunger in position.

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

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

NOTE: Gentle pressure on the lever, coupled with a pulling action on the locking pin should enable the pin to be easily released.

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

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

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

(j) Prior to the second wash the eluate from the first wash can be concentrated using the Filta-Max® apparatus according to Section 12.3.3.2.1 or the eluate can be decanted into a 2-L pooling beaker and set aside.

12.3.2.2.2 Second wash

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

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

(c) The eluate can be concentrated using the Filta-Max® apparatus according to Section 12.3.3.2.2 or the eluate can be decanted into the 2-L pooling beaker containing the eluate from the first wash and

concentrated using centrifugation, as described in Section 12.3.3.3.

12.3.2.3 Stomacher elution procedure

12.3.2.3.1 First wash

(a) Place the filter module in the stomacher bag then use the allen key to remove the bolt from the filter module, allowing the rings to expand. Remove the end caps from the stomacher bag and rinse with PBST into the stomacher bag.

(b) Add 600 mL of PBST to stomacher bag containing the filter pads. Place bag in stomacher and wash for 5 minutes on a normal setting.

(c) Remove the bag from the stomacher and decant the eluate into a 2-L pooling beaker.

12.3.2.3.2 Second wash

(a) Add a second 600-mL aliquot of PBST to the stomacher bag. Place bag in stomacher and wash for 5 minutes on a normal setting. Remove the bag from the stomacher and decant the eluate from the stomacher bag into the 2-L pooling beaker. Wring the stomacher bag by hand to remove eluate from the foam filter and add to the pooling beaker. Remove the foam filter from the bag and using a squirt bottle, rinse the stomacher bag with reagent water and add the rinse to the pooling beaker.

(b) Proceed to concentration (Section 12.3.3).

12.3.3 Concentration

12.3.3.1 The eluate can be concentrated using the Filta-Max® concentrator apparatus, which pulls most of the eluate through a membrane filter leaving the oocysts concentrated in a small volume of the remaining eluting solution (Section 12.3..2), or by directly centrifuging all of the eluting solution used to wash the filter (Section 12.3.2.3).

12.3.3.2 The Filta-Max® concentrator procedure

12.3.3.2.1 Concentration of first wash

(a) If the stomacher was used to elute the sample (Section 12.3.2.3), transfer 600 mL of eluate from the pooling beaker to the concentrator tube. Otherwise proceed to Step (b).

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

NOTE: *The force of the vacuum should not exceed 30 cmHg.*

(c) Allow the liquid to drain until it is approximately level with the middle of the stirrer bar then close the valve. Remove the magnetic stirrer, and rinse it with

PBST or distilled water to recover all oocysts. Decant the concentrate into a 50-mL tube, then rinse the sides of the concentration tube and add the rinsate to the 50-mL tube.

12.3.3.2.2 Concentration of second wash

(a) If the stomacher was used to elute the sample (Section 12.3.2.3), transfer the remaining 600 mL of eluate from the pooling beaker to the concentrator tube. Otherwise proceed to Step (b).

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

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

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

12.3.3.2.3 Membrane elution. The membrane can be washed manually or using a stomacher:

- **Manual wash.** Add 5 mL of PBST to the bag containing the membrane. Rub the surface of the membrane through the bag until the membrane appears clean. Using a pipette, transfer the eluate to a 50-mL tube. Repeat the membrane wash with another 5 mL of PBST and transfer the eluate to the 50-mL tube. (Optional: Perform a third wash using another 5 mL of PBST, by hand-kneading an additional minute or placing the bag on a flat-headed vortexer and vortexing for one minute. Transfer the eluate to the 50-mL tube.)

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

- **Stomacher wash.** Add 5 mL of PBST to the bag containing the membrane. Place the bag containing the membrane into a small stomacher and stomach for 3 minutes. Using a pipette transfer the eluate to a 50-mL tube. Repeat the wash two times using the stomacher and 5-mL aliquots of PBST. (Optional: Perform a fourth wash using another 5 mL of PBST, by hand-kneading an additional minute or placing the bag on a flat-headed vortexer and vortexing for one minute. Transfer the eluate to the 50-mL tube.)

12.3.3.2.4 If the membrane filter clogs before concentration is complete, there are two possible options for completion

of concentration. One option is replacing the membrane as often as necessary. Filter membranes may be placed smooth side up during the second concentration step. Another option is concentrating the remaining eluate using centrifugation. Both options are provided below.

- **Using multiple membranes.** Disassemble the concentrator tube and pour any remaining eluate back into the pooling beaker. Remove the membrane using membrane forceps, placing it in the bag provided. Place a new membrane in the concentrator tube smooth side up, reassemble, return the eluate to the concentrator tube, rinse the pooling beaker and add rinse to the eluate, and continue the concentration. Replace the membrane as often as necessary.
- **Centrifuging remaining volume.** Decant the remaining eluate into a 2-L pooling beaker. Rinse the sides of the concentrator tube and add to the pooling beaker. Remove the filter membrane and place it in the bag provided. Wash the membrane as described in Section 12.3.3.2.3, then concentrate the sample as described in Section 12.3.3.3.1.

12.3.3.3 If the Filta-Max® concentrator is not used for sample concentration, or if the membrane filter clogs before sample concentration is complete, then the procedures described in Section 12.3.3.3.1 should be used to concentrate the sample. If less than 50 mL of concentrate has been generated, the sample can be further concentrated, as described in Section 12.3.3.3.2, to reduce the volume of sample to be processed through IMS.

NOTE: The volume must not be reduced to less than 5 mL above the packed pellet. The maximum amount of pellet that should be processed through IMS is 0.5 mL. If the packed pellet is greater than 0.5 mL then the pellet may be subsampled as described in Section 13.2.4.

12.3.3.3.1 Centrifugation of greater than 50 mL of eluate

(a) Decant the eluate from the 2-L pooling beaker into 250-mL conical centrifuge tubes. Make sure that the centrifuge tubes are balanced.

(b) Centrifuge the 250-mL centrifuge tubes containing the eluate at 1500 × G for 15 minutes. Allow the centrifuge to coast to a stop.

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

(d) Vortex each 250-mL tube vigorously until pellet is completely resuspended. Swirl the centrifuge tube gently to reduce any foaming after vortexing. Combine the contents of each 250-mL centrifuge tube into a 50-mL centrifuge tube. Rinse each of the

250-mL centrifuge tubes with PBST and add the rinse to the 50-mL tube.

(e) Proceed to Section 12.3.3.3.2.

12.3.3.3.2 Centrifugation of less than 50 mL of eluate

(a) Centrifuge the 50-mL centrifuge tube containing the combined concentrate at 1500 x G for 15 minutes. Allow the centrifuge to coast to a stop. Record the initial pellet volume (volume of solids) and the date and time that concentration was completed on a bench sheet.

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

12.3.4 Maintenance and cleaning

12.3.4.1 Maintenance of O-rings

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

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

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

12.3.4.2 Cleaning

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

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

12.4 Sample collection (filtration and concentration) using portable continuous-flow centrifugation. Please follow manufacturer's instructions. This procedure was validated for the detection of *Cryptosporidium* using 50-L sample volumes. Alternate sample volumes may be used, provided the laboratory demonstrates acceptable performance on initial and ongoing spiked reagent water and source water samples (Section 9.1.2).

13.0 Sample Concentration and Separation (Purification)

13.1 During concentration and separation, the filter eluate is concentrated through centrifugation, and the oocysts in the sample are separated from other particulates through immunomagnetic separation (IMS). Alternate procedures and products may be used if the laboratory first demonstrates equivalent or superior performance as per Section 9.1.2.

13.2 Adjustment of pellet volume

13.2.1 Centrifuge the 250-mL centrifuge tube containing the capsule filter eluate at 1500 × G for 15 minutes. Allow the centrifuge to coast to a stop—do not use the brake. Record the pellet volume (volume of solids) on the bench sheet.

NOTE: Recoveries may be improved if centrifugation force is increased to 2000 × G. However, do not use this higher force if the sample contains sand or other gritty material that may degrade the condition of any oocysts in the sample.

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

13.2.3 **If the packed pellet volume is ≤ 0.5 mL,** vortex the tube vigorously until pellet is completely resuspended. Swirl the centrifuge tube gently to reduce any foaming after vortexing. Record the resuspended pellet volume on the bench sheet. Proceed to Section 13.3.

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

13.2.4 **If the packed pellet volume is > 0.5 mL,** the concentrate must be separated into multiple subsamples (a subsample is equivalent to no greater than 0.5 mL of packed pellet material, the recommended maximum amount of particulate material to process through the subsequent purification and examination steps in the method). Use the following formula to determine the total volume required in the centrifuge tube before separating the concentrate into two or more subsamples:

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

(For example, if the packed pellet volume is 1.2 mL, the total volume required is 12 mL.) Add reagent water to the centrifuge tube to bring the total volume to the level calculated above.

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

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

13.2.4.1.1 **Calculate number of subsamples:** Divide the total volume in the centrifuge tube by 5 mL and round up to the nearest integer (for example, if the resuspended volume in Section 13.2.4 is 12 mL, then the number of subsamples would be 12 mL / 5 mL = 2.4, rounded = 3 subsamples).

13.2.4.1.2 **Determine volume of resuspended concentrate per subsample.** Divide the total volume in the centrifuge tube by the calculated number of subsamples (for example, if the resuspended volume in Section 13.2.4 is 12 mL, then the volume to use for each subsample = 12 mL / 3 subsamples = 4 mL).

13.2.4.1.3 **Process subsamples through IMS.** Vortex the tube vigorously for 10 to 15 seconds to completely resuspend the pellet. Record the resuspended pellet volume on the

bench sheet. Proceed immediately to Section 13.3, and transfer aliquots of the resuspended concentrate equivalent to the volume in the previous step to multiple, flat-sided sample tubes in Section 13.3.2.1. Process the sample as multiple, independent subsamples from Section 13.3 onward, including the preparation and examination of separate slides for each aliquot. Record the volume of resuspended concentrate transferred to IMS on the bench sheet (this will be equal to the volume recorded in Section 13.2.4). Also record the number of subsamples processed independently through the method on the bench sheet.

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

$$\text{percent examined} = \frac{\text{total volume of resuspended concentrate transferred to IMS}}{\text{total volume of resuspended concentrate in Section 13.2.4}} \times 100\%$$

Then multiply the volume filtered (Section 12.2.5.2) by this percentage to determine the volume analyzed.

13.3 IMS procedure (adapted from Reference 20.13)

NOTE: The IMS procedure should be performed on a bench top with all materials at room temperature, ranging from 15°C to 25°C.

13.3.1 Preparation and addition of reagents

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

13.3.1.2 For each 10mL sample or subsample (Section 13.2) to be processed through IMS, add 1 mL of the 10X SL-buffer-A (supplied—not the diluted 1X SL-buffer-A) to a flat-sided tube (Section 6.5.4).

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

13.3.2 Oocyst and cyst capture

13.3.2.1 Use a graduated, 10-mL pipette that has been pre-rinsed with elution buffer to transfer the water sample concentrate from Section 13.2 to the flat-sided tube(s) containing the SL-buffers. If all of the concentrate is used, rinse the centrifuge tube twice with reagent water and add the rinsate to the flat-sided tube containing the concentrate (or to the tube

containing the first subsample, if multiple subsamples will be processed). Each of the two rinses should be half the volume needed to bring the total volume in the flat-sided sample tube to 12 mL (including the buffers added in Sections 13.3.1.2 and 13.3.1.3). (For example, if the tube contained 1 mL of SL-buffer-A and 1 mL of SL-buffer-B, and 5 mL of sample was transferred after resuspension of the pellet, for a total of 7 mL, the centrifuge tube would be rinsed twice with 2.5 mL of reagent water to bring the total volume in the flat-sided tube to 12 mL.) Visually inspect the centrifuge tube after completing the transfer to ensure that no concentrate remains. If multiple subsamples will be processed, bring the volume in the remaining flat-sided tubes to 12 mL with reagent water. Label the flat-sided tube(s) with the sample number (and subsample letters).

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

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

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

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

13.3.2.6 Without removing the sample tube from the MPC®-1, place the magnet side of the MPC®-1 downwards, so the tube is horizontal and the flat side of the tube is facing down.

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

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

13.3.2.9 Return the MPC®-1 to the upright position, sample tube vertical, with cap at top. Immediately remove the cap and, keeping the flat side of the tube on top, pour off all of the supernatant from the tube held in the MPC®-1 into a suitable container. Do not shake the tube and do not remove the tube from MPC®-1 during this step. Allow more supernatant to settle; aspirate additional supernatant with pipette.

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

13.3.2.11 Quantitatively transfer (transfer followed by two rinses) all the liquid from the sample tube to a labeled, 1.5-mL microcentrifuge tube. Use 0.5

mL of 1X SL-buffer-A to perform the first rinse and 0.5 mL of 1X SL-buffer-A for the second rinse. Allow the flat-sided sample tube to sit for a minimum of 1 minute after transfer of the second rinse volume, then use a pipette to collect any residual volume that drips down to the bottom of the tube to ensure that as much sample volume is recovered as possible. Ensure that all of the liquid and beads are transferred.

13.3.2.12 Place the microcentrifuge tube into the second magnetic particle concentrator (MPC®-M or MPC®-S), with its magnetic strip in place.

13.3.2.13 Without removing the microcentrifuge tube from MPC®-M, gently rock/roll the tube through 180° by hand. Continue for approximately 1 minute with approximately one 180° roll/rock per second. At the end of this step, the beads should produce a distinct brown dot at the back of the tube.

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

13.3.3 Dissociation of beads/oocyst complex

NOTE: Two acid dissociations are required.

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

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

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

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

13.3.3.4 Vortex vigorously for approximately 30 seconds.

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

13.3.3.6 Replace magnetic strip in MPC®-M and allow the tube to stand undisturbed for a minimum of 10 seconds.

13.3.3.7 Prepare a well slide for sample screening and label the slide.

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

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

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

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

NOTE: The wells on Dynal Spot-On slides are likely to be too small to accommodate the volumes from both dissociations.

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

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

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

- Make sure the resuspended pellet is fully homogenized before placing the tube in the MPC®-1 or MPC®-M to avoid trapping "clumps" or a dirty layer between the beads and the side of the tube.
- When using the MPC®-1 magnet, make sure that the tube is snugged flat against the magnet. Push the tube flat if necessary. Sometimes the magnet is not flush with the outside of the holder and, therefore, the attraction between the beads and the magnet is not as strong as it should be. However, it can be difficult to determine this if you do not have more than one MPC®-1 to make comparisons.
- After the supernatant has been poured off at Section 13.3.2.11, leave the tube in the MPC®-1 and allow time for any supernatant remaining in the tube to settle down to the bottom. Then aspirate the settled supernatant and associated particles from the bottom of the tube. The same can be done at Section 13.3.2.16 with the microcentrifuge tube.
- An additional rinse can also be performed at Section 13.3.2.11. After the supernatant has been poured off and any settled material is aspirated off the bottom, leave the tube in the MPC®-1 and add an additional 10 mL of reagent water or PBS to the tube and repeat Sections 13.3.2.9 and 13.3.2.11. Although labs have reported successfully using this technique to reduce carryover, because the attraction between the MPC®-1 and the beads is not as great as the attraction between the MPC®-M and the beads, the chances would be greater for loss of oocysts doing the rinse at this step instead of at Section 13.3.2.16.
- After the supernatant has been aspirated from the tube at Section 13.3.2.16, add 0.1 mL of PBS, remove the tube from the MPC®-M, and resuspend. Repeat Sections 13.3.2.15 and 13.3.2.16.
- Use a slide with the largest diameter well available to spread out the sample as much as possible.

14.0 Sample Staining

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

14.1 Prepare positive and negative controls.

14.1.1 For the positive control, pipette 10 µL of positive antigen or 200 to 400 intact oocysts to the center of a well.

14.1.2 For the negative control, pipette 50 µL of PBS (Section 7.4.2.1) into the center of a well and spread it over the well area with a pipette tip.

14.1.3 Air-dry the control slides (see Section 13.3.3.12 for guidance).

NOTE: If the laboratory has a large batch of slides that will be examined over several days, and is concerned that a single positive control may fade, due to multiple examinations, the laboratory should prepare multiple control slides with the batch of field slides and alternate between the positive controls when performing the positive control check.

14.2 Follow manufacturer's instructions in applying stain to slides.

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

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

14.5 Apply one drop of wash buffer (prepared according to the manufacturer's instructions [Section 7.6]) to each well. Tilt each slide on a clean paper towel, long edge down. Gently aspirate the excess detection reagent from below the well using a clean Pasteur pipette or absorb with paper towel or other absorbent material placed at edge of slide. Avoid disturbing the sample.

14.6 Apply 50 µL of 4',6-diamidino-2-phenylindole (DAPI) staining solution (Section 7.7.2) to each well. Allow to stand at room temperature for a minimum of 1 minute. (The solution concentration may be increased up to 1 µg/mL if fading/diffusion of DAPI staining is encountered, but the staining solution must be tested first on expendable environmental samples to confirm that staining intensity is appropriate.)

14.7 Apply one drop of wash buffer (prepared according to the manufacturer's instructions [Section 7.6]) to each well. Tilt each slide on a clean paper towel, long edge down. Gently aspirate the excess DAPI staining solution from below the well using a clean Pasteur pipette or absorb with paper towel or other absorbent material placed at edge of slide. Avoid disturbing the sample.

NOTE: If using the MeriFluor® Cryptosporidium/Giardia (Section 7.6.1), do not allow slides to dry completely.

14.8 Add mounting medium (Section 7.8) to each well.

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

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

15.0 Examination

NOTE: Although immunofluorescence assay (FA) and 4',6-diamidino-2-phenylindole (DAPI) and differential interference contrast (DIC) microscopy examination should be 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. However, if fading/diffusion of FITC or DAPI fluorescence is noticed, the laboratory must reduce this holding time. In addition the laboratory may adjust the concentration of the DAPI staining solution (Sections 7.7.2) so that fading/diffusion does not occur.

15.1 Scanning technique: Scan each well in a systematic fashion. An up-and-down or a side-to-side scanning pattern may be used (Figure 4).

15.2 Examination using immunofluorescence assay (FA), 4',6-diamidino-2-phenylindole (DAPI) staining characteristics, and differential interference contrast (DIC) microscopy. The minimum magnification requirements for each type of examination are noted below.

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

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

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

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

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

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

15.2.2 Sample examination—*Cryptosporidium*

15.2.2.1 **FITC examination** (the analyst must use a minimum of 200X total magnification). Use epifluorescence to scan the entire well for apple-green fluorescence of oocyst and cyst shapes. When brilliant apple-green fluorescing ovoid or spherical objects 4 to 6 µm in diameter are observed with brightly highlighted edges, increase magnification to 400X and

switch the microscope to the UV filter block for DAPI (Section 15.2.2.2), then to DIC (Section 15.2.2.3) at 1000X.

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

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

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

15.2.2.3 **DIC examination** (the analyst must use a minimum of 1000X total magnification [oil immersion lens]). Using DIC, look for external or internal morphological characteristics atypical of *Cryptosporidium* oocysts (e.g., spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.) (adapted from Reference 20.10). If atypical structures are not observed, then categorize each apple-green fluorescing object as:

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

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

15.2.2.4 A positive result is a *Cryptosporidium* oocyst which exhibits typical IFA fluorescence, typical size and shape and exhibits nothing atypical on IFA, DAPI fluorescence, or DIC microscopy. A positive result must be characterized and assigned to one of the DAPI and DIC categories in Sections 15.2.2.2 and 15.2.2.3.

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

15.2.4 Report *Cryptosporidium* concentrations as oocysts/L.

15.2.5 Record analyst name.

16.0 Analysis of Complex Samples

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

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

16.3 If the holding times for the sample and for microscopic examination of the cleaned up retentate/eluate have been exceeded, the site should be re-sampled. If this is not possible, the results should be qualified accordingly.

16.4 Some samples may adhere to the centrifuge tube walls. The use of siliconized or low-adhesion centrifuge tubes (Fisherbrand siliconized/low retention microcentrifuge tubes, 02-681-320 or equivalent) may reduce adhesion. Alternately, rinse centrifuge tubes with PBST elution buffer or Sigmacote® prior to use.

17.0 Method Performance

17.1 Method acceptance criteria are shown in Tables 3 and 4 in Section 21.0. The initial and ongoing precision and recovery criteria are based on the results of spiked reagent water samples analyzed during the Information Collection Rule Supplemental Surveys (Reference 20.11). The matrix spike and matrix spike duplicate criteria are based on spiked source water data generated during the interlaboratory validation study of Method 1622 involving 11 laboratories and 11 raw surface water matrices across the U.S. (Reference 20.14).

NOTE: Some sample matrices may prevent the MS acceptance criteria in Tables 3 and 4 to be met. An assessment of the distribution of MS recoveries across 430 MS samples from 87 sites during the ICR Supplemental Surveys is provided in Table 5.

18.0 Pollution Prevention

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

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

19.0 Waste Management

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

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

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

20.0 References

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21.0 Tables and Figures

Table 1. Method Holding Times (See Section 8.2 for details)

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

Table 2. Tier 1 and Tier 2 Validation/Equivalency Demonstration Requirements

Test	Description	Tier 1 modification(1)	Tier 2 modification(2)
IPR (Section 9.4)	4 replicates of spiked reagent water	Required. Must be accompanied by a method blank.	Required per laboratory
Method blank (Section 9.6)	Unspiked reagent water	Required	Required per laboratory
MS (Section 9.5.1)	Spiked matrix water	Required on each water to which the modification will be applied and on every 20th sample of that water thereafter. Must be accompanied by an unspiked field sample collected at the same time as the MS sample	Not required
MS/MSD (Section 9.5)	2 replicates of spiked matrix water	Recommended, but not required. Must be accompanied by an unspiked field sample collected at the same time as the MS sample	Required per laboratory. Each laboratory must analyze a different water.

(1) If a modification will be used only in one laboratory, these tests must be performed and the results must meet all of the QC acceptance criteria in the method (these tests also are required the first time a laboratory uses the validated version of the method)

(2) If nationwide approval of a modification is sought for one type of water matrix (such as surface water), a minimum of 3 laboratories must perform the tests and the results from each lab individually must meet all QC acceptance criteria in the method. If more than 3 laboratories are used in a study, a minimum of 75% of the laboratories must meet all QC acceptance criteria.

NOTE: The initial precision and recovery and ongoing precision and recovery (OPR) acceptance criteria listed in Table 3 are based on results from 293 Cryptosporidium OPR samples analyzed by six laboratories during the Information Collection Rule Supplemental Surveys (Reference 20.15). The matrix spike acceptance criteria are based on data generated through interlaboratory validation of Method 1623 (Reference 20.14).

Table 3. Quality Control Acceptance Criteria for *Cryptosporidium*

Performance test	Section	Acceptance criteria
Initial precision and recovery	9.4	
Mean recovery (percent)	9.4.3	24 - 100
Precision (as maximum relative standard deviation)	9.4.3	55
Ongoing precision and recovery (percent)	9.7	11 - 100
Matrix spike/matrix spike duplicate (for method modifications)	9.5	
Mean recovery[1, 2] (as percent)	9.5.2.2	13 - 143
Precision (as maximum relative percent difference)	9.5.2.3	67

(1) The acceptance criteria for mean MS/MSD recovery serves as the acceptance criteria for MS recovery during routine use of the method (Section 9.5.1).

(2) Some sample matrices may prevent the acceptance criteria from being met. An assessment of the distribution of MS recoveries from multiple MS samples from 87 sites during the ICR Supplemental Surveys is provided in Table 4.

Table 4. Distribution of Matrix Spike Recoveries from Multiple Samples Collected from 87 Source Waters During the ICR Supplemental Surveys (Adapted from Reference 20.16)

MS Recovery Range	Percent of 430 *Cryptosporidium* MS Samples in Recovery Range
<10%	6.7%
>10% - 20%	6.3%
>20% - 30%	14.9%
>30% - 40%	14.2%
>40% - 50%	18.4%
>50% - 60%	17.4%
>60% - 70%	11.2%
>70% - 80%	8.4%
>80% - 90%	2.3%
>90%	0.2%

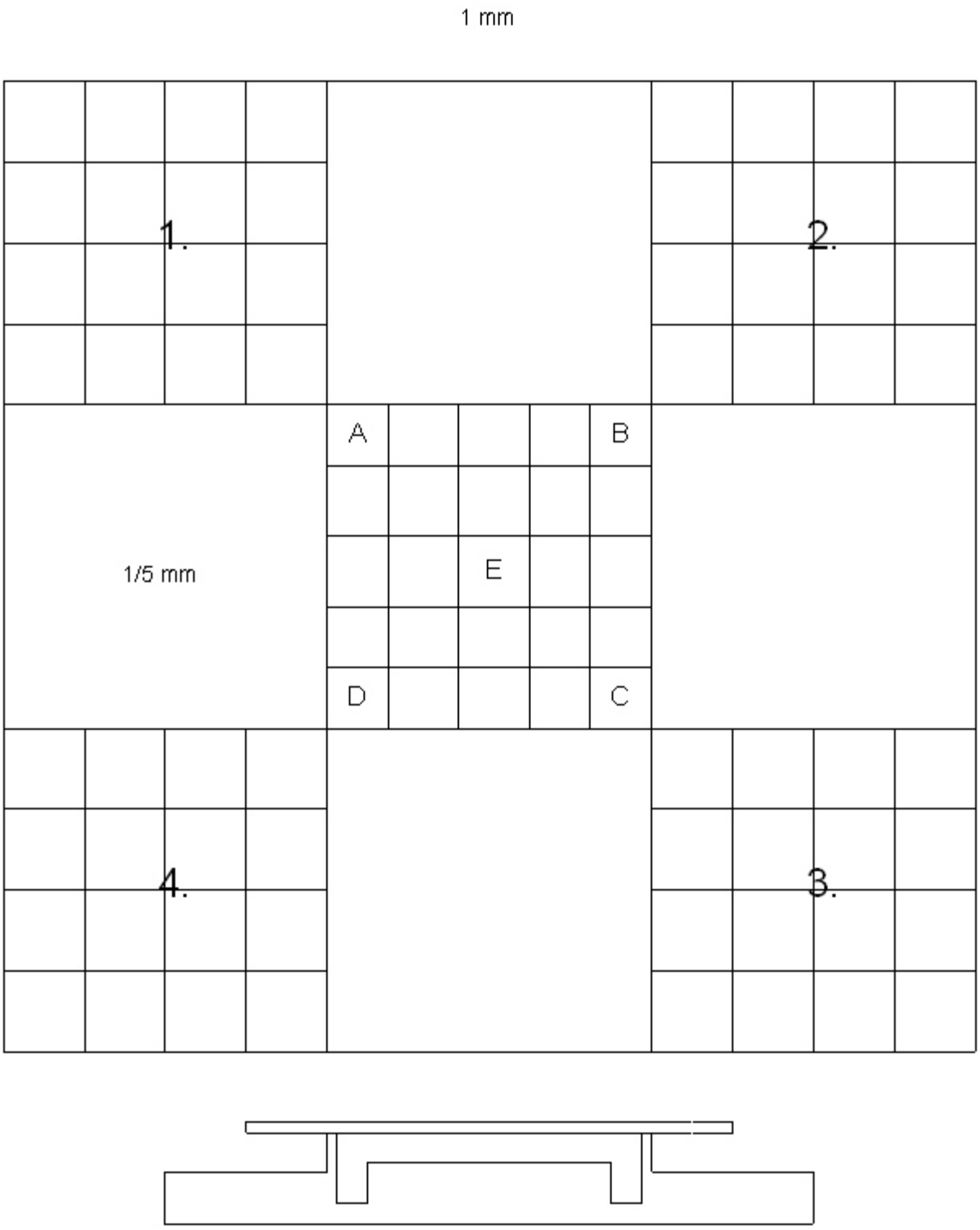

Figure 1. Hemacytometer Platform Ruling. Squares 1, 2, 3, and 4 are used to count stock suspensions of *Cryptosporidium* oocysts (after Miale, 1967)

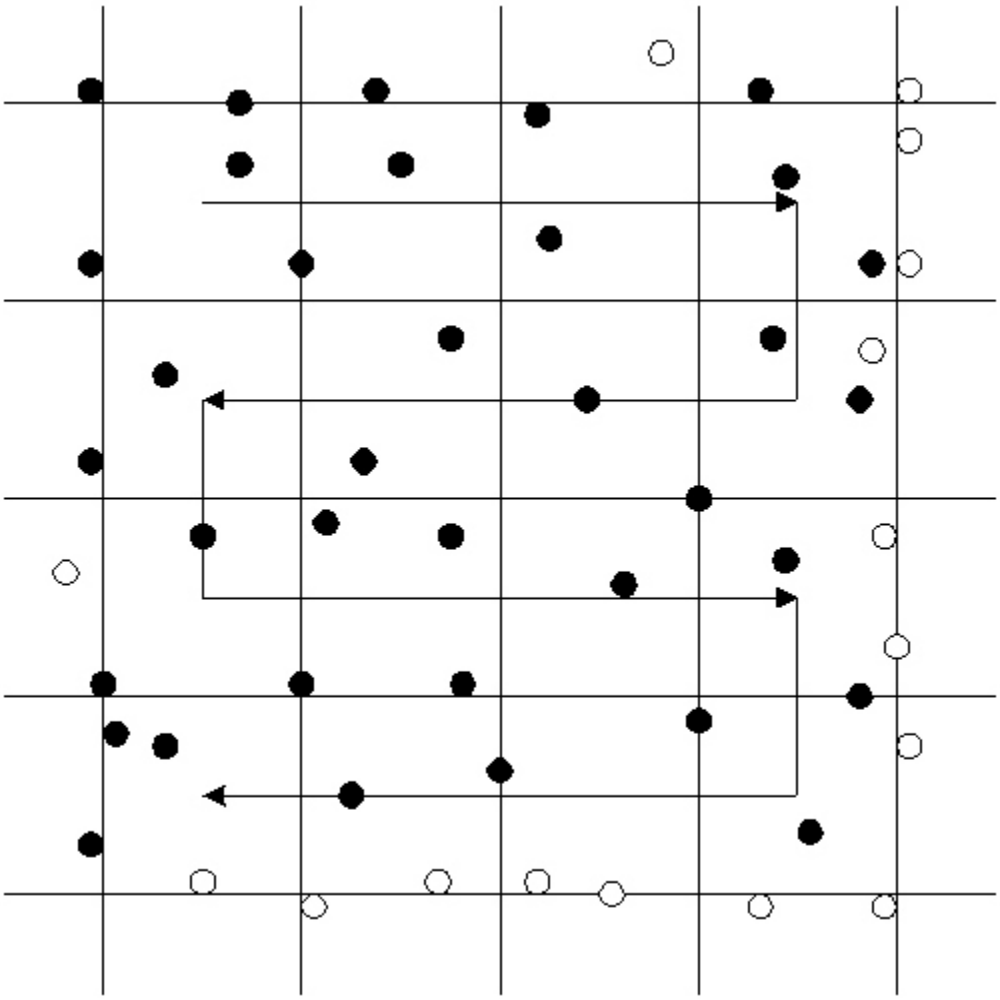

Figure 2. Manner of Counting Oocysts in 1 Square mm. Dark organisms are counted and light organisms are omitted (after Miale, 1967).

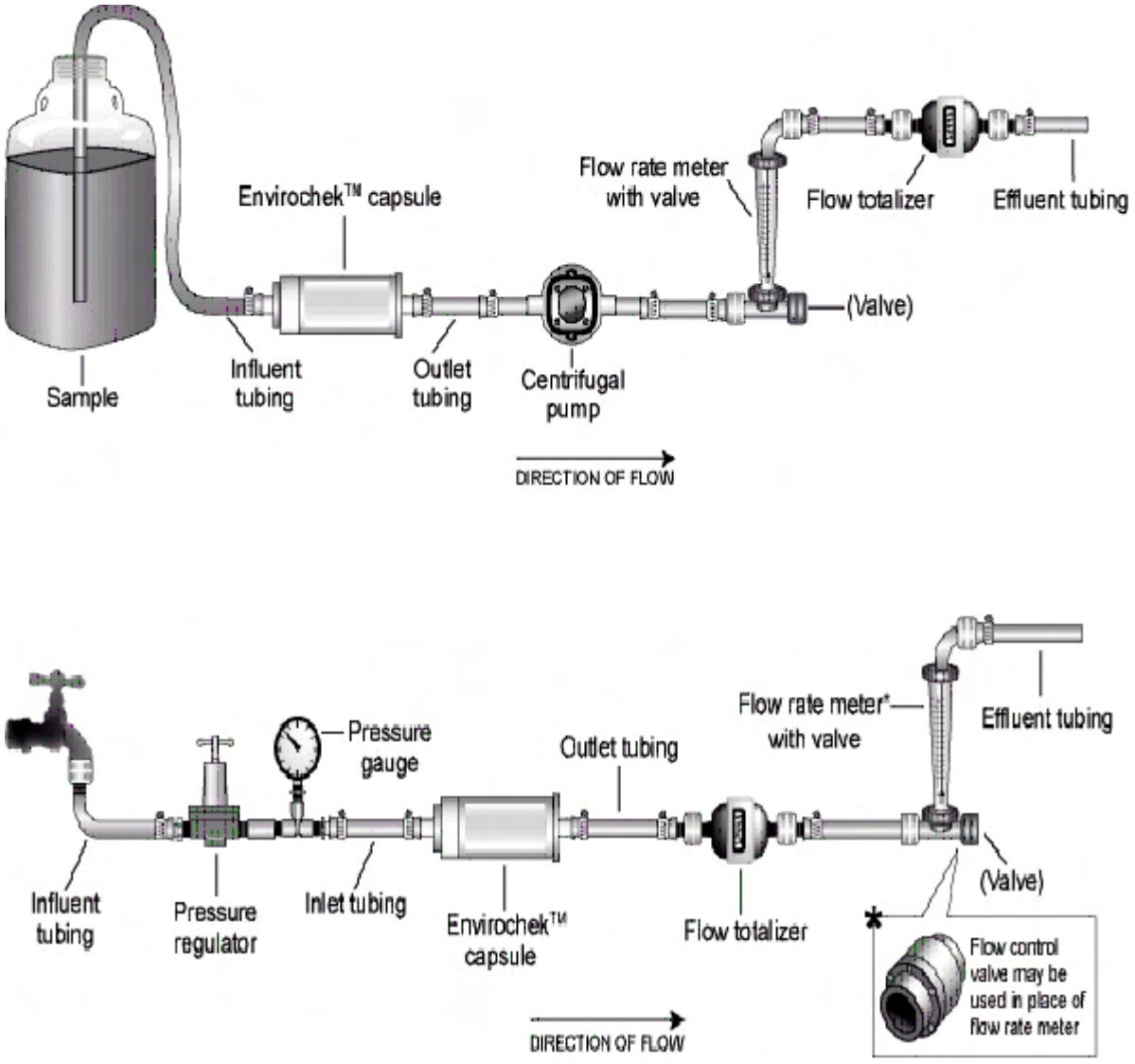

Figure 3a. Filtration Systems for Envirochek™ or Envirochek™HV Capsule (unpressurized source - top, pressurized source - bottom)

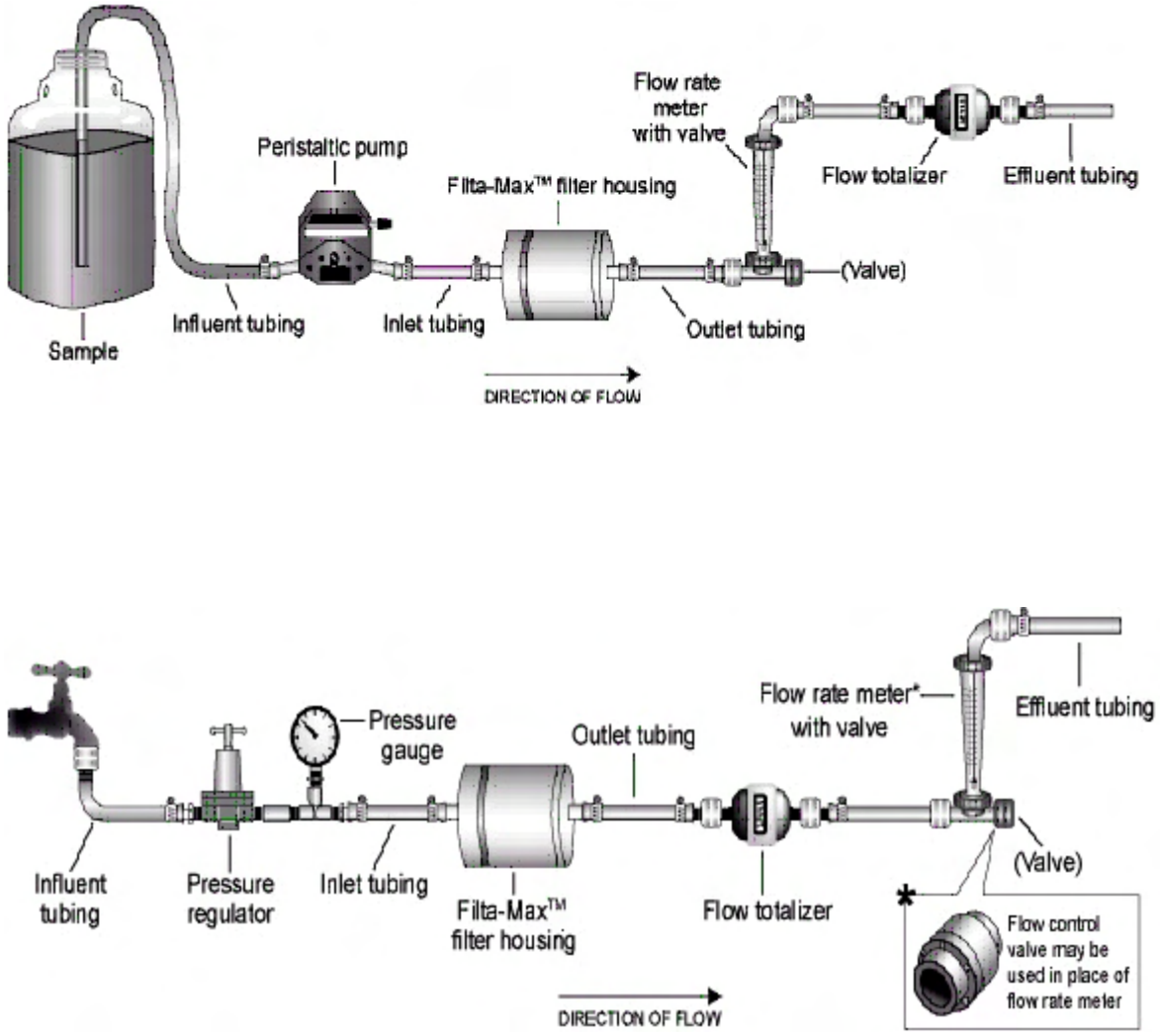

Figure 3b. Filtration Systems for Filta-Max® filters (unpressurized source - top, pressurized source - bottom)

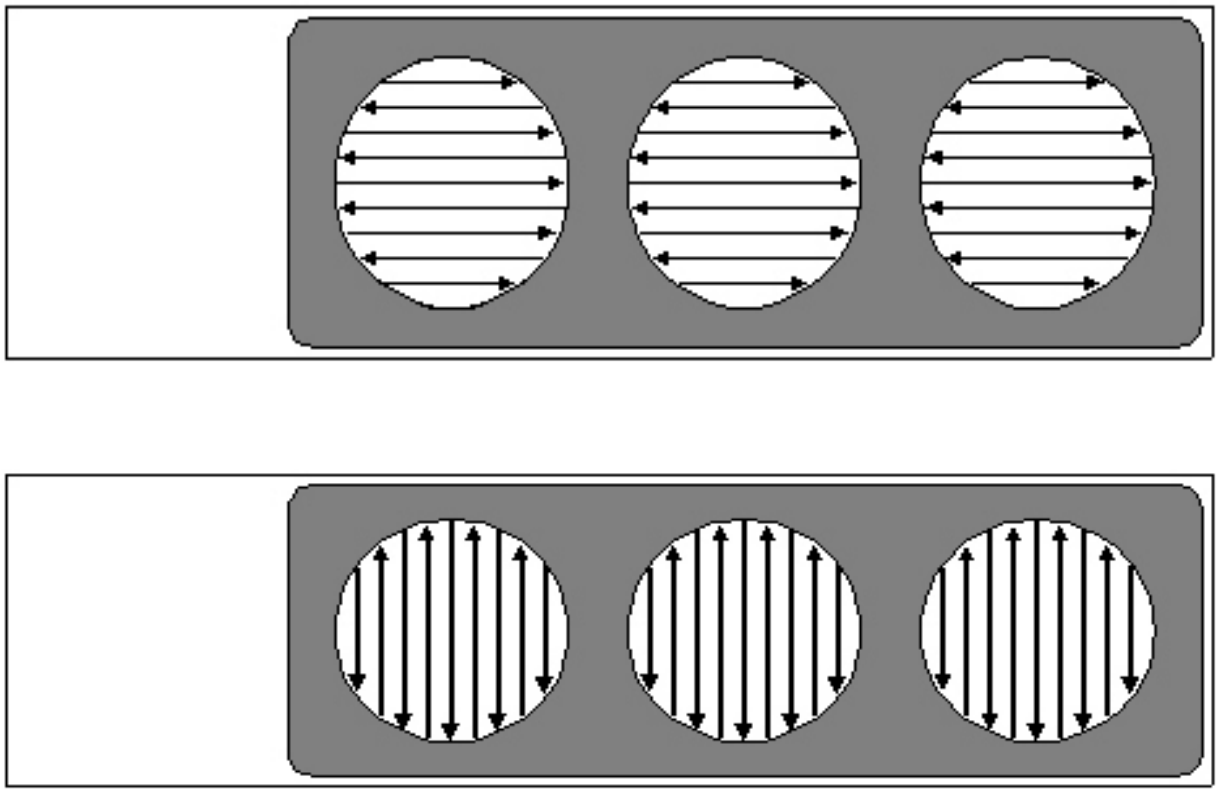

Figure 4. Methods for Scanning a Well Slide

22.0 Glossary of Definitions and Purposes

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

22.1 Units of weight and measure and their abbreviations

22.1.1 Symbols

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

22.1.2 Alphabetical characters

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

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

Analyst—The analyst should have at least 2 years of college in microbiology or equivalent or closely related field. The analyst also should have a minimum of 6 months of continuous bench experience with *Cryptosporidium* and IFA microscopy. The analyst should have a minimum of 3 months experience using EPA Method 1622 and/or EPA Method 1623 and should have successfully analyzed a minimum of 50 samples using EPA Method 1622 and/or EPA Method 1623.

Analyte—A protozoan parasite tested for by this method. The analyte in this method is *Cryptosporidium*.

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

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

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

Initial precision and recovery (IPR)—Four aliquots of spiking suspension analyzed to establish the ability to generate acceptable precision and accuracy. An IPR is performed prior to the first time this method is used and any time the method or instrumentation is modified.

Laboratory blank—See Method blank

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

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

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

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

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

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

Negative control—See Method blank

Nucleus—A membrane-bound organelle containing genetic material. Nuclei are a prominent internal structure seen both in *Cryptosporidium* oocysts. In *Cryptosporidium* oocysts, there is one nucleus per sporozoite.

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

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

Oocyst and cyst spiking suspension—See Spiking suspension

Oocyst and cyst stock suspension—See Stock suspension

Positive control—See Ongoing precision and recovery standard

Principal analyst—The principal analyst (may not be applicable to all monitoring programs) should have a BS/BA in microbiology or closely related field and a minimum of 1 year of continuous bench experience with *Cryptosporidium* and IFA microscopy. The principal analyst also should have a minimum of 6 months experience using EPA Method 1622 and/or EPA

Method 1623 and should have analyzed a minimum of 100 samples using EPA Method 1622 and/or EPA Method 1623.

PTFE—Polytetrafluoroethylene

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

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

Reagent water blank—see Method blank

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

RSD—See Relative standard deviation

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

Spiking suspension—Diluted stock suspension containing the organism(s) of interest at a concentration appropriate for spiking samples.

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

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

Technician—The technician filters samples, performs centrifugation, elution, concentration, and purification using IMS, and places purified samples on slides for microscopic examination, but does not perform microscopic protozoan detection and identification. No minimum education or experience requirements with *Cryptosporidium* and IFA microscopy apply to the technician. The technician should have at least 3 months of experience in filter extraction and processing of protozoa samples by EPA Method 1622/1623 and should have successfully processed a minimum of 50 samples using EPA Method 1622/1623.

Appendix C

Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA

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EPA
United States
Environmental Protection
Agency

Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA

December 2005

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Cryptosporidium cover photo courtesy of the U.S. Centers for Disease Control

Giardia cover photo courtesy of CH Diagnostic & Consulting Service, Inc.

Disclaimer

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

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Introduction

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

Method Development and Validation

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

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

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

Changes in the April 2001 Versions of the Methods

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

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

- Modified IMS acid dissociation procedure
- Updated QC acceptance criteria for IPR and OPR tests
- Addition of a troubleshooting section for QC failures
- Modified holding times
- Inclusion of flow cytometry–sorted spiking suspensions

Changes in the June 2003 Versions of the Methods

Both methods were revised again in June 2003 to support proposal of EPA's Long Term 2 Enhanced Surface Water Treatment Rule. Changes incorporated into the December 2002 versions include:

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

Changes in the December 2005 Versions of the Methods

Both methods were revised again in 2005 to support promulgation of EPA's Long Term 2 Enhanced Surface Water Treatment Rule. Changes incorporated into the June 2003 versions include:

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

Performance-Based Method Concept and Modifications Approved for Nationwide Use

EPA Method 1623 is a performance-based method applicable to the determination of *Cryptosporidium* and *Giardia* in aqueous matrices. EPA Method 1623 requires filtration, immunomagnetic separation of the oocysts and cysts from the material captured, and enumeration of the target organisms based on the results of immunofluorescence assay, 4',6-diamidino-2-phenylindole (DAPI) staining results, and differential interference contrast microscopy.

The interlaboratory validation of EPA Method 1623 conducted by EPA used the Pall Gelman capsule filtration procedure, Dynal immunomagnetic separation (IMS) procedure, and Meridian sample staining procedure described in this document. Alternate procedures are allowed, provided that required quality control tests are performed and all quality control acceptance criteria in this method are met.

Since the interlaboratory validation of EPA Method 1623, interlaboratory validation studies have been performed to demonstrate the equivalency of modified versions of the method using the following components:

- Whatman Nuclepore CryptTest™ filter (no longer available)
- IDEXX Filta-Max® filter
- Pall Gelman Envirochek™ HV filter
- Portable Continuous-Flow Centrifugation (PCFC)
- Waterborne Aqua-Glo™ G/C Direct FL antibody stain
- Waterborne Crypt-a-Glo™ and Giardi-a-Glo™ antibody stains
- BTF EasyStain™ antibody stain
- BTF EasySeed™ irradiated oocysts and cysts for use in routine QC samples

The validation studies for these modified versions of the method met EPA's performance-based measurement system Tier 2 validation for nationwide use (see Section 9.1.2 for details), and have been accepted by EPA as equivalent in performance to the original version of the method validated by EPA. The equipment and reagents used in these modified versions of the method are noted in Sections 6 and 7 of the method.

Because this is a performance-based method, other alternative components not listed in the method may be available for evaluation and use by the laboratory. Confirming the acceptable performance of a modified version of the method using alternate components in a single laboratory does not require that an interlaboratory validation study be conducted. However, method modifications validated only in a single laboratory have not undergone sufficient testing to merit inclusion in the method. Only those modified versions of the method that have been demonstrated as equivalent at multiple laboratories on multiple water sources through a Tier 2 interlaboratory study will be cited in the method.

Table of Contents

Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA

1.0 Scope and Application

1.1 This method is for the detection of *Cryptosporidium* (CAS Registry number 137259-50-8) and *Giardia* (CAS Registry number 137259-49-5) in water by concentration, immunomagnetic separation (IMS), and immunofluorescence assay (FA) microscopy. *Cryptosporidium* and *Giardia* may be verified using 4',6-diamidino-2-phenylindole (DAPI) staining and differential interference contrast (DIC) microscopy. The method has been validated in surface water, but may be used in other waters, provided the laboratory demonstrates that the method's performance acceptance criteria are met.

1.2 This method is designed to meet the survey and monitoring requirements of the U.S. Environmental Protection Agency (EPA). It is based on laboratory testing of recommendations by a panel of experts convened by EPA. The panel was charged with recommending an improved protocol for recovery and detection of protozoa that could be tested and implemented with minimal additional research.

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

1.4 This method is for use only by persons experienced in the determination of *Cryptosporidium* and *Giardia* by filtration, IMS, and FA. Experienced persons are defined in Section 22.2 as analysts or principal analysts. Laboratories unfamiliar with analyses of environmental samples by the techniques in this method should gain experience using water filtration techniques, IMS, fluorescent antibody staining with monoclonal antibodies, and microscopic examination of biological particulates using bright-field and DIC microscopy.

1.5 Any modification of the method beyond those expressly permitted is subject to the application and approval of alternative test procedures under 40 *CFR* Part 141.27.

2.0 Summary of Method

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

2.2 Elution and separation

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

2.2.2 The oocysts and cysts are magnetized by attachment of magnetic beads conjugated to anti-*Cryptosporidium* and anti-*Giardia* antibodies. The magnetized oocysts and cysts are separated from the extraneous materials using a magnet, and the extraneous materials are discarded. The magnetic bead complex is then detached from the oocysts and cysts.

2.3 Enumeration

2.3.1 The oocysts and cysts are stained on well slides with fluorescently labeled monoclonal antibodies and 4',6-diamidino-2-phenylindole (DAPI). The stained sample is examined using fluorescence and differential interference contrast (DIC) microscopy.

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

2.3.3 Quantitative analysis is performed by counting the total number of objects on the slide confirmed as oocysts or cysts.

2.4 Quality is assured through reproducible calibration and testing of the filtration, immunomagnetic separation (IMS), staining, and microscopy systems. Detailed information on these tests is provided in Section 9.0.

3.0 Definitions

3.1 *Cryptosporidium* is a genus of protozoan parasites potentially found in water and other media. The recent taxonomy of the genus *Cryptosporidium* includes the following species and their potential hosts: *C. hominis* (humans; formerly *C. parvum* genotype I; Reference 20.1); *C. parvum* (bovine and other mammals including humans; formerly genotype II;); *C. baileyi* and *C. meleagridis* (birds); *C. muris* (rodents); *C. canis* (dogs); *C. felis* (cats); *C. serpentis* (reptiles); and *C. nasorum* (fish). *Cryptosporidium* oocysts are defined in this method as objects exhibiting brilliant apple green fluorescence under UV light (FA-positive), typical size (4 to 6 µm) and shape (round to oval), and no atypical characteristics by FA, DAPI fluorescence, or DIC microscopy. Examination and characterization using fluorescence (FITC and DAPI stain) and DIC microscopy are required for exclusion of atypical organisms (e.g., those possessing spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.).

3.2 *Giardia* is a genus of protozoan parasites potentially found in water and other media. The recent taxonomy of the genus *Giardia* includes the following species and their potential hosts: *G. lamblia* (also called *G. intestinalis* or *G. duodenalis*; humans and other mammals); *G. muris* (rodents); *G. agilis* (amphibians); *G. psittaci* and *G. ardeae* (birds). Recent molecular studies suggest the division of *G. lamblia* into multiple genotypes (Reference 20.2). *Giardia* cysts are defined in this method as objects exhibiting brilliant apple green fluorescence under UV light (FA-positive), typical size (8 to 18 µm long by 5 to 15 µm wide) and shape (oval to round), and no atypical characteristics by FA, DAPI fluorescence, or DIC microscopy. Examination and characterization by fluorescence (FITC and DAPI stain) and DIC microscopy are required for exclusion of atypical organisms (e.g., those possessing spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.).

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

4.0 Contamination, Interferences, and Organism Degradation

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

4.2 Organisms and debris that autofluoresce or demonstrate non-specific immunofluorescence, such as algal and yeast cells, when examined by epifluorescent microscopy, may interfere with the detection of oocysts and cysts and contribute to false positives by immunofluorescence assay (FA) (Reference 20.3).

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

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

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

5.0 Safety

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

5.2 The toxicity or carcinogenicity of each compound or reagent used in this method has not been precisely determined; however, each chemical compound should be treated as a potential health hazard. Exposure to these compounds should be reduced to the lowest possible level. The laboratory is responsible for maintaining current knowledge of Occupational Safety and Health Administration regulations regarding the safe handling of the chemicals specified in this method. A reference file of material safety data sheets should be made available to all personnel involved in these analyses. Additional information on laboratory safety can be found in References 20.4 through 20.7.

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

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

5.5 Centers for Disease Control (CDC) regulations (42 CFR 72) prohibit interstate shipment of more than 4 L of solution known to contain infectious materials (see http://www.cdc.gov/od/ohs/biosfty/shipregs.htm for details). State regulations may contain similar regulations for intrastate commerce. Unless the sample is known or suspected to contain *Cryptosporidium*, *Giardia*, or other infectious agents (e.g., during an outbreak), samples should be shipped as noninfectious and should not be marked as infectious. If a sample is known or suspected to be infectious, and the sample must be shipped to a laboratory by a transportation means affected by CDC or state regulations, the sample should be shipped in accordance with these regulations.

6.0 Equipment and Supplies

NOTE: Brand names, suppliers, and part numbers are for illustrative purposes only. No endorsement is implied. Equivalent performance may be achieved using apparatus and materials other than those specified here, but demonstration of equivalent performance that meets the requirements of this method is the responsibility of the laboratory.

6.1 Sample collection equipment for shipment of bulk water samples for laboratory filtration.

Collapsible LDPE cubitainer for collection of 10-L bulk sample(s)—Cole Parmer cat. no. U-06100-30 or equivalent. Fill completely to ensure collection of a full 10-L sample. Discard after one use.

6.2 Equipment for sample filtration. Four options have been demonstrated to be acceptable for use with Method 1623. Other options may be used if their acceptability is demonstrated according to the procedures outlined in Section 9.1.2.

6.2.1 Cubitainer spigot to facilitate laboratory filtration of sample (for use with any filtration option)—Cole Parmer cat. no. U-06061-01, or equivalent.

6.2.2 Original Envirochek™ sampling capsule or Envirochek™ HV sampling capsule equipment requirements (for use with the procedure described in Section 12.2). The versions of the method using these filters were validated using 10-L and 50-L sample volumes, respectively. Alternate sample volumes may be used, provided the laboratory demonstrates acceptable performance on initial and ongoing spiked reagent water and source water samples (Section 9.1.2).

6.2.2.1 Sampling capsule

6.2.2.1.1 Envirochek™, Pall Corporation, Ann Arbor, MI, part no. 12110 (individual filter) and or part no.12107 (box of 25 filters) (www.pall.com or (800) 521-1520 ext. 2)

6.2.2.1.2 Envirochek™ HV, Pall Corporation, Ann Arbor, MI, part no. 12099 (individual filter) or part no.12098 (box of 25 filters) (www.pall.com or (800) 521-1520 ext. 2)

6.2.2.2 Laboratory shaker with arms for agitation of sampling capsules

6.2.2.2.1 Laboratory shaker—Lab-Line model 3589 (available through VWR Scientific cat. no. 57039-055), Pall Corporation part no. 4821, Fisher cat. no. 14260-11, or equivalent

6.2.2.2.2 Side arms for laboratory shaker—Lab-Line Model 3587-4 (available through VWR Scientific cat. no. 57039-045), Fisher cat. no. 14260-13, or equivalent

6.2.3 Filta-Max® foam filter equipment requirements (for use with the procedure described in Section 12.3). The version of the method using this filter was validated using 50-L sample volumes; alternate sample volumes may be used, provided the laboratory demonstrates acceptable performance on initial and ongoing spiked reagent water and matrix samples (Section 9.1.2).

6.2.3.1 Foam filter—Filta-Max®, IDEXX, Westbrook, ME. Filter module cat. no. FMC 10603

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

6.2.3.2 Filter processing equipment—Filta-Max® starter kit, IDEXX, Westbrook, ME, cat. no. FMC 11002. Starter kit includes manual wash station with clamp set (FMC 10101 or 10106) including plunger head (FMC 12001), tubing set (FMC 10307), vacuum set (FMC 10401), MKII filter housing with hose-tail fittings (FMC 10504) and green housing tools (FMC 10506). In addition, processing requires magnetic stirrer (FMC 10901) and filter membranes, 100 pk, (FMC 10800).

6.2.4 Portable Continuous-Flow Centrifuge (PCFC) requirements (for use with procedures described in Section 12.4). The version of the method using this technique was validated for *Cryptosporidium* in 50-L sample volumes; alternate sample volumes may be used, provided the laboratory demonstrates acceptable performance on initial and ongoing spiked reagent water and matrix samples (Section 9.1.2). Individual laboratories are also permitted to demonstrate acceptable performance for *Giardia* in their laboratory. The technique is based on technology from Haemonetics Corporation, Braintree, MA.

6.3 Ancillary sampling equipment

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

6.3.2 Flow control valve—0.5 gpm (0.03 L/s), Bertram Controls, Plast-O-Matic cat. no. FC050B½-PV, or equivalent; or 0.4- to 4-Lpm flow meter with valve, Alamo Water Treatment, San Antonio, TX, cat. no. R5310, or equivalent

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

6.3.4 Flow meter—SaMeCo cold water totalizer, E. Clark and Associates, Northboro, MA, product no. WFU 10.110; Omega flow meter, Stamford, CT, model FTB4105; or equivalent. Alternatively, use a graduated carboy(s) (See Section 6.18)

6.4 Equipment for spiking samples in the laboratory

6.4.1 Collapsible 10-L LDPE cubitainer with cubitainer spigot—Cole Parmer cat. no. U-06100-30 or equivalent and Cole Parmer cat. no. U-06061-01, or equivalent. Discard after one use to eliminate possible contamination. Alternatively, use clean, 10-L carboy

with bottom delivery port (½"), Cole-Palmer cat. no. 06080-42, or equivalent; calibrate to 10.0 L and mark level with waterproof marker

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

6.4.3 Stir plate—Fisher cat. no. 11-510-49S, S50461HP, or equivalent

6.4.4 Hemacytometer—Neubauer type, Hausser Scientific, Horsham, PA, product no. 3200 or 1475, or equivalent

6.4.5 Hemacytometer coverslip—Hausser Scientific, product no. 5000 (for hemacytometer cat. no. 3200) or 1461 (for hemacytometer cat. no 1475), or equivalent

6.4.6 Lens paper without silicone—Fisher cat. no. 11-995, or equivalent

6.4.7 Polystyrene or polypropylene conical tubes with screw caps—15- and 50-mL

6.4.8 Equipment required for enumeration of spiking suspensions using membrane filters

6.4.8.1 Glass microanalysis filter holder—25-mm-diameter, with fritted glass support, Fisher cat. no. 09-753E, or equivalent. Replace stopper with size 8, one-hole rubber stopper, Fisher Cat. No. 14-135M, or equivalent.

6.4.8.2 Three-port vacuum filtration manifold and vacuum source—Fisher Cat. No. 09-753-39A, or equivalent

6.4.8.3 Cellulose acetate support membrane—1.2-µm-pore-size, 25-mm-diameter, Fisher cat. no. A12SP02500, or equivalent

6.4.8.4 Polycarbonate track-etch hydrophilic membrane filter—1-µm-pore-size, 25-mm-diameter, Fisher cat. no. K10CP02500, or equivalent

6.4.8.5 100 × 15 mm polystyrene petri dishes (bottoms only)

6.4.8.6 60 × 15 mm polystyrene petri dishes

6.4.8.7 Glass microscope slides—1 in. × 3 in or 2 in. × 3 in.

6.4.8.8 Coverslips—25 mm^2

6.5 Immunomagnetic separation (IMS) apparatus

6.5.1 Sample mixer—Dynal Inc., Lake Success, NY, cat. no. 947.01, or equivalent

6.5.2 Magnetic particle concentrator for 10-mL test tubes—Dynal MPC®-1 , cat. no. 120.01 or MPC®-6, cat. No 120.02, or equivalent

6.5.3 Magnetic particle concentrator for microcentrifuge tubes—Dynal MPC®-M, cat. no. 120.09 (no longer available); Dynal MPC®-S, cat. no. 120.20, or equivalent

6.5.4 Flat-sided sample tubes—16 × 125 mm Leighton-type tubes with 60 × 10 mm flat-sided magnetic capture area, Dynal L10, cat. no. 740.03, or equivalent

6.6 Powder-free latex gloves—Fisher cat no. 113945B, or equivalent

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

6.8 Centrifuges

6.8.1 Centrifuge capable of accepting 15- to 250-mL conical centrifuge tubes and achieving 1500 × G—International Equipment Company, Needham Heights, MA, Centrifuge Size 2, Model K with swinging bucket, or equivalent

6.8.2 Centrifuge tubes—Conical, graduated, 1.5-, 50-, and 250-mL

6.9 Microscope

6.9.1 Epifluorescence/differential interference contrast (DIC) with stage and ocular micrometers and 20X (N.A.=0.4) to 100X (N.A.=1.3) objectives—Zeiss™ Axioskop, Olympus™ BH, or equivalent. Hoffman Modulation Contrast optics may be equivalent.

6.9.2 Excitation/band-pass filters for immunofluorescence assay (FA)—Zeiss™ 487909 or equivalent, including, 450- to 490-nm exciter filter, 510-nm dicroic beam-splitting mirror, and 515- to 520-nm barrier or suppression filter

6.9.3 Excitation/band-pass filters for DAPI—Filters cited below (Chroma Technology, Brattleboro, VT), or equivalent

Microscope model	Fluoro-chrome	Excitation filter (nm)	Dichroic beam- splitting mirror (nm)	Barrier or suppression filter (nm)	Chroma catalog number
Zeiss™ - Axioskop	DAPI (UV)	340-380	400	420	CZ902
Zeiss™ -IM35	DAPI (UV)	340-380	400	420	CZ702
Olympus™ BH	DAPI (UV)	340-380	400	420	11000
	Filter holder				91002
Olympus™ BX	DAPI (UV)	340-380	400	420	11000
	Filter holder				91008
Olympus™ IMT2	DAPI (UV)	340-380	400	420	11000
	Filter holder				91003

6.10 Ancillary equipment for microscopy

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

6.10.2 Glass coverslips—22 × 50 mm

6.10.3 Nonfluorescing immersion oil—Type FF, Cargille cat. no. 16212, or equivalent

6.10.4 Micropipette, adjustable: 0- to 10-µL with 0- to 10-µL tips
10- to 100-µL, with 10- to 200-µL tips
100- to 1000-µL with 100- to 1000-µL tips

6.10.5 Forceps—Splinter, fine tip

6.10.6 Forceps—Blunt-end

6.10.7 Desiccant—Drierite™ Absorbent, Fisher cat. no. 07-577-1A, or equivalent

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

6.11 Pipettes—Glass or plastic

6.11.1 5-, 10-, and 25-mL

6.11.2 Pasteur, disposable

6.12 Balances

6.12.1 Analytical—Capable of weighing 0.1 mg

6.12.2 Top loading—Capable of weighing 10 mg

6.13 pH meter

6.14 Incubator—Fisher Scientific Isotemp™, or equivalent

6.15 Vortex mixer—Fisons Whirlmixer, or equivalent

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

6.17 Miscellaneous labware and supplies

6.17.1 Test tubes and rack

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

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

6.17.4 Lint-free tissues

6.18 10- to 15-L graduated container—Fisher cat. no. 02-961-50B, or equivalent; calibrate to 9.0, 9.5, 10.0, 10.5, and 11.0 L and mark levels with waterproof marker

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

7.0 Reagents and Standards

7.1 Reagents for adjusting pH

7.1.1 Sodium hydroxide (NaOH)—ACS reagent grade, 6.0 N and 1.0 N in reagent water

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

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

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

7.3 Reagent water—Water in which oocysts and cysts and interfering materials and substances, including magnetic minerals, are not detected by this method. See Reference 20.8 (Section 9020) for reagent water requirements.

7.4 Reagents for eluting filters

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

7.4.1 Reagents for eluting Envirochek™ and Envirochek™ HV sampling capsules (Section 6.2.2)

7.4.1.1 Laureth-12—PPG Industries, Gurnee, IL, cat. no. 06194, or equivalent. Store Laureth-12 as a 10% solution in reagent water. Weigh 10 g of Laureth-12 and dissolve using a microwave or hot plate in 90 mL of reagent water. Dispense 10-mL aliquots into sterile vials and store at room temperature for up to 2 months, or in the freezer for up to a year.

7.4.1.2 1 M Tris, pH 7.4—Dissolve 121.1 g Tris (Fisher cat. no. BP152) in 700 mL of reagent water and adjust pH to 7.4 with 1 N HCl or NaOH. Dilute to a final 1000 mL with reagent water and adjust the final pH. Filter-sterilize through a 0.2-µm membrane into a sterile plastic container and store at room temperature. Alternatively, use prepared TRIS, Sigma T6066 or equivalent.

7.4.1.3 0.5 M EDTA, 2 Na, pH 8.0—Dissolve 186.1 g ethylenediamine tetraacetic acid, disodium salt dihydrate (Fisher cat. no. S311) in 800 mL of reagent water and adjust pH to 8.0 with 6.0 N HCl or NaOH. Dilute to a final volume of 1000 mL with reagent water and adjust to pH 8.0 with 1.0 N HCl or NaOH. Alternatively, use prepared EDTA, Sigma E5134 or equivalent.

7.4.1.4 Antifoam A—Sigma Chemical Co. cat. no. A5758, or equivalent

7.4.1.5 Preparation of elution buffer solution—Add the contents of a pre-prepared Laureth-12 vial (Section 7.4.1.1) to a 1000-mL graduated

cylinder. Rinse the vial several times to ensure the transfer of the detergent to the cylinder. Add 10 mL of Tris solution (Section 7.4.1.2), 2 mL of EDTA solution (Section 7.4.1.3), and 150 µL Antifoam A (Section 7.4.1.4). Dilute to 1000 mL with reagent water.

7.4.2 Reagents for eluting Filta-Max® foam filters (Section 6.2.3)

7.4.2.1 Phosphate buffered saline (PBS), pH 7.4—Sigma Chemical Co. cat. no. P-3813, or equivalent. Alternately, prepare PBS by adding the following to 1 L of reagent water: 8 g NaCl; 0.2 g KCl; 1.15 g Na_2HPO_4, anhydrous; and 0.2 g KH_2PO_4.

7.4.2.2 Tween® 20 —Sigma Chemical Co. cat. no. P-7949, or equivalent

7.4.2.3 High-vacuum grease—BDH/Merck. cat. no. 636082B, or equivalent

7.4.2.4 Preparation of PBST elution buffer. Add 100 µL of Tween® 20 to prepared PBS (Section 7.4.2.1). Alternatively, add the contents of one packet of PBS to 1.0 L of reagent water. Dissolve by stirring for 30 minutes. Add 100 µL of Tween® 20 . Mix by stirring for 5 minutes.

7.4.3 Reagents for Portable Continuous-Flow Centrifuge (Section 6.2.4)

7.4.3.1 Sodium dodecyl sulfate—Sigma Chemical Co. cat. no. 71730 or equivalent

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

7.4.3.3 Antifoam A—Sigma Chemical Co. cat. no. A5758, or equivalent

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

7.5 Reagents for immunomagnetic separation (IMS)—Dynabeads® GC-Combo, Dynal cat. nos. 730.02/730.12, or equivalent

7.6 Direct antibody labeling reagents for detection of oocysts and cysts. Store reagents between 1°C and 10°C and return promptly to this temperature after each use. Do not allow any of the reagents to freeze. The reagents should be protected from exposure to light. Diluted, unused working reagents should be discarded after 48 hours. Discard reagents after the expiration date is reached. The labeling reagents in Sections 7.6.1-7.6.3 have been approved for use with this method.

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

7.6.2 Aqua-Glo™ G/C Direct FL, Waterborne cat. no. A100FLR, New Orleans, LA, or equivalent

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

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

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NOTE: If a laboratory will use multiple types of labeling reagents, the laboratory must demonstrate acceptable performance through an initial precision and recovery test (Section 9.4) for each type, and must perform positive and negative staining controls for each batch of slides stained using each product. However, the laboratory is not required to analyze additional ongoing precision and recovery samples or method blank samples for each type. The performance of each labeling reagent used also should be monitored in each source water type.

7.6.5 Diluent for labeling reagents—Phosphate buffered saline (PBS) (Section 7.4.2).

7.7 4',6-diamidino-2-phenylindole (DAPI) stain—Sigma Chemical Co. cat. no. D9542, or equivalent

7.7.1 Stock solution—Dissolve 2 mg/mL DAPI in absolute methanol. Prepare volume consistent with minimum use. Store between 1°C and 10°C in the dark. Do not allow to freeze. Discard unused solution when positive staining control fails or after specified time determined by laboratory.

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

7.8 Mounting medium

7.8.1 DABCO/glycerol mounting medium (2%)—Dissolve 2 g of DABCO (Sigma Chemical Co. cat no. D-2522, or equivalent) in 95 mL of warm glycerol/PBS (60% glycerol, 40% PBS). After the DABCO has dissolved completely, adjust the solution volume to 100 mL by adding an appropriate volume of glycerol/PBS solution. Alternately, dissolve the DABCO in 40 mL of PBS, then add azide (1 mL of 100X, or 10% solution), then 60 mL of glycerol.

7.8.2 Mounting medium supplied with MeriFluor® *Cryptosporidium/Giardia*, Meridian Diagnostics cat. no. 250050, or equivalent (Section 7.6.1)

7.8.3 Mounting medium supplied with Aqua-Glo™ G/C Direct FL kit, Waterborne cat. no. A100FLR, cat. no. M101, or equivalent (Section 7.6.2)

7.8.4 Mounting medium supplied with EasyStain™C&G, BTF Pty Limited or equivalent (Section 7.6.4)

7.8.5 Elvanol or equivalent permanent, non-fade archiving mounting medium

7.9 Clear fingernail polish or clear fixative, PGC Scientifics, Gaithersburg, MD, cat. no. 60-4890-00, or equivalent

7.10 Oocyst and cyst suspensions for spiking

7.10.1 Enumerated spiking suspensions prepared by flow cytometer—not formalin fixed.

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

7.10.1.2 Irradiated, flow cytometer–sorted oocysts and cysts—flow cytometer–sorted oocysts and cysts—BTF EasySeed™ (contact@btfbio.com), or equivalent

7.10.2 Materials for manual enumeration of spiking suspensions

7.10.2.1 Purified *Cryptosporidium* oocyst stock suspension for manual enumeration—not formalin-fixed: Sterling Parasitology Laboratory, University of Arizona, Tucson, or equivalent

7.10.2.2 Purified *Giardia* cyst stock suspension for manual enumeration—not formalin-fixed: Waterborne, Inc., New Orleans, LA; Hyperion Research, Medicine Hat, Alberta, Canada; or equivalent

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

7.10.3 Storage procedure—Store oocyst and cyst suspensions between 1°C and 10°C, until ready to use; do not allow to freeze

7.11 Additional reagents for enumeration of spiking suspensions using membrane filtration (Section 11.3.6)—Sigmacote® Sigma Company Product No. SL-2, or equivalent

8.0 Sample Collection and Storage

8.1 Sample collection, shipment, and receipt

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

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

NOTE: See transportation precautions in Section 5.5.

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

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

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

8.1.2.4 Public water systems shipping samples to off-site laboratories for analysis should include in the shipping container a means for monitoring the temperature of the sample during shipping to verify that the sample did not freeze or exceed 20°C. Suggested approaches for monitoring sample temperature during shipping are discussed in Section 8.1.4.

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

8.1.4 **Suggestions on measuring sample temperature.** Given the importance of maintaining sample temperatures for *Cryptosporidium* and *Giardia* determination, laboratories performing analyses using this method must establish acceptance criteria for receipt of samples transported to their laboratory. Several options are available to measure sample temperature upon receipt at the laboratory and, in some cases, during shipment:

8.1.4.1 **Temperature sample.** One option, for filtered samples only (not for 10-L bulk samples), is for the sampler to fill a small, inexpensive sample bottle

with water and pack this "temperature sample" next to the filtered sample. The temperature of this extra sample volume is measured upon receipt to estimate the temperature of the filter. Temperature sample bottles are not appropriate for use with bulk samples because of the potential effect that the difference in sample volume may have in temperature equilibration in the sample cooler. *Example product:* Cole Parmer cat. no. U-06252-20.

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

8.1.4.3 **iButton.** Measures the sample temperature during shipment and upon receipt. An iButton is a small, waterproof device that contains a computer chip that can be programmed to record temperature at different time intervals. The information is then downloaded from the iButton onto a computer. The iButton should be placed in a temperature sample, rather than placed loose in the cooler, or attached to the sample container. This option is appropriate for use with both filtered and bulk samples. Information on Thermocron® iButtons is available from http://www.ibutton.com/. Distributors include http://www.pointsix.com/, http://www.rdsdistributing.com, and http://www.scigiene.com/.

8.1.4.4 **Stick-on temperature strips.** Another option is for the laboratory to apply a stick-on temperature strip to the outside of the sample container upon receipt at the laboratory. This option does not measure temperature as precisely as the other options, but provides an indication of sample temperature to verify that the sample temperature is acceptable. This option is appropriate for use with both filtered and bulk samples. *Example product:* Cole Parmer cat. no. U-90316-00.

8.1.4.5 **Infrared thermometers.** A final option is to measure the temperature of the surface of the sample container or filter using an infrared thermometer. The thermometer is pointed at the sample, and measures the temperature without coming in contact with the sample volume. This option is appropriate for use with both filtered and bulk samples. *Example product*: Cole Parmer cat. no. EW-39641-00.

As with other laboratory equipment, all temperature measurement devices must be calibrated routinely to ensure accurate measurements. See the EPA *Manual for the Certification of Laboratories Analyzing Drinking Water* (Reference 20.9) for more information.

8.2 **Sample holding times.** Samples must be processed or examined within each of the holding times specified in Sections 8.2.1 through 8.2.4. Sample processing should be completed as soon as possible by the laboratory. The laboratory should complete sample filtration, elution, concentration, purification, and staining the day the sample is received wherever possible. However, the laboratory is permitted to split up the sample processing steps if processing a sample completely in one day is not possible. If this is necessary, sample processing can be halted after filtration, application of the purified sample onto the slide, or staining. **Table 1**, in Section 21.0 provides a breakdown of the holding times for each set of steps. Sections 8.2.1 through 8.2.4 provide descriptions of these holding times.

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

8.2.2 **Sample elution, concentration, and purification.** The laboratory must complete elution, concentration, and purification (Sections 12.2.6 through 13.3.3.11) in one work day. It is critical that these steps be completed in one work day to minimize the time that any target organisms present in the sample sit in eluate or concentrated matrix. This process ends with the application of the purified sample on the slide for drying.

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

8.2.4 **Examination.** Although immunofluorescence assay (FA) and 4',6-diamidino-2-phenylindole (DAPI) and differential interference contrast (DIC) microscopy examination and characterization should be performed immediately after staining is complete, laboratories have up to 168 hours (7 days) from the completion of sample staining to perform the examination and verification of samples. However, if fading/diffusion of FITC or DAPI staining is noticed, the laboratory must reduce this holding time. In addition the laboratory may adjust the concentration of the DAPI staining solution (Sections 7.7.2) so that fading/diffusion does not occur.

8.3 **Spiking suspension enumeration holding times.** Flow-cytometer-sorted spiking suspensions (Sections 7.10.1 and 11.2) used for spiked quality control (QC) samples (Section 9) must be used within the expiration date noted on the suspension. Manually enumerated spiking suspensions must be used within 24 hours of enumeration of the spiking suspension if the hemacytometer chamber technique is used (Section 11.3.4); or within 24 hours of application of the spiking suspension to the slides if the well slide or membrane filter enumeration technique is used (Sections 11.3.5 and 11.3.6). Oocyst and cyst suspensions must be stored between 1°C and 10°C, until ready to use; do not allow to freeze.

9.0 Quality Control

9.1 Each laboratory that uses this method is required to operate a formal quality assurance (QA) program that addresses and documents data quality, instrument and equipment maintenance and performance, reagent quality and performance, analyst training and certification, and records storage and retrieval. General requirements and recommendations for QA and quality control (QC) procedures for microbiology laboratories are provided in References 20.8, 20.9, 20.10. The minimum analytical requirements of this program consist of an initial demonstration of laboratory capability (IDC) through performance of the initial precision and recovery (IPR) test (Section 9.4), and ongoing demonstration of laboratory capability and method performance through the matrix spike (MS) test (Section 9.5.1), the method blank test (Section 9.6), the ongoing precision and recovery (OPR) test (Section 9.7), staining controls (Section 14.1 and 15.2.1), and analyst verification tests (Section 10.6). Laboratory performance is compared to established performance criteria to determine if the results of analyses meet the performance characteristics of the method.

9.1.1 A test of the microscope used for detection of oocysts and cysts is performed prior to examination of slides. This test is described in Section 10.0.

9.1.2 In recognition of advances that are occurring in analytical technology, the laboratory is permitted to modify certain method procedures to improve recovery or lower the costs of measurements, provided that all required quality control (QC) tests are performed and all QC acceptance criteria are met. Method procedures that can be modified include front-end techniques, such as filtration or immunomagnetic separation (IMS). The laboratory is not permitted to use an alternate determinative technique to replace immunofluorescence assay in this method (the use of different determinative techniques are considered to be different methods, rather than modified version of this method).

However, the laboratory is permitted to modify the immunofluorescence assay procedure, provided that all required QC tests are performed (Section 9.1.2.1) and all QC acceptance criteria are met (see guidance on the use of multiple labeling reagents in Section 7.6).

NOTE: *Method modifications should be considered only to improve method performance, reduce cost, or reduce sample processing time. Method modifications that reduce cost or sample processing time, but that result in poorer method performance should not be used.*

9.1.2.1 Method modification validation/equivalency demonstration requirements

9.1.2.1.1 **Method modifications at a single laboratory.** Each time a modification is made to this method for use in a single laboratory, the laboratory must, at a minimum, validate the modification according to Tier 1 of EPA's performance-based measurement system (PBMS) (Table 2) to demonstrate that the modification produces results equivalent or superior to results produced by this method as written. Briefly, each time a modification is made to this method, the laboratory is required to demonstrate acceptable modified method performance through the IPR test (Section 9.4). IPR results must meet the QC acceptance criteria in Tables 3 and 4 in Section 21.0, and should be comparable to previous results using the unmodified procedure. Although not required, the laboratory also should perform a matrix spike/matrix spike duplicate (MS/MSD) test to demonstrate the performance of the modified method in at least one real-world matrix before analyzing field samples using the modified method. The laboratory is required to perform MS samples using the modified method at the frequency noted in Section 9.1.8. If the modified method involves changes that cannot be adequately evaluated through these tests, additional tests may be required to demonstrate acceptability.

9.1.2.1.2 **Method modifications for nationwide approval.** If the laboratory or a manufacturer seeks EPA approval of a method modification for nationwide use, the laboratory or manufacturer must, at a minimum, validate the modification according to Tier 2 of EPA's PBMS (Table 2). Briefly, at least three laboratories must perform IPR tests (Section 9.4) and MS/MSD (Section 9.5) tests using the modified method, and all tests must meet the QC acceptance criteria specified in Tables 3 and 4 in Section 21.0. Upon nationwide approval, laboratories electing to use the modified method still must demonstrate acceptable performance in their own laboratory according to the requirements in Section 9.1.2.1.1. If the modified method involves changes that cannot be adequately evaluated through these tests, additional tests may be required to demonstrate acceptability.

9.1.2.2 The laboratory is required to maintain records of modifications made to this method. These records include the following, at a minimum:

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

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

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

9.1.2.2.4 Results from all QC tests comparing the modified method to this method, including:

(a) IPR (Section 9.4)
(b) MS/MSD (Section 9.5)
(c) Analysis of method blanks (Section 9.6)

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

(a) Sample numbers and other identifiers
(b) Source of spiking suspensions, as well as lot number and date received (Section 7.10)
(c) Spike enumeration date and time
(d) All spiking suspension enumeration counts and calculations (Section 11.0)
(e) Sample spiking dates and times
(f) Volume filtered (Section 12.2.5.2)
(g) Filtration and elution dates and times
(h) Pellet volume, resuspended concentrate volume, resuspended concentrate volume transferred to IMS, and all calculations required to verify the percent of concentrate examined (Section 13.2)
(i) Purification completion dates and times (Section 13.3.3.11)
(j) Staining completion dates and times (Section 14.10)
(k) Staining control results (Section 15.2.1)
(l) All required examination information (Section 15.2.2)
(m) Examination completion dates and times (Section 15.2.4)
(n) Analysis sequence/run chronology
(o) Lot numbers of elution, IMS, and staining reagents
(p) Copies of bench sheets, logbooks, and other recordings of raw data
(q) Data system outputs, and other data to link the raw data to the results reported

9.1.3 The laboratory shall spike a separate sample aliquot from the same source to monitor method performance. The frequency of the MS test is described in Section 9.1.8 and the procedures are described in Section 9.5.1.

9.1.4 Analysis of method blanks is required to demonstrate freedom from contamination. The frequency of the analysis of method blanks is described in Section 9.1.7 and the procedures and criteria for analysis of a method blank are described in Section 9.6.

9.1.5 The laboratory shall, on an ongoing basis, demonstrate through analysis of the ongoing precision and recovery (OPR) sample that the analysis system is in control. Frequency of OPR samples is described in Section 9.1.7 and the procedures are described in Section 9.7.

9.1.6 The laboratory shall maintain records to define the quality of data that are generated. Development of accuracy statements is described in Sections 9.5.1.4 and 9.7.6.

9.1.7 The laboratory shall analyze one method blank (Section 9.6) and one OPR sample (Section 9.7) each week (7 day or 168 hours time period which begins with processing the OPR) in which samples are analyzed if 20 or fewer field samples are analyzed during this period. The laboratory shall analyze one laboratory blank and one OPR sample for every 20 samples if more than 20 samples are analyzed in a one week (7 day or 168 hours) period.

9.1.8 The laboratory shall analyze MS samples (Section 9.5.1) at a minimum frequency of 1 MS sample per 20 field samples from each source analyzed. The laboratory should analyze an MS sample when samples are first received from a PWS for which the laboratory has never before analyzed samples to identify potential method performance issues with the matrix (Section 9.5.1; Tables 3 and 4). If an MS sample cannot be analyzed on the first sampling event, the first MS sample should be analyzed as soon as possible to identify potential method performance issues with the matrix.

9.2 Micropipette calibration

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

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

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

9.2.4 If the weight of the reagent water is outside the acceptable limits, consult the manufacturer's instruction manual troubleshooting section and repeat steps described in Section 9.2.3. If problems with the pipette persist, the laboratory must send the pipette to the manufacturer for recalibration.

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

9.4 Initial precision and recovery (IPR)—To establish the ability to demonstrate control over the analytical system and to generate acceptable precision and recovery, the laboratory shall perform the following operations:

9.4.1 Using the spiking procedure in Section 11.4 and enumerated spiking suspensions (Section 7.10.1 or Section 11.3), spike, filter, elute, concentrate, separate (purify), stain,

and examine the four reagent water samples spiked with ~100-500 oocysts and ~100-500 cysts.

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

9.4.1.2 If more than one modification will be used for filtration and/or separation of samples, a separate set of IPR samples must be prepared for each modification.

9.4.1.3 The set of four IPR samples must be accompanied by analysis of an acceptable method blank (Section 9.6).

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

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

where:

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

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

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

9.4.4 Compare the mean and RSD to the corresponding method performance acceptance criteria for initial precision and recovery in Tables 3 and 4 in Section 21.0. If the mean and RSD for recovery meet the acceptance criteria, system performance is acceptable and analysis of blanks and samples may begin. If the mean or RSD falls outside the range for recovery, system performance is unacceptable. In this event, trouble-shoot the problem by starting at the end of the method (see guidance in Section 9.7.5), correct the problem and repeat the IPR test (Section 9.4.1).

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

9.4.6 Using 200X to 400X magnification, more than 50% of the oocysts or cysts must appear undamaged and morphologically intact; otherwise, the organisms in the spiking suspension may be of unacceptable quality or the analytical process may be damaging the organisms. If the quality of the organisms on the IPR test slides is unacceptable, examine the spiking suspension organisms directly (by centrifuging, if possible, to concentrate the organisms in a volume that can be applied directly to a slide). If the

unprocessed organisms appear undamaged and morphologically intact under DIC, determine the step or reagent that is causing damage to the organisms. Correct the problem (see Section 9.7.5) and repeat the IPR test.

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

9.5.1 Matrix spike— The laboratory shall spike and analyze a separate field sample aliquot to determine the effect of the matrix on the method's oocyst and cyst recovery. The MS and field sample must be that was collected from the same sampling location as split samples or as samples sequentially collected immediately after one another. The MS sample volume analyzed must be within 10% of the field sample volume. The MS shall be analyzed according to the frequency in Section 9.1.8.

9.5.1.1 Analyze an unspiked field sample according to the procedures in Sections 12.0 to 15.0. Using the spiking procedure in Section 11.4 and enumerated spiking suspensions (Section 7.10.1 or Section 11.3), spike, filter, elute, concentrate, separate (purify), stain, and examine a second field sample aliquot with a similar number of organisms as that used in the IPR or OPR tests (Sections 9.4 and 9.7).

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

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

where

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

9.5.1.3 Compare the recovery for each organism with the acceptance criteria in Tables 3 and 4 in Section 21.0.

NOTE: Some sample matrices may prevent the acceptance criteria in Tables 3 and 4 from being met. An assessment of the distribution of MS recoveries across 430 MS samples from 87 sites during the ICR Supplemental Surveys is provided in Table 5.

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

9.5.2 Matrix spike duplicate—MSD analysis is required as part of Tier 2 or nationwide approval of a modified version of this method to demonstrate that the modified version of this method produces results equal or superior to results produced by the method as written (Section 9.1.2.1.2). At the same time the laboratory spikes and analyzes the second field sample aliquot in Section 9.5.1.1, the laboratory shall spike and analyze a third, identical field sample aliquot.

NOTE: Matrix spike duplicate samples are only required for Tier 2 validation studies. They are recommended for Tier 1 validation, but not required.

9.5.2.1 For each organism, calculate the percent recovery (R) using the equation in Section 9.5.1.2.

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

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

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

where

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

9.5.2.4 Compare the mean MS/MSD recovery and RPD with the acceptance criteria in Tables 3 and 4 in Section 21.0 for each organism.

9.6 Method blank (negative control sample, laboratory blank)—Reagent water blanks are routinely analyzed to demonstrate freedom from contamination. Analyze the blank immediately after analysis of the IPR test (Section 9.4) and OPR test (Section 9.7) and prior to analysis of samples for the week to demonstrate freedom from contamination.

9.6.1 Filter, elute, concentrate, separate (purify), stain, and examine at least one reagent water method blank per week (Section 9.1.7) according to the procedures in Sections 12.0 to 15.0. A method blank must be analyzed each week (7 day or 168 hours time period that begins with processing the OPR) in which samples are analyzed if 20 or fewer field samples are analyzed during this period. If more than 20 samples are analyzed in a week (7 days or 168 hours), process and analyze one reagent water method blank for every 20 samples.

9.6.2 Actions

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

9.6.2.2 If *Cryptosporidium* oocysts, *Giardia* cysts (as defined in Section 3), or any potentially interfering organism or materials that may be misidentified as oocysts or cysts are found in the method blank, the method blank test is unacceptable. Any field sample in a batch associated with an unacceptable method blank is assumed to be contaminated and should be recollected. Analysis of additional samples is halted until the source of contamination is eliminated, the method blank test is performed again, and no evidence of contamination is detected.

9.7 Ongoing precision and recovery (OPR; positive control sample; laboratory control sample)—Using the spiking procedure in Section 11.4 and enumerated spiking suspensions (Section 7.10.1 or Section 11.3), filter, elute, concentrate, separate (purify), stain, and examine at least one reagent water sample spiked with ~100 to 500 oocysts and ~100 to 500 cysts each week

to verify all performance criteria. The laboratory must analyze one OPR sample for every 20 samples if more than 20 samples are analyzed in a week. If multiple method variations are used, separate OPR samples must be prepared for each method variation. Adjustment and/or recalibration of the analytical system shall be performed until all performance criteria are met. Only after all performance criteria are met should samples be analyzed.

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

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

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

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

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

where:

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

9.7.3 Compare the recovery with the acceptance criteria for ongoing precision and recovery in Tables 3 and 4 in Section 21.0.

9.7.4 Actions

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

9.7.4.2 If the recovery for *Cryptosporidium* or *Giardia* falls outside of the criteria, system performance is unacceptable. Any sample in a batch associated with an unacceptable OPR sample is unacceptable. Analysis of additional samples is halted until the analytical system is brought under control. Troubleshoot the problem using the procedures at Section 9.7.5 as a guide. After assessing the issue, perform another OPR test and verify that *Cryptosporidium* and *Giardia* recoveries meet the acceptance criteria.

9.7.5 **Troubleshooting.** If an OPR sample has failed, and the cause of the failure is not known, the laboratory generally should identify the problem working backward in the analytical process from the microscopic examination to filtration.

9.7.5.1 **Quality of spiked organisms.** Examine the spiking suspension organisms directly (by centrifuging, if possible, to concentrate the organisms in a volume that can be applied directly to a slide). If the organisms appear damaged under DIC, obtain fresh spiking materials. If the organisms appear undamaged and morphologically intact, determined whether the problem is associated with the microscope system or antibody stain (Section 9.7.5.2).

9.7.5.2 **Microscope system and antibody stain:** To determine if the failure of the OPR test is due to changes in the microscope or problems with the antibody stain, re-examine the positive staining control (Section 15.2.1), check Köhler illumination, and check the fluorescence of the fluorescein-labeled monoclonal antibodies (Mabs) and 4',6-diamidino-2-phenylindole (DAPI). If results are unacceptable, re-examine a previously-prepared positive staining control to determine whether the problem is associated with the microscope or the antibody stain.

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

9.7.5.4 **Filtration/elution/concentration system:** If the failure of the OPR test is attributable to the filtration/elution/concentration system, check system performance by processing spiked reagent water according to the procedures in Section 12.2 through 13.2.2, and filter, stain, and examine the sample concentrate according to Section 11.3.6.

9.7.6 The laboratory should add results that pass the specifications in Section 9.7.3 to initial and previous ongoing data and update the QC chart to form a graphic representation of continued laboratory performance. The laboratory should develop a statement of laboratory accuracy (reagent water, raw surface water) by calculating the mean percent recovery (R) and the standard deviation of percent recovery (s_r). Express the accuracy as a recovery interval from R – 2 s_r to R + 2 s_r. For example, if R = 95% and s_r = 25%, the accuracy is 45% to 145%.

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

9.9 The specifications contained in this method can be met if the analytical system is under control. The standards used for initial (Section 9.4) and ongoing (Section 9.7) precision and recovery should be identical, so that the most precise results will be obtained. The microscope in particular will provide the most reproducible results if dedicated to the settings and conditions required for the determination of *Cryptosporidium* and *Giardia* by this method.

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

10.0 Microscope Calibration and Analyst Verification

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

10.2 Using the manuals provided with the microscope, all analysts must familiarize themselves with operation of the microscope.

10.3 Microscope adjustment and calibration (adapted from Reference 20.10)

10.3.1 Preparations for adjustment

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

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

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

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

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

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

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

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

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

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

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

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

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

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

10.3.2.8 Reattach the objective to the nosepiece.

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

10.3.2.10 Turn off the transmitted light and replace the card with a slide of fluorescent material. Check the field for even fluorescent illumination. Adjustment of the diffuser lens probably will be required. Additional slight adjustments as in Section 10.3.2.7 above may be required.

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

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

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

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

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

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

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

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

10.3.4 Adjustment of the interpupillary distance and oculars for each eye: These adjustments are necessary so that eye strain is reduced to a minimum, and must be made for each individual using the microscope. Section 10.3.4.2 assumes use of a microscope with both oculars adjustable; Section 10.3.4.3 assumes use of a microscope with a single adjustable ocular. The procedure must be followed each time an analyst uses the microscope.

10.3.4.1 Interpupillary distance

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

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

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

10.3.4.2.1 Place a card between the right ocular and eye keeping both eyes open. Adjust the correction (focusing) collar on

the left ocular by focusing the left ocular until it reads the same as the interpupillary distance. Bring an image located in the center of the field of view into as sharp a focus as possible.

10.3.4.2.2 Transfer the card to between the left eye and ocular.

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

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

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

10.3.4.3.2 Transfer the card to between the left eye and ocular.

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

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

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

10.3.5.2 Adjust the stage and ocular with the micrometer so the “0" line on the ocular micrometer is exactly superimposed on the “0" line on the stage micrometer.

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

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

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

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

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

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

10.3.5.7 Follow the procedure below for each objective. Record the information as shown in the example below and keep the information available at the microscope.

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

[1]1000 µm/mm
[2](Stage micrometer length in mm × (1000 µm/mm)) ÷ no. ocular micrometer spaces
[3]N.A. refers to numerical aperature. The numerical aperature value is engraved on the barrel of the objective.

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

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

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

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

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

radiant field diaphragm leaves, open the radiant field diaphragm until the leaves just disappear from view.

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

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

10.4 Microscope cleaning procedure

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

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

10.4.3 Protocol for cleaning oculars and condenser

10.4.3.1 Use a new, clean Q-tip dampened with MCS to clean each lense. Start at the center of the lens and spiral the Q-tip outward using little to no pressure. Rotate the Q-tip head while spiraling to ensure a clean surface is always contacting the lens.

10.4.3.2 Repeat the procedure using a new, dry Q-tip.

10.4.3.3 Repeat Sections 10.4.3.1 and 10.4.3.2.

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

10.4.4 Protocol for cleaning objective lenses

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

10.4.4.2 Hold a new Q-tip dampened with MCS at a 45° angle on the objective and twirl.

10.4.4.3 Repeat Sections 10.4.4.2 with a new, dry Q-tip.

10.4.4.4 Repeat Sections 10.4.4.2 and 10.4.4.3.

10.4.4.5 Clean all objectives whether they are used or not.

10.4.5 Protocol for cleaning light source lens and filters

10.4.5.1 Using a Kimwipe dampened with microscope cleaning solution, wipe off the surface of each lens and filter.

10.4.5.2 Repeat the procedure using a dry Kimwipe.

10.4.5.3 Repeat Sections 10.4.5.1 and 10.4.5.2.

10.4.6 Protocol for cleaning microscope stage

10.4.6.1 Using a Kimwipe dampened with microscope cleaning solution, wipe off the stage and stage clip. Be sure to clean off any residual immersion oil or fingernail polish. Remove the stage clip if necessary to ensure that it is thoroughly cleaned.

10.4.7 Use 409 and a paper towel to clean the bench top surrounding the microscope.

10.4.8 Frequency

10.4.8.1 Perform Sections 10.4.2, 10.4.3, 10.4.4, 10.4.5 and 10.4.7 after each

microscope session.

10.4.8.2 Perform complete cleaning each week.

10.5 Protozoa libraries: Each laboratory is encouraged to develop libraries of photographs and drawings for identification of protozoa.

10.5.1 Take color photographs of *Cryptosporidium* oocysts and *Giardia* cysts by FA, 4',6-diamidino-2-phenylindole (DAPI), and DIC that the analysts (Section 22.2) determine are accurate (Section 15.2).

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

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

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

10.6.2 Each analyst shall determine the total number of oocysts and cysts detected by FITC on the entire slide meeting the criteria in 10.6.1. For the same 10 oocysts and 10 cysts, each analyst shall determine the DAPI category (DAPI negative, DAPI positive internal intense blue and DAPI positive number of nuclei) and the DIC category (empty, containing amorphous structures, or containing identifiable internal structures) of each. The DAPI/DIC comparisons may be performed on the slide prepared in 10.6.1, OPR slide, MS slide, or a positive staining control slide.

10.6.3 Requirements for laboratories with multiple analysts

10.6.3.1 The total number of oocysts and cysts determined by each analyst (Section 10.6.2.) must be within ±10% of each other. If the number is not within this range, the analysts must identify the source of any variability between analysts' examination criteria, prepare a new slide, and repeat the performance verification (Sections 10.6.1 to 10.6.2). It is recommended that the DAPI and DIC categorization of the same 10 oocysts and 10 cysts occur with all analysts at the same time, i.e. each analyst determines the categorizations independently, then the differences in the DAPI and DIC categorizations among analysts are discussed and resolved, and these resolutions documented. Alternatively, organism coordinates may be recorded for each analyst to locate and categorize the organisms at different times. Differences among analysts must be discussed and resolved.

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

10.6.3.3 Only after an analyst has passed the criteria in Section 10.6.3, may oocysts and cysts in QC samples and field samples be identified and enumerated.

10.6.4 Laboratories with only one analyst should maintain a protozoa library (Section 10.5) and compare the results of the examinations performed in Sections 10.6.1 and 10.6.2 to photographs of oocysts and cysts and interfering organisms to verify that examination results are consistent with these references. These laboratories also should perform repetitive counts of a single verification slide for FITC. These laboratories should also coordinate with other laboratories to share slides and compare counts.

11.0 Oocyst and Cyst Suspension Enumeration and Sample Spiking

11.1 This method requires routine analysis of spiked QC samples to demonstrate acceptable initial and ongoing laboratory and method performance (initial precision and recovery samples [Section 9.4], matrix spike and matrix spike duplicate samples [Section 9.5], and ongoing precision and recovery samples [Section 9.7]). The organisms used for these samples must be enumerated to calculate recoveries (and precision) and monitor method performance. EPA recommends that flow cytometry be used for this enumeration, rather than manual techniques. Flow cytometer–sorted spikes generally are characterized by a relative standard deviation of ≤2.5%, versus greater variability for manual enumeration techniques (Reference 20.11). Guidance on preparing spiking suspensions using a flow cytometer is provided in Section 11.2. Manual enumeration procedures are provided in Section 11.3. The procedure for spiking bulk samples in the laboratory is provided in Section 11.4.

11.2 **Flow cytometry enumeration guidelines.** Although it is unlikely that many laboratories performing Method 1623 will have direct access to a flow cytometer for preparing spiking suspensions, flow-sorted suspensions are available from commercial vendors and other sources (Section 7.10.1). The information provided in Sections 11.2.1 through 11.2.4 is simply meant as a guideline for preparing spiking suspensions using a flow cytometer. Laboratories performing flow cytometry must develop and implement detailed standardized protocols for calibration and operation of the flow cytometer.

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

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

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

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

11.2.5 **Method blanks.** Depending on the operation of the flow cytometer, method blanks should be prepared and examined at the same frequency as the ongoing calibration

samples (Section 11.2.4).

11.2.6 Holding time criteria. Flow-cytometer-sorted spiking suspensions (Sections 7.10.1 and 11.2) used for spiked quality control (QC) samples (Section 9) must be used within the expiration date noted on the suspension. The holding time specified by the flow cytometry laboratory should be determined based on a holding time study.

11.3 Manual enumeration procedures. Two sets of manual enumerations are required per organism before purified *Cryptosporidium* oocyst and *Giardia* cyst stock suspensions (Section 7.10.2) received from suppliers can be used to spike samples in the laboratory. First, the stock suspension must be diluted and enumerated (Section 11.3.3) to yield a suspension at the appropriate oocyst or cyst concentration for spiking (spiking suspension). Then, 10 aliquots of spiking suspension must be enumerated to calculate a mean spike dose. Spiking suspensions can be enumerated using hemacytometer chamber counting (Section 11.3.4), well slide counting (Section 11.3.5), or membrane filter counting (Section 11.3.6).

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

11.3.2 Holding time criteria. Manually enumerated spiking suspensions must be used within 24 hours of enumeration of the spiking suspension if the hemacytometer chamber technique is used (Section 11.3.4); or within 24 hours of application of the spiking suspension or membrane filter to the slides if the well slide or membrane filter enumeration technique is used (Sections 11.3.5 and 11.3.6).

11.3.3 Enumerating and diluting stock suspensions

11.3.3.1 Purified, concentrated stock suspensions (Sections 7.10.2.1 and 7.10.2.2) must be diluted and enumerated before the diluted suspensions are used to spike samples in the laboratory. Stock suspensions should be diluted with reagent water/Tween® 20 , 0.01% (Section 7.10.2.3), to a concentration of 20 to 50 organisms per large hemacytometer square before proceeding to Section 11.3.3.2.

11.3.3.2 Apply a clean hemacytometer coverslip (Section 6.4.5) to the hemacytometer and load the hemacytometer chamber with 10 µL of vortexed suspension per chamber. If this operation has been properly executed, the liquid should amply fill the entire chamber without bubbles or overflowing into the surrounding moats. Repeat this step with a clean, dry hemacytometer and coverslip if loading has been incorrectly performed. See Section 11.3.3.13, below, for the hemacytometer cleaning procedure.

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

11.3.3.4 Use 200X magnification.

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

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

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

11.3.3.8 At each of the four corners of the chamber is a 1-square-mm area divided into 16 squares in which organisms are to be counted (Figure 1).

Beginning with the top row of four squares, count with a hand-tally counter in the directions indicated in Figure 2. Avoid counting organisms twice by counting only those touching the top and left boundary lines. Count each square millimeter in this fashion.

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

$$\frac{\text{number of organisms counted}}{\text{number of mm}^2\text{ counted}} \times \frac{10}{1\text{ mm}} \times \frac{\text{dilution factor}}{1} \times \frac{1\text{ mm}^3}{1\text{ µL}} = \frac{\text{number of organisms}}{\text{µL}}$$

11.3.3.10 Record the result on a hemacytometer data sheet.

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

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

NOTE: If the diluted stock suspensions (the spiking suspensions) will be enumerated using hemacytometer chamber counts (Section 11.3.4) or membrane filter counts (Section 11.3.6), then the stock suspensions should be diluted with 0.01% Tween® 20 . If the spiking suspensions will be enumerated using well slide counts (Section 11.3.5), then the stock suspensions should be diluted in reagent water.

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

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

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

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

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

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

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

11.3.3.13 After each use, the hemacytometer and coverslip must be cleaned immediately to prevent the organisms and debris from drying on it. Since this apparatus is precisely machined, abrasives cannot be used to clean it,

as they will disturb the flooding and volume relationships.

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

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

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

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

11.3.4 Enumerating spiking suspensions using a hemacytometer chamber

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

11.3.4.1 Vortex the tube containing the spiking suspension (diluted stock suspension; Section 11.3.3) for a minimum of 2 minutes. Gently invert the tube three times.

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

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

11.3.4.4 While the stir bar is still spinning, remove a 10-µL aliquot and carefully load one side of the hemacytometer. Count all organisms on the platform, at 200X magnification using phase-contrast or darkfield microscopy. The count must include the entire area under the hemacytometer, not just the four outer 1-mm^2 squares. Repeat this procedure nine times. This step allows confirmation of the number of organisms per 10 µL (Section 11.3.3.12). Based on the 10 counts, calculate the mean, standard deviation, and RSD of the counts. Record the counts and the calculations on a spiking suspension enumeration form. The relative standard deviation (RSD) of the calculated mean spike dose must be ≤16% for *Cryptosporidium* and ≤19% for *Giardia* before proceeding. If the RSD is unacceptable, or the mean number is outside the expected range, add

additional oocysts from stock suspension or dilute the contents of the beaker appropriately with reagent water. Repeat the process to confirm counts. Refer to Section 11.3.3.14 for factors that may introduce errors.

11.3.5 Enumerating spiking suspensions using well slides

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

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

11.3.5.2 Vortex the tube containing the spiking suspension (diluted stock suspension; Section 11.3.3) for a minimum of 2 minutes. Gently invert the tube three times.

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

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

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

11.3.5.6 Positive and negative controls must be prepared.

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

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

11.3.5.6.3 Air-dry the control slides.

11.3.5.7 Follow the manufacturer's instructions (Section 7.6) in applying the stain to the slide.

11.3.5.8 Place the slides in a humid chamber in the dark and incubate according to manufacturer's directions. The humid chamber consists of a tightly sealed plastic container containing damp paper towels on top of which the slides are placed.

11.3.5.9 Apply one drop of wash buffer (prepared according to the manufacturer's instructions [Section 7.6]) to each well. Tilt each slide on a clean paper towel, long edge down. Gently aspirate the excess detection reagent from below the well using a clean Pasteur pipette or absorb with a paper towel or other absorbent material. Avoid disturbing the sample.

NOTE: If using the MeriFluor® Cryptosporidium/Giardia stain (Section 7.6.1), do not allow slides to dry completely.

11.3.5.10 Add mounting medium (Section 7.8) to each well.

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

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

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

11.3.6 Enumeration of spiking suspensions using membrane filters

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

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

11.3.6.2 Place foil around the bottoms of the 100 × 15 mm petri dishes.

11.3.6.3 Filter-sterilize (Section 6.19) approximately 10 mL of PBS (Section 7.4.2.1). Dilute detection reagent (Section 7.6) as per manufacturer's instructions using sterile PBS. Multiply the anticipated number of filters to be stained by 100 mL to calculate total volume of stain required. Divide the total volume required by 5 to obtain the microliters of antibody necessary. Subtract the volume of antibody from the total stain volume to obtain the required microliters of sterile PBS to add to the antibody.

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

11.3.6.5 Place a decontaminated and cleaned filter holder base (Section 6.4.8.1) into each of the three ports of the vacuum manifold (Section 6.4.8.2).

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

11.3.6.7 Using forceps, moisten a 1.2-µm cellulose-acetate support membrane (Section 6.4.8.3) in the 0.01% Tween® 20 and place it on the fritted glass support of one of the filter bases. Moisten a polycarbonate filter (Section 6.4.8.4) the same way and position it on top of the cellulose-

acetate support membrane. Carefully clamp the glass funnel to the loaded filter support. Repeat for the other two filters.

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

11.3.6.9 Vortex the tube containing the spiking suspension (diluted stock suspension; Section 11.3.3) for a minimum of 2 minutes. Gently invert the tube three times.

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

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

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

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

11.3.6.14 Repeat Sections 11.3.6.4 through 11.3.6.10 for the next three samples (if that the diluted spiking suspension has sat less than 15 minutes, reduce the suspension vortex time to 60 seconds). Ten, 10-µL spiking suspension aliquots must be prepared and counted, and the counts averaged, to sufficiently enumerate the spike dose. Include a filter blank sample at a frequency of every five samples; rotate the position of filter blank to eventually include all three filter placements.

11.3.6.15 Repeat Sections 11.3.6.4 through 11.3.6.10 until the 10-µL spiking suspensions have been filtered. The last batch should include a 10-µL 0.01 Tween® 20 blank control and 20 µL of positive control antigen as a positive staining control.

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

11.3.6.17 To each slide, add 20 µL of mounting medium (Section 7.8).

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

11.3.6.19 Record the date and time that staining was completed. If slides will not be read immediately, store sealed slides in a closed container in the dark between 1°C and 10°C until ready for examination.

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

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

11.4 Procedure for spiking samples in the laboratory with enumerated spiking suspensions.

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

11.4.2 For initial precision and recovery (Section 9.4) and ongoing precision and recovery (Section 9.7) samples, fill the container with 10 L of reagent water or a volume of reagent water equal to the volume of the field samples analyzed in the analytical batch. For matrix spike samples (Section 9.5), fill the container with the field sample to be spiked. Continuously mix the sample (using a stir bar and stir plate for smaller-volume samples and alternate means for larger-volume samples).

11.4.3 Follow the procedures in Section 11.4.3.1 or manufacturer's instructions for flow cytometer–enumerated suspensions and the procedures in Section 11.4.3.2 for manually enumerated suspensions.

11.4.3.1 For flow cytometer–enumerated suspensions (where the entire volume of a spiking suspension tube will be used):

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

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

11.4.3.1.3 Pour the suspension into the sample container.

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

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

11.4.3.1.6 Record the estimated number of organisms spiked, the date and time the sample was spiked, and the sample volume spiked on a bench sheet. Proceed to Section 11.4.4.

11.4.3.2 For manually enumerated spiking suspensions:

11.4.3.2.1 Vortex the spiking suspension(s) (Section 11.2 or Section 11.3) for a minimum of 30 seconds.

11.4.3.2.2 Rinse a pipette tip with 0.01% Tween® 20 once, then repeatedly pipette the well-mixed spiking suspension a

minimum of five times before withdrawing an aliquot to spike the sample.

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

11.4.3.2.4 Record the estimated number of organisms spiked, the date and time the sample was spiked, and the sample volume spiked on a bench sheet. Proceed to Section 11.4.4

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

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

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

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

11.4.8 Proceed to filter disassembly.

12.0 Sample Filtration and Elution

12.1 A water sample is filtered according to the procedures in Section 12.2, 12.3, or 12.4. Alternate procedures may be used if the laboratory first demonstrates that the alternate procedure provides equivalent or superior performance per Section 9.1.2.

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

12.2 Capsule filtration (adapted from Reference 20.12). This procedure was validated using 10-L sample volumes (for the original Envirochek ™ filter) and 50-L sample volumes (for the Envirochek™ HV filter). Alternate sample volumes may be used, provided the laboratory demonstrates acceptable performance on initial and ongoing spiked reagent water and source water samples (Section 9.1.2).

12.2.1 Flow rate adjustment

12.2.1.1 Connect the sampling system, minus the capsule, to a carboy filled with reagent water (Figure 3).

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

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

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

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

12.2.4 Filtration

12.2.4.1 Mix the sample well by shaking, add stir bar and place on stir plate. Turn on stir plate to lowest setting needed to keep sample thoroughly mixed. Connect the sampling system to the field carboy of sample water, or

transfer the sample water to the laboratory carboy used in Section 12.2.1.1. If the sample will be filtered from a field carboy, a spigot (Section 6.2.1) can be used with the carboy to facilitate sample filtration.

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

12.2.4.2 Place the drain end of the sampling system tubing into an empty graduated container with a capacity of 10 to 15 L, calibrated at 9.0, 9.5, 10.0, 10.5, and 11.0 L (Section 6.18). This container will be used to determine the sample volume filtered. Alternately, connect a flow meter (Section 6.3.4) downstream of the filter, and record the initial meter reading.

12.2.4.3 Allow the carboy discharge tube and capsule to fill with sample water by gravity. Vent residual air using the bleed valve/vent port, gently shaking or tapping the capsule, if necessary. Turn on the pump to start water flowing through the filter. Verify that the flow rate is 2 L/min.

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

12.2.4.5 Turn off stir plate; add 1 L PBST or reagent water rinse (to 10-L carboy) or 5 L PBST or reagent water rinse (to 50-L carboy). Swirl or shake the carboy to rinse down the side walls.

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

12.2.5 Disassembly

12.2.5.1 Disconnect the inlet end of the capsule filter assembly while maintaining the level of the inlet fitting above the level of the outlet fitting to prevent backwashing and the loss of oocysts and cysts from the filter. Restart the pump and allow as much water to drain as possible. Turn off the pump.

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

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

12.2.6 Elution

NOTE: The laboratory must complete the elution, concentration, and purification (Sections 12.2.6 through 13.3.3.11) in one work day. It is critical that these steps be completed in one work day to minimize the time that any target organisms present in the sample sit in eluate or concentrated matrix. This process ends with the application of the purified sample on the slide for drying.

12.2.6.1 Setup

12.2.6.1.1 Assemble the laboratory shaker with the clamps aligned vertically so that the filters will be aligned horizontally. Extend the clamp arms to their maximum distance from the horizontal shaker rods to maximize the shaking action.

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

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

12.2.6.2 Elution

12.2.6.2.1 Record the elution date and time on the bench sheet. Using a ring stand or other means, clamp each capsule in a vertical position with the inlet end up.

12.2.6.2.2 Remove the inlet cap, pour elution buffer through the inlet fitting, and allow the liquid level to stabilize. Sufficient elution buffer must be added to cover the pleated white membrane with buffer solution or elution buffer may be measured to ensure the use of one 250-mL centrifuge tube. Replace the inlet cap.

12.2.6.2.3 Securely clamp the capsule in one of the clamps on the laboratory shaker with the bleed valve positioned at the top on a vertical axis (in the 12 o'clock position). Turn on the shaker and set the speed to maximum (approximately 900 rpm or per manufacturer's instructions). Agitate the capsule for approximately 5 minutes. Time the agitation using a lab timer, rather than the timer on the shaker to ensure accurate time measurement.

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

12.2.6.2.5 Clamp the capsule vertically with the inlet end up and add sufficient volume of elution buffer through the inlet fitting to cover the pleated membrane. Replace the inlet cap.

12.2.6.2.6 Return the capsule to the shaker with the bleed valve positioned at the 4 o'clock position. Turn on the shaker and agitate the capsule for approximately 5 minutes.

12.2.6.2.7 Remove the filter from the shaker, but leave the elution buffer in the capsule. Re-clamp the capsule to the shaker at the 8 o'clock position. Turn on the shaker and agitate the capsule for a final 5 minutes.

12.2.6.2.8 Remove the filter from the shaker and pour the contents into the 250-mL centrifuge tube. Rinse down the inside of the capsule filter walls with reagent water or elution buffer using a squirt bottle inserted in the inlet end of the capsule. Invert the capsule filter over the centrifuge tube and ensure that as much of the eluate as possible has been transferred.

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

12.3 Sample filtration using the Filta-Max® foam filter. This procedure was validated using 50-L sample volumes. Alternate sample volumes may be used, provided the laboratory demonstrates

acceptable performance on initial and ongoing spiked reagent water and source water samples (Section 9.1.2).

NOTE: The filtration procedures specified in Sections 12.3.1.2 - 12.3.1.6.3 are specific to laboratory filtration of a bulk sample. These procedures may require modification if samples will be filtered in the field.

12.3.1 Filtration

12.3.1.1 Flow rate adjustment

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

12.3.1.1.2 Place the peristaltic pump upstream of the filter housing.

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

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

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

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

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

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

12.3.1.5 Filtration

12.3.1.5.1 Connect the sampling system to the field carboy of sample water, or transfer the sample water to the laboratory carboy used in Section 12.3.1.1.1. If the sample will be filtered from a field carboy, a spigot can be used with the carboy to facilitate sample filtration.

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

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

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

12.3.1.5.4 After all of the sample has passed through the filter, turn off the pump. Allow the pressure to decrease until flow stops.

12.3.1.6 Disassembly

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

12.3.1.6.2 Based on the water level in the graduated container or the meter reading, record the volume filtered on a bench sheet to the nearest quarter liter.

12.3.1.6.3 Loosen the outlet fitting, the filter housing should be sealed with rubber plugs.

NOTE: Filters should be prevented from drying out, as this can impair their ability to expand when decompressed.

12.3.2 Elution

12.3.2.1 The filter is eluted to wash the oocysts from the filter. This can be accomplished using the Filta-Max® wash station, which moves a plunger up and down a tube containing the filter and eluting solution (Section 12.3.2.2), or a stomacher, which uses paddles to agitate the stomacher bag containing the foam filter in the eluting solution (Section 12.3.2.3). If the Filta-Max® automatic wash station is used please see the manufacturer's operator's guide for instructions on its use. If Filta-Max® Quick Connect kit is used please follow manufacturer's instructions.

12.3.2.2 Filta-Max® wash station elution procedure

12.3.2.2.1 First wash

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

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

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

(d) Remove the filter module from the filter housing or transportation container. Pour excess liquid into the assembled concentrator, then rinse the housing or

container with PBST and add the rinse to the concentrator tube. Screw the filter module onto the base of the plunger. Locate the elution tube base in the jaws of the wash station and screw the elution tube (the shorter of the two tubes) firmly in place.

(e) Pull the plunger down until the filter module sits at the bottom of the elution tube; the locking pin (at the top left of the wash station) should "click" to lock the plunger in position.

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

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

NOTE: Gentle pressure on the lever, coupled with a pulling action on the locking pin should enable the pin to be easily released.

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

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

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

(j) Prior to the second wash the eluate from the first wash can be concentrated using the Filta-Max® apparatus according to Section 12.3.3.2.1 or the eluate can be decanted into a 2-L pooling beaker and set aside.

12.3.2.2.2 Second wash

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

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

(c) The eluate can be concentrated using the Filta-Max® apparatus according to Section 12.3.3.2.2 or the eluate can be decanted into the 2-L pooling beaker containing the eluate from the first wash and concentrated using centrifugation, as described in Section 12.3.3.3.

12.3.2.3 Stomacher elution procedure

12.3.2.3.1 First wash

(a) Place the filter module in the stomacher bag then use the allen key to remove the bolt from the filter module, allowing the rings to expand. Remove the end caps from the stomacher bag and rinse with PBST into the stomacher bag.

(b) Add 600 mL of PBST to stomacher bag containing the filter pads. Place bag in stomacher and wash for 5 minutes on a normal setting.

(c) Remove the bag from the stomacher and decant the eluate into a 2-L pooling beaker.

12.3.2.3.2 Second wash

(a) Add a second 600-mL aliquot of PBST to the stomacher bag. Place bag in stomacher and wash for 5 minutes on a normal setting. Remove the bag from the stomacher and decant the eluate from the stomacher bag into the 2-L pooling beaker. Wring the stomacher bag by hand to remove eluate from the foam filter and add to the pooling beaker. Remove the foam filter from the bag and using a squirt bottle, rinse the stomacher bag with reagent water and add the rinse to the pooling beaker.

(b) Proceed to concentration (Section 12.3.3).

12.3.3 Concentration

12.3.3.1 The eluate can be concentrated using the Filta-Max® concentrator apparatus, which pulls most of the eluate through a membrane filter leaving the oocysts concentrated in a small volume of the remaining eluting solution (Section 12.3..2), or by directly centrifuging all of the eluting solution used to wash the filter (Section 12.3.2.3).

12.3.3.2 The Filta-Max® concentrator procedure

12.3.3.2.1 Concentration of first wash

(a) If the stomacher was used to elute the sample (Section 12.3.2.3), transfer 600 mL of eluate from the pooling beaker to the concentrator tube. Otherwise proceed to Step (b).

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

NOTE: *The force of the vacuum should not exceed 30 cmHg.*

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

12.3.3.2.2 Concentration of second wash

(a) If the stomacher was used to elute the sample (Section 12.3.2.3), transfer the remaining 600 mL of eluate from the pooling beaker to the concentrator tube. Otherwise proceed to Step (b).

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

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

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

12.3.3.2.3 Membrane elution. The membrane can be washed manually or using a stomacher:

- **Manual wash.** Add 5 mL of PBST to the bag containing the membrane. Rub the surface of the membrane through the bag until the membrane appears clean. Using a pipette, transfer the eluate to a 50-mL tube. Repeat the membrane wash with another 5 mL of PBST and transfer the eluate to the 50-mL tube. (Optional: Perform a third wash using another 5 mL of PBST, by hand-kneading an additional minute or placing the bag on a flat-headed vortexer and vortexing for one minute. Transfer the eluate to the 50-mL tube.)

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

- **Stomacher wash.** Add 5 mL of PBST to the bag containing the membrane. Place the bag containing the membrane into a small stomacher and stomach for 3 minutes. Using a pipette transfer the eluate to a 50-mL tube. Repeat the wash two times using the stomacher and 5-mL aliquots of PBST. (Optional:

Perform a fourth wash using another 5 mL of PBST, by hand-kneading an additional minute or placing the bag on a flat-headed vortexer and vortexing for one minute. Transfer the eluate to the 50-mL tube.)

12.3.3.2.4 If the membrane filter clogs before concentration is complete, there are two possible options for completion of concentration. One option is replacing the membrane as often as necessary. Filter membranes may be placed smooth side up during the second concentration step. Another option is concentrating the remaining eluate using centrifugation. Both options are provided below.

- **Using multiple membranes.** Disassemble the concentrator tube and pour any remaining eluate back into the pooling beaker. Remove the membrane using membrane forceps, placing it in the bag provided. Place a new membrane in the concentrator tube smooth side up, reassemble, return the eluate to the concentrator tube, rinse the pooling beaker and add rinse to the eluate, and continue the concentration. Replace the membrane as often as necessary.
- **Centrifuging remaining volume.** Decant the remaining eluate into a 2-L pooling beaker. Rinse the sides of the concentrator tube and add to the pooling beaker. Remove the filter membrane and place it in the bag provided. Wash the membrane as described in Section 12.3.3.2.3, then concentrate the sample as described in Section 12.3.3.3.1.

12.3.3.3 If the Filta-Max® concentrator is not used for sample concentration, or if the membrane filter clogs before sample concentration is complete, then the procedures described in Section 12.3.3.3.1 should be used to concentrate the sample. If less than 50 mL of concentrate has been generated, the sample can be further concentrated, as described in Section 12.3.3.3.2, to reduce the volume of sample to be processed through IMS.

NOTE: The volume must not be reduced to less than 5 mL above the packed pellet. The maximum amount of pellet that should be processed through IMS is 0.5 mL. If the packed pellet is greater than 0.5 mL then the pellet may be subsampled as described in Section 13.2.4.

12.3.3.3.1 Centrifugation of greater than 50 mL of eluate

(a) Decant the eluate from the 2-L pooling beaker into 250-mL conical centrifuge tubes. Make sure that the centrifuge tubes are balanced.

(b) Centrifuge the 250-mL centrifuge tubes containing the eluate at 1500 × G for 15 minutes. Allow the centrifuge to coast to a stop.

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

(d) Vortex each 250-mL tube vigorously until pellet is completely resuspended. Swirl the centrifuge tube gently to reduce any foaming after vortexing. Combine the contents of each 250-mL centrifuge tube into a 50-mL centrifuge tube. Rinse each of the 250-mL centrifuge tubes with PBST and add the rinse to the 50-mL tube.

(e) Proceed to Section 12.3.3.3.2.

12.3.3.3.2 Centrifugation of less than 50 mL of eluate

(a) Centrifuge the 50-mL centrifuge tube containing the combined concentrate at 1500 x G for 15 minutes. Allow the centrifuge to coast to a stop. Record the initial pellet volume (volume of solids) and the date and time that concentration was completed on a bench sheet.

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

12.3.4 Maintenance and cleaning

12.3.4.1 Maintenance of O-rings

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

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

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

12.3.4.2 Cleaning

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

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

12.4 Sample collection (filtration and concentration) using portable continuous-flow centrifugation.

Please follow manufacturer's instructions. This procedure was validated for the detection of *Cryptosporidium* using 50-L sample volumes. Alternate sample volumes may be used, provided the laboratory demonstrates acceptable performance on initial and ongoing spiked reagent water and source water samples (Section 9.1.2). Laboratories are permitted to demonstrate acceptable performance for *Giardia* in their individual laboratory.

13.0 Sample Concentration and Separation (Purification)

13.1 During concentration and separation, the filter eluate is concentrated through centrifugation, and the oocysts and cysts in the sample are separated from other particulates through immunomagnetic separation (IMS). Alternate procedures and products may be used if the laboratory first demonstrates equivalent or superior performance as per Section 9.1.2.

13.2 Adjustment of pellet volume

13.2.1 Centrifuge the 250-mL centrifuge tube containing the capsule filter eluate at 1500 × G for 15 minutes. Allow the centrifuge to coast to a stop—do not use the brake. Record the pellet volume (volume of solids) on the bench sheet.

NOTE: *Recoveries may be improved if centrifugation force is increased to 2000 × G. However, do not use this higher force if the sample contains sand or other gritty material that may degrade the condition of any oocysts and/or cysts in the sample.*

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

13.2.3 **If the packed pellet volume is ≤ 0.5 mL,** vortex the tube vigorously until pellet is completely resuspended. Swirl the centrifuge tube gently to reduce any foaming after vortexing. Record the resuspended pellet volume on the bench sheet. Proceed to Section 13.3.

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

13.2.4 **If the packed pellet volume is > 0.5 mL,** the concentrate must be separated into multiple subsamples (a subsample is equivalent to no greater than 0.5 mL of packed pellet material, the recommended maximum amount of particulate material to process through the subsequent purification and examination steps in the method). Use the following formula to determine the total volume required in the centrifuge tube before separating the concentrate into two or more subsamples:

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

(For example, if the packed pellet volume is 1.2 mL, the total volume required is 12 mL.) Add reagent water to the centrifuge tube to bring the total volume to the level calculated above.

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

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

13.2.4.1.1 **Calculate number of subsamples:** Divide the total volume in the centrifuge tube by 5 mL and round up to the nearest integer (for example, if the resuspended volume in Section 13.2.4 is 12 mL, then the number of

subsamples would be 12 mL / 5 mL = 2.4, rounded = 3 subsamples).

13.2.4.1.2 **Determine volume of resuspended concentrate per subsample.** Divide the total volume in the centrifuge tube by the calculated number of subsamples (for example, if the resuspended volume in Section 13.2.4 is 12 mL, then the volume to use for each subsample = 12 mL / 3 subsamples = 4 mL).

13.2.4.1.3 **Process subsamples through IMS.** Vortex the tube vigorously for 10 to 15 seconds to completely resuspend the pellet. Record the resuspended pellet volume on the bench sheet. Proceed immediately to Section 13.3, and transfer aliquots of the resuspended concentrate equivalent to the volume in the previous step to multiple, flat-sided sample tubes in Section 13.3.2.1. Process the sample as multiple, independent subsamples from Section 13.3 onward, including the preparation and examination of separate slides for each aliquot. Record the volume of resuspended concentrate transferred to IMS on the bench sheet (this will be equal to the volume recorded in Section 13.2.4). Also record the number of subsamples processed independently through the method on the bench sheet.

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

$$\text{percent examined} = \frac{\text{total volume of resuspended concentrate transferred to IMS}}{\text{total volume of resuspended concentrate in Section 13.2.4}} \times 100\%$$

Then multiply the volume filtered (Section 12.2.5.2) by this percentage to determine the volume analyzed.

13.3 IMS procedure (adapted from Reference 20.13)

NOTE: The IMS procedure should be performed on a bench top with all materials at room temperature, ranging from 15°C to 25°C.

13.3.1 Preparation and addition of reagents

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

13.3.1.2 For each 10mL sample or subsample (Section 13.2) to be processed through IMS, add 1 mL of the 10X SL-buffer-A (supplied—not the diluted 1X SL-buffer-A) to a flat-sided tube (Section 6.5.4).

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

13.3.2 Oocyst and cyst capture

13.3.2.1 Use a graduated, 10-mL pipette that has been pre-rinsed with elution buffer to transfer the water sample concentrate from Section 13.2 to the flat-sided tube(s) containing the SL-buffers. If all of the concentrate is used, rinse the centrifuge tube twice with reagent water and add the rinsate to the flat-sided tube containing the concentrate (or to the tube containing the first subsample, if multiple subsamples will be processed). Each of the two rinses should be half the volume needed to bring the total volume in the flat-sided sample tube to 12 mL (including the buffers added in Sections 13.3.1.2 and 13.3.1.3). (For example, if the tube contained 1 mL of SL-buffer-A and 1 mL of SL-buffer-B, and 5 mL of sample was transferred after resuspension of the pellet, for a total of 7 mL, the centrifuge tube would be rinsed twice with 2.5 mL of reagent water to bring the total volume in the flat-sided tube to 12 mL.) Visually inspect the centrifuge tube after completing the transfer to ensure that no concentrate remains. If multiple subsamples will be processed, bring the volume in the remaining flat-sided tubes to 12 mL with reagent water. Label the flat-sided tube(s) with the sample number (and subsample letters).

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

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

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

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

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

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

13.3.2.8 Without removing the sample tube from the MPC®-1, place the magnet side of the MPC®-1 downwards, so the tube is horizontal and the flat side of the tube is facing down.

13.3.2.9 Gently rock the sample tube by hand end-to-end through approximately 90°, tilting the cap-end and base-end of the tube up and down in turn.

Continue the tilting action for 2 minutes with approximately one tilt per second.

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

13.3.2.11 Return the MPC®-1 to the upright position, sample tube vertical, with cap at top. Immediately remove the cap and, keeping the flat side of the tube on top, pour off all of the supernatant from the tube held in the MPC®-1 into a suitable container. Do not shake the tube and do not remove the tube from MPC®-1 during this step. Allow more supernatant to settle; aspirate additional supernatant with pipette.

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

13.3.2.13 Quantitatively transfer (transfer followed by two rinses) all the liquid from the sample tube to a labeled, 1.5-mL microcentrifuge tube. Use 0.5 mL of 1X SL-buffer-A to perform the first rinse and 0.5 mL of 1X SL-buffer-A for the second rinse. Allow the flat-sided sample tube to sit for a minimum of 1 minute after transfer of the second rinse volume, then use a pipette to collect any residual volume that drips down to the bottom of the tube to ensure that as much sample volume is recovered as possible. Ensure that all of the liquid and beads are transferred.

13.3.2.14 Place the microcentrifuge tube into the second magnetic particle concentrator (MPC®-M or MPC®-S), with its magnetic strip in place.

13.3.2.15 Without removing the microcentrifuge tube from MPC®-M, gently rock/roll the tube through 180° by hand. Continue for approximately 1 minute with approximately one 180° roll/rock per second. At the end of this step, the beads should produce a distinct brown dot at the back of the tube.

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

13.3.3 Dissociation of beads/oocyst/cyst complex

NOTE: Two acid dissociations are required.

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

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

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

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

13.3.3.4 Vortex vigorously for approximately 30 seconds.

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

13.3.3.6 Replace magnetic strip in MPC®-M and allow the tube to stand undisturbed for a minimum of 10 seconds.

13.3.3.7 Prepare a well slide for sample screening and label the slide.

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

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

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

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

NOTE: *The wells on Dynal Spot-On slides are likely to be too small to accommodate the volumes from both dissociations.*

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

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

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

- Make sure the resuspended pellet is fully homogenized before placing the tube in the MPC®-1 or MPC®-M to avoid trapping "clumps" or a dirty layer between the beads and the side of the tube.
- When using the MPC®-1 magnet, make sure that the tube is snugged flat against the magnet. Push the tube flat if necessary. Sometimes the magnet is not flush with the outside of the holder and, therefore, the attraction between the beads and the magnet is not as strong as it should be. However, it can be difficult to determine this if you do not have more than one MPC®-1 to make comparisons.
- After the supernatant has been poured off at Section 13.3.2.11, leave the tube in the MPC®-1 and allow time for any supernatant remaining in the tube to settle down to the bottom. Then aspirate the settled supernatant and associated particles from the bottom of the tube. The same can be done at Section 13.3.2.16 with the microcentrifuge tube.

- An additional rinse can also be performed at Section 13.3.2.11. After the supernatant has been poured off and any settled material is aspirated off the bottom, leave the tube in the MPC®-1 and add an additional 10 mL of reagent water or PBS to the tube and repeat Sections 13.3.2.9 and 13.3.2.11. Although labs have reported successfully using this technique to reduce carryover, because the attraction between the MPC®-1 and the beads is not as great as the attraction between the MPC®-M and the beads, the chances would be greater for loss of cysts and oocysts doing the rinse at this step instead of at Section 13.3.2.16.
- After the supernatant has been aspirated from the tube at Section 13.3.2.16, add 0.1 mL of PBS, remove the tube from the MPC®-M, and resuspend. Repeat Sections 13.3.2.15 and 13.3.2.16.
- Use a slide with the largest diameter well available to spread out the sample as much as possible.

14.0 Sample Staining

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

14.1 Prepare positive and negative controls.

14.1.1 For the positive control, pipette 10 µL of positive antigen or 200 to 400 intact oocysts and 200 to 400 cysts to the center of a well.

14.1.2 For the negative control, pipette 50 µL of PBS (Section 7.4.2.1) into the center of a well and spread it over the well area with a pipette tip.

14.1.3 Air-dry the control slides (see Section 13.3.3.12 for guidance).

NOTE: If the laboratory has a large batch of slides that will be examined over several days, and is concerned that a single positive control may fade, due to multiple examinations, the laboratory should prepare multiple control slides with the batch of field slides and alternate between the positive controls when performing the positive control check.

14.2 Follow manufacturer's instructions in applying stain to slides.

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

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

14.5 Apply one drop of wash buffer (prepared according to the manufacturer's instructions [Section 7.6]) to each well. Tilt each slide on a clean paper towel, long edge down. Gently aspirate the excess detection reagent from below the well using a clean Pasteur pipette or absorb with paper towel or other absorbent material placed at edge of slide. Avoid disturbing the sample.

14.6 Apply 50 µL of 4',6-diamidino-2-phenylindole (DAPI) staining solution (Section 7.7.2) to each well. Allow to stand at room temperature for a minimum of 1 minute. (The solution concentration may be increased up to 1 µg/mL if fading/diffusion of DAPI staining is encountered, but the staining solution must be tested first on expendable environmental samples to confirm that staining intensity is appropriate.)

14.7 Apply one drop of wash buffer (prepared according to the manufacturer's instructions [Section 7.6]) to each well. Tilt each slide on a clean paper towel, long edge down. Gently aspirate the excess DAPI staining solution from below the well using a clean Pasteur pipette or absorb with paper towel or other absorbent material placed at edge of slide. Avoid disturbing the sample.

NOTE: *If using the MeriFluor® Cryptosporidium/Giardia (Section 7.6.1), do not allow slides to dry completely.*

14.8 Add mounting medium (Section 7.8) to each well.

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

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

15.0 Examination

NOTE: *Although immunofluorescence assay (FA) and 4',6-diamidino-2-phenylindole (DAPI) and differential interference contrast (DIC) microscopy examination should be 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. However, if fading/diffusion of FITC or DAPI fluorescence is noticed, the laboratory must reduce this holding time. In addition the laboratory may adjust the concentration of the DAPI staining solution (Sections 7.7.2) so that fading/diffusion does not occur.*

15.1 Scanning technique: Scan each well in a systematic fashion. An up-and-down or a side-to-side scanning pattern may be used (Figure 4).

15.2 Examination using immunofluorescence assay (FA), 4',6-diamidino-2-phenylindole (DAPI) staining characteristics, and differential interference contrast (DIC) microscopy. The minimum magnification requirements for each type of examination are noted below.

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

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

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

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

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

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

15.2.2 Sample examination—*Cryptosporidium*

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

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

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

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

15.2.2.3 **DIC examination** (the analyst must use a minimum of 1000X total magnification [oil immersion lens]). Using DIC, look for external or internal morphological characteristics atypical of *Cryptosporidium* oocysts (e.g., spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.) (adapted from Reference 20.10). If atypical structures are not observed, then categorize each apple-green fluorescing object as:

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

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

15.2.2.4 A positive result is a *Cryptosporidium* oocyst which exhibits typical IFA fluorescence, typical size and shape and exhibits nothing atypical on IFA, DAPI fluorescence, or DIC microscopy. A positive result must be characterized and assigned to one of the DAPI and DIC categories in Sections 15.2.2.2 and 15.2.2.3.

15.2.3 Sample examination—*Giardia*

15.2.3.1 **FITC examination** (the analyst must use a minimum of 200X total magnification). When brilliant apple-green fluorescing round to ovoid

objects (8 - 18 µm long by 5 - 15 µm wide) are observed with brightly highlighted edges, increase magnification to 400X and switch the microscope to the UV filter block for DAPI (Section 15.2.3.2) then to DIC (Section 15.2.3.3) at 1000X.

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

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

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

15.2.3.3 **DIC examination** (the analyst must use a minimum of 1000X total magnification [oil immersion lens]). Using DIC microscopy, look for external or internal morphological characteristics atypical of *Giardia* cysts (e.g., spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.) (adapted from Reference 20.10). If atypical structures are not observed, then categorize each object meeting the criteria specified in Sections 15.2.3.1 through 15.2.3.3 as one of the following, based on DIC examination:

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

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

15.2.3.4 A positive result is a *Giardia* cyst which exhibits typical IFA fluorescence, typical size and shape and exhibits nothing atypical on IFA, DAPI fluorescence, or DIC microscopy. A positive result must be characterized and assigned to one of the DAPI and DIC categories in Section 15.2.3.2 and 15.2.3.3.

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

15.2.5 Report *Cryptosporidium* and *Giardia* concentrations as oocysts/L and cysts/L, respectively.

15.2.6 Record analyst name

16.0 Analysis of Complex Samples

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

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

16.3 If the holding times for the sample and for microscopic examination of the cleaned up retentate/eluate have been exceeded, the site should be re-sampled. If this is not possible, the results should be qualified accordingly.

16.4 Some samples may adhere to the centrifuge tube walls. The use of siliconized or low-adhesion centrifuge tubes (Fisherbrand siliconized/low retention microcentrifuge tubes, 02-681-320 or equivalent) may reduce adhesion. Alternately, rinse centrifuge tubes with PBST elution buffer or Sigmacote® prior to use.

17.0 Method Performance

17.1 Method acceptance criteria are shown in Tables 3 and 4 in Section 21.0. The initial and ongoing precision and recovery criteria are based on the results of spiked reagent water samples analyzed during the Information Collection Rule Supplemental Surveys (Reference 20.11). The matrix spike and matrix spike duplicate criteria are based on spiked source water data generated during the interlaboratory validation study of Method 1623 involving 11 laboratories and 11 raw surface water matrices across the U.S. (Reference 20.14).

NOTE: Some sample matrices may prevent the MS acceptance criteria in Tables 3 and 4 to be met. An assessment of the distribution of MS recoveries across 430 MS samples from 87 sites during the ICR Supplemental Surveys is provided in Table 5.

18.0 Pollution Prevention

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

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

19.0 Waste Management

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

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

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

20.0 References

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21.0 Tables and Figures

Table 1. Method Holding Times (See Section 8.2 for details)

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

Table 2. Tier 1 and Tier 2 Validation/Equivalency Demonstration Requirements

Test	Description	Tier 1 modification(1)	Tier 2 modification(2)
IPR (Section 9.4)	4 replicates of spiked reagent water	Required. Must be accompanied by a method blank.	Required per laboratory
Method blank (Section 9.6)	Unspiked reagent water	Required	Required per laboratory
MS (Section 9.5.1)	Spiked matrix water	Required on each water to which the modification will be applied and on every 20th sample of that water thereafter. Must be accompanied by an unspiked field sample collected at the same time as the MS sample	Not required
MS/MSD (Section 9.5)	2 replicates of spiked matrix water	Recommended, but not required. Must be accompanied by an unspiked field sample collected at the same time as the MS sample	Required per laboratory. Each laboratory must analyze a different water.

(1) If a modification will be used only in one laboratory, these tests must be performed and the results must meet all of the QC acceptance criteria in the method (these tests also are required the first time a laboratory uses the validated version of the method)

(2) If nationwide approval of a modification is sought for one type of water matrix (such as surface water), a minimum of 3 laboratories must perform the tests and the results from each lab individually must meet all QC acceptance criteria in the method. If more than 3 laboratories are used in a study, a minimum of 75% of the laboratories must meet all QC acceptance criteria.

NOTE: The initial precision and recovery and ongoing precision and recovery (OPR) acceptance criteria listed in Tables 3 and 4 are based on results from 293 Cryptosporidium OPR samples and 186 Giardia OPR samples analyzed by six laboratories during the Information Collection Rule Supplemental Surveys (Reference 20.15). The matrix spike acceptance criteria are based on data generated through interlaboratory validation of Method 1623 (Reference 20.14).

Table 3. Quality Control Acceptance Criteria for *Cryptosporidium*

Performance test	Section	Acceptance criteria
Initial precision and recovery	9.4	
Mean recovery (percent)	9.4.3	24 - 100
Precision (as maximum relative standard deviation)	9.4.3	55
Ongoing precision and recovery (percent)	9.7	11 - 100
Matrix spike/matrix spike duplicate (for method modifications)	9.5	
Mean recovery[1, 2] (as percent)	9.5.2.2	13 - 111
Precision (as maximum relative percent difference)	9.5.2.3	61

(1) The acceptance criteria for mean MS/MSD recovery serves as the acceptance criteria for MS recovery during routine use of the method (Section 9.5.1).
(2) Some sample matrices may prevent the acceptance criteria from being met. An assessment of the distribution of MS recoveries from multiple MS samples from 87 sites during the ICR Supplemental Surveys is provided in Table 5.

Table 4. Quality Control Acceptance Criteria for *Giardia*

Performance test	Section	Acceptance criteria
Initial precision and recovery	9.4	
Mean recovery (percent)	9.4.3	24 - 100
Precision (as maximum relative standard deviation)	9.4.3	49
Ongoing precision and recovery (percent)	9.7	14 - 100
Matrix spike/matrix spike duplicate (for method modifications)	9.5	
Mean recovery[1,2] (as percent)	9.5.2.2	15 - 118
Precision (as maximum relative percent difference)	9.5.2.3	30

(1) The acceptance criteria for mean MS/MSD recovery serves as the acceptance criteria for MS recovery during routine use of the method (Section 9.5.1).
(2) Some sample matrices may prevent the acceptance criteria from being met. An assessment of the distribution of MS recoveries across multiple MS samples from 87 sites during the ICR Supplemental Surveys is provided in Table 5.

Table 5. Distribution of Matrix Spike Recoveries from Multiple Samples Collected from 87 Source Waters During the ICR Supplemental Surveys (Adapted from Reference 20.16)

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

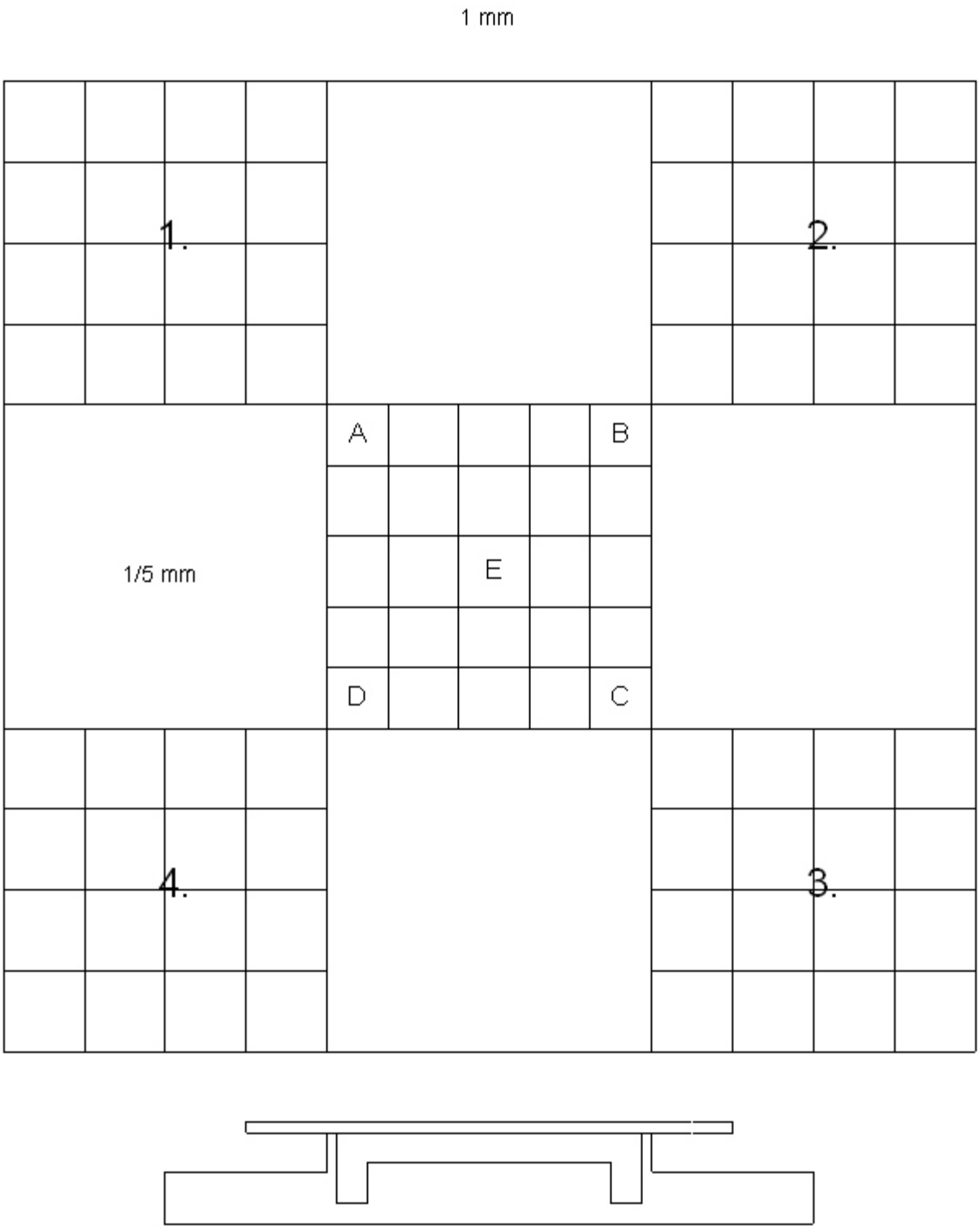

Figure 1. Hemacytometer Platform Ruling. Squares 1, 2, 3, and 4 are used to count stock suspensions of *Cryptosporidium* oocysts and *Giardia* cysts (after Miale, 1967)

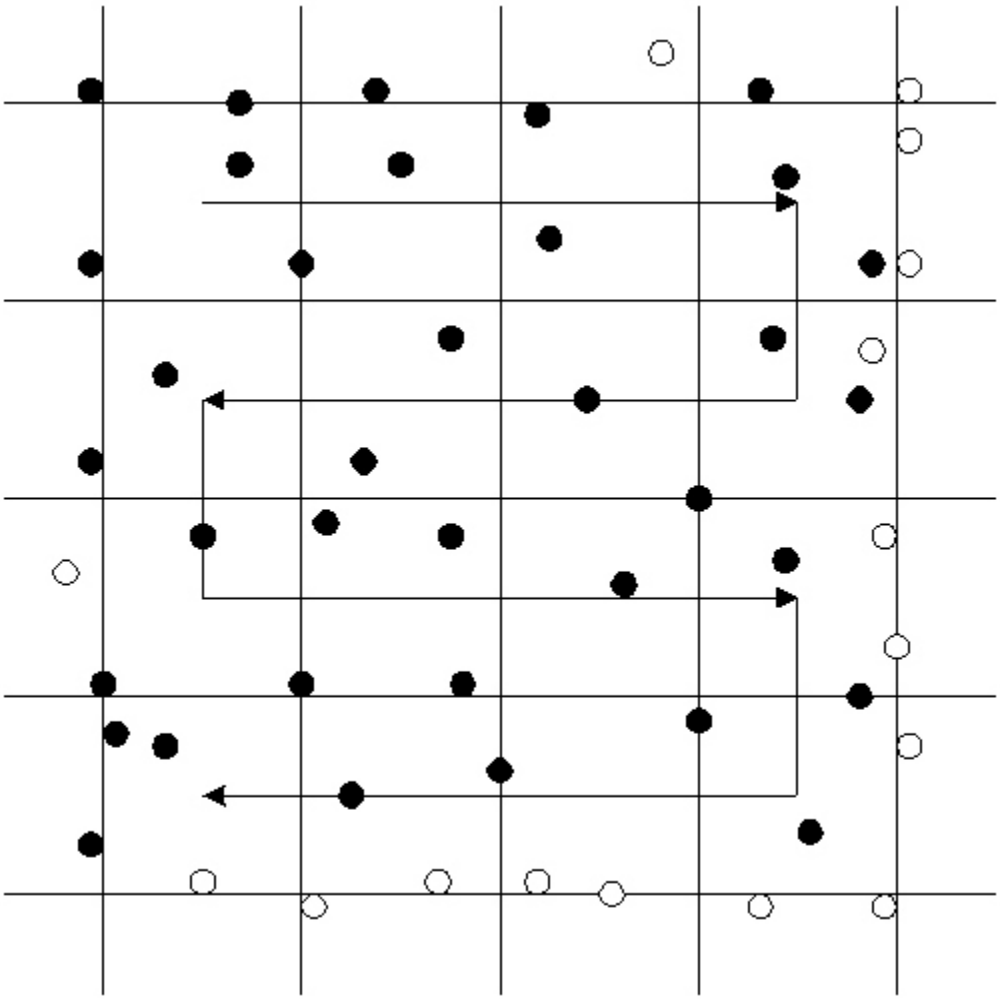

Figure 2. Manner of Counting Oocysts and Cysts in 1 Square mm. Dark organisms are counted and light organisms are omitted (after Miale, 1967).

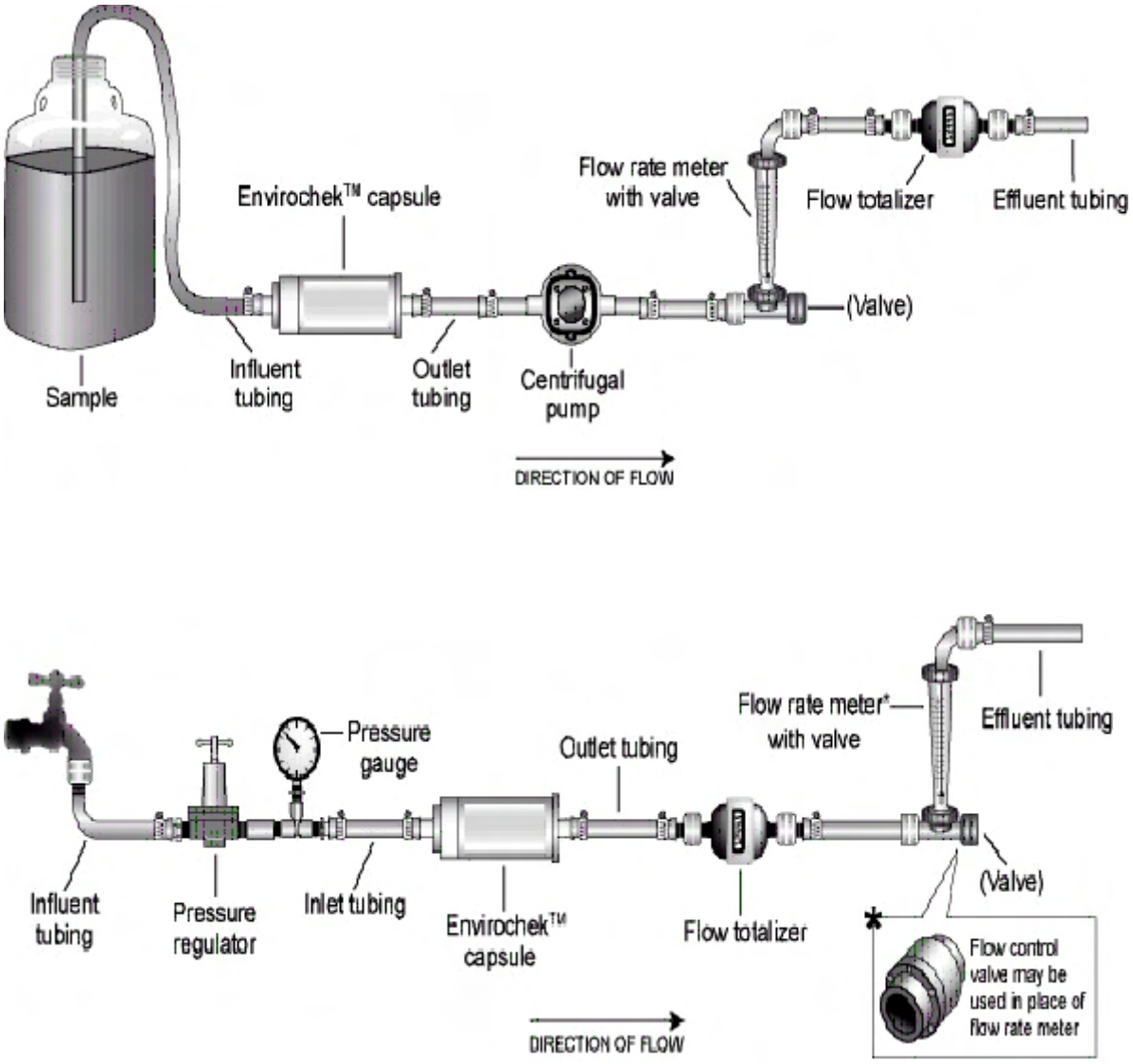

Figure 3a. Filtration Systems for Envirochek™ or Envirochek™HV Capsule (unpressurized source - top, pressurized source - bottom)

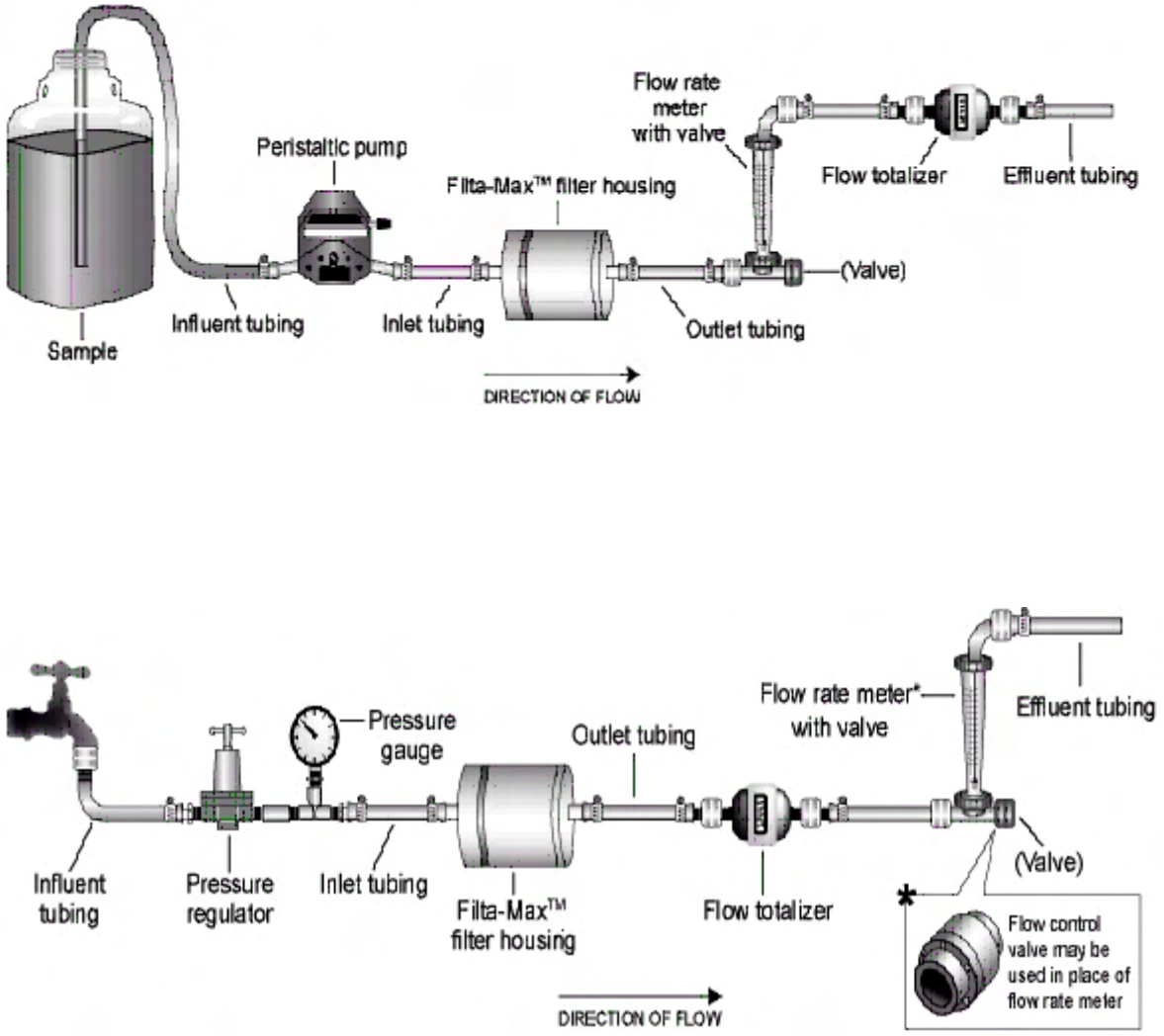

Figure 3b. Filtration Systems for Filta-Max® filters (unpressurized source - top, pressurized source - bottom)

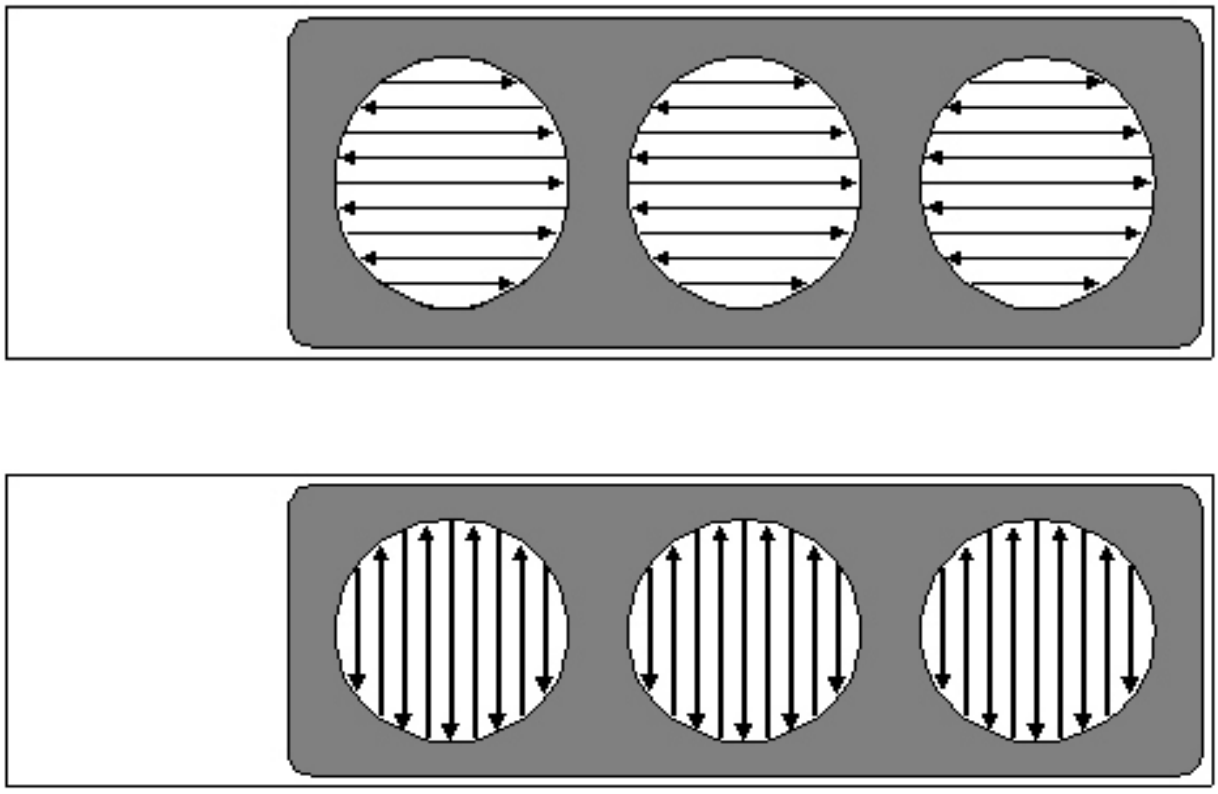

Figure 4. Methods for Scanning a Well Slide

22.0 Glossary of Definitions and Purposes

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

22.1 Units of weight and measure and their abbreviations

22.1.1 Symbols

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

22.1.2 Alphabetical characters

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

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

Analyst—The analyst should have at least 2 years of college in microbiology or equivalent or closely related field. The analyst also should have a minimum of 6 months of continuous bench experience with *Cryptosporidium* and IFA microscopy. The analyst should have a minimum of 3 months experience using EPA Method 1622 and/or EPA Method 1623 and should have successfully analyzed a minimum of 50 samples using EPA Method 1622 and/or EPA Method 1623.

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

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

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

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

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

Initial precision and recovery (IPR)—Four aliquots of spiking suspension analyzed to establish the ability to generate acceptable precision and accuracy. An IPR is performed prior to the first time this method is used and any time the method or instrumentation is modified.

Laboratory blank—See Method blank

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

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

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

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

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

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

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

Negative control—See Method blank

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

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

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

Oocyst and cyst spiking suspension—See Spiking suspension

Oocyst and cyst stock suspension—See Stock suspension

Positive control—See Ongoing precision and recovery standard

Principal analyst—The principal analyst (may not be applicable to all monitoring programs) should have a BS/BA in microbiology or closely related field and a minimum of 1 year of continuous bench experience with *Cryptosporidium* and IFA microscopy. The principal analyst also should have a minimum of 6 months experience using EPA Method 1622 and/or EPA Method 1623 and should have analyzed a minimum of 100 samples using EPA Method 1622 and/or EPA Method 1623.

PTFE—Polytetrafluoroethylene

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

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

Reagent water blank—see Method blank

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

RSD—See Relative standard deviation

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

Spiking suspension—Diluted stock suspension containing the organism(s) of interest at a concentration appropriate for spiking samples.

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

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

Technician—The technician filters samples, performs centrifugation, elution, concentration, and purification using IMS, and places purified samples on slides for microscopic examination, but does not perform microscopic protozoan detection and identification. No minimum education or experience requirements with *Cryptosporidium* and IFA microscopy apply to the technician. The technician should have at least 3 months of experience in filter extraction and processing of protozoa samples by EPA Method 1622/1623 and should have successfully processed a minimum of 50 samples using EPA Method 1622/1623.

Appendix D

Abbreviated Checklist for *Cryptosporidium* Sample Results

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Abbreviated Checklist *for Cryptosporidium* Sample Results

Sample analyzed by EPA Method 1622 or 1623		✔
Laboratory has current approval status through Lab QA Evaluation Program		✔
Quality Control (QC) Requirements Before Sample Processing		
Frequency	The ongoing precision and recovery (OPR) and method blank samples associated with the sample were performed within the same week and associated with no more than 19 other samples	
Method blank results	No *Cryptosporidium* oocysts or potentially interfering materials were found in the method blank	
OPR sample results	Recovery for the OPR sample was at least 11%)	
Spike for OPR	The associated OPR was spiked with no more than 500 oocysts as counted by flow-cytometry	
Analyst Performance	Analysts' examination criteria was verified monthly	
Key Sample Requirements		
Location/Schedule	Description of sampling location and schedule submitted to the State	
Temperature	The sample temperature upon receipt was ≤20°C, and not frozen	
Volume analyzed	The volume analyzed for the sample was at least 10 L *OR* 2 mL of packed pellet volume *OR* as much volume as can be filtered by 2 EPA-approved filters	
Staining Controls	Positive staining control for this sample had appropriate fluorescence and characterization of FITC, size, shape, DIC and DAPI characteristics for 3 oocysts was recorded. The negative staining control for this sample did not contain any oocysts or potentially interfering materials	
Holding Time Requirements		
Sample collection	The elution step for the sample was initiated within 96 hours of sample collection (if shipped to the laboratory as a bulk sample) or filtration (if filtered in the field)	
Sample processing	The sample was processed (eluted, concentrated, purified, and applied to the slide) in 1 working day	
Sample drying	The slide(s) for the sample were stained within 72 hours of application of the sample to the slide	
Sample examination	The slide(s) for the sample were examined within 168 hours (7 days) of staining	
Matrix Spike (MS) Sample Requirements		
Sample volume	The same sample volume (within 10%) was analyzed for the MS sample and the associated, unspiked field sample	
MS Frequency	MS sample analyzed with first field sample and at least every 20 samples thereafter	
Method version	The same method version (type of filter, IMS kit, and staining kit) was used for the MS sample and the associated, unspiked field sample	
Spike for MS	The MS sample was spiked with no more than 500 oocysts as counted by flow-cytometry	

Appendix E

Cryptosporidium Laboratory Quality Assurance Program Application

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OMB Control Number: 2040-0246

United States Environmental Protection Agency
Office of Ground Water and Drinking Water
Standards and Risk Management Division

February 21, 2006

Dear Laboratory Manager:

Thank you for your interest in the U.S. EPA's Laboratory Quality Assurance Evaluation Program for Analysis of *Cryptosporidium* under the Safe Drinking Water Act (Lab QA Program). This is a voluntary program open to laboratories analyzing *Cryptosporidium* in water using EPA Method 1622 and EPA Method 1623. To increase the likelihood that laboratories analyzing water samples for *Cryptosporidium* generate reliable data, EPA has established the following process for evaluating laboratory performance and quality assurance practices:

Step 1. **Application.** Laboratories must first submit the Lab QA Program application. The application forms are enclosed with this letter, and the application process is described in detail below. EPA will evaluate laboratory applications to confirm the following: (1) the laboratory has the equipment required in EPA Method 1622 and/or EPA Method 1623 (December 2005 version), (2) laboratory personnel have the recommended experience to analyze samples, and (3) the laboratory has successfully completed the initial precision and recovery and matrix spike/matrix spike duplicate tests specified in the method. Laboratories will be requested to correct any deficiencies in these areas before proceeding to the next step in the evaluation process.

Step 2. **Proficiency testing.** After an application has been accepted, the laboratory will be sent a set of eight initial proficiency testing (IPT) samples consisting of a suspension of oocysts in a concentrated matrix. Laboratories will resuspend these spikes in reagent water to produce simulated source water samples, and analyze the samples using the 2005 version of Method 1622/1623 that the laboratory plans to use for routine *Cryptosporidium* analyses. If a laboratory wishes to be evaluated for more than one variation of the method, the laboratory will receive a set of eight proficiency test (PT) samples for each variation. Laboratory IPT data will be evaluated against the mean recovery and precision (as relative standard deviation) for the IPT samples from other laboratories.

Laboratories already participating in the EPA *Cryptosporidium* PT Program, may use the initial round of samples from the PT program to meet the IPT sample requirement.

Step 3. **On-site evaluation.** After a laboratory completes the IPT, an on-site evaluation of the laboratory will be scheduled. The on-site evaluation will include two separate but concurrent assessments: (1) assessment of the laboratory's sample processing and analysis procedures, including microscopic examination, and (2) evaluation of the laboratory's personnel qualifications, quality control program, equipment, and record keeping procedures.

Each laboratory will receive an audit report, which will document deficiencies, if any, that should be corrected by the laboratory. After a laboratory has corrected any deficiencies noted in the audit report, EPA will confirm that the laboratory is approved under the Lab QA Program.

Laboratories that meet the program performance criteria will also receive a set of three ongoing proficiency testing (OPT) samples approximately every four months that must be analyzed in the same manner as the IPT samples. EPA will evaluate the precision and recovery data for OPT samples to determine if the laboratory continues to meet the performance criteria of the Laboratory QA Program.

Application Requirements

The first step in the laboratory evaluation process is submission of a laboratory application package. The following materials should be submitted for each laboratory application package:

1. Signed, completed application form (attached).

2. Completed self-audit checklist (attached). This checklist is similar to the checklist that will be used to audit your laboratory during the on-site evaluation.

3. Resumes detailing qualifications of your laboratory's proposed principal analyst/supervisor and each analyst and technician listed on the application form and documentation of the training, including the list of samples analyzed by each and the time period during which the samples were performed (the list for each analyst and technician should include at a minimum the number of samples specified below for personnel prerequisites).

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

   Principal Analyst/Supervisor (one required per laboratory)
   - BS/BA in microbiology or closely related field
   - A minimum of 1 year of continuous bench experience with *Cryptosporidium* and IFA microscopy
   - A minimum of 6 months experience using EPA Method 1622 and/or EPA Method 1623
   - A minimum of 100 samples analyzed using EPA Method 1622 and/or EPA Method 1623 (minimum 50 samples if the person was an approved analyst for *Cryptosporidium* under the Information Collection Rule(ICR))

   Other Analysts (no minimum requirement per laboratory)
   - Two years of college in microbiology or equivalent or closely related field
   - A minimum of 6 months of continuous bench experience with *Cryptosporidium* and IFA microscopy
   - A minimum of 3 months experience using EPA Method 1622 and/or EPA Method 1623
   - A minimum of 50 samples analyzed using EPA Method 1622 and/or EPA Method 1623 (minimum 25 samples if the person was an ICR-approved analyst)

   Technician (no minimum requirement per laboratory)
   - Three months experience with the specific parts of the procedure he/she will be performing
   - A minimum of 50 samples analyzed using EPA Method 1622 and/or EPA Method 1623 (minimum 25 samples if the person was an ICR-approved technician) for the specific analytical procedures they will be using.

4. Detailed laboratory standard operating procedures (SOP) for each variation of the method your laboratory plans on using for routine *Cryptosporidium* analyses. SOP's for the following should be included:

   - Performance of each method step including, sample spiking, filtration, elution, concentration, purification, slide preparation, sample staining and examination
   - Dividing pellets greater than 0.5mL
   - Preparation of reagents
   - Dishwashing
   - Staff training
   - Corrective action procedures for failing to meet OPR, method blank, staining controls, sample acceptance, and performance verification criteria
   - Sampling procedures to be followed by field or utility personnel
   - Procedures for data recording, checking manual calculations, and checking accuracy of all data transcriptions

5. EPA Method 1622/1623 initial demonstration of capability (IDC) data which include initial precision and recovery (IPR) test results and matrix spike and matrix spike duplicate (MS/MSD) test results for *Cryptosporidium*. The IPR test consists of four reagent water samples spiked with between 100 - 500 oocysts and one method blank. The MS/MSD test consists of one unspiked and two spiked source water samples. These tests are described in Section 9 of EPA Method 1622/1623 and the results should meet the criteria in the method (December 2005 version). The following data should be submitted:

   - Completed EPA Method 1622/1623 bench sheets and report forms for each of the eight samples (attached)
   - Initial demonstration of capability summary form (attached)
   - Spiking suspension preparation data. This should include completed flow-cytometer calibration forms.

   Laboratories wishing to be evaluated for more than one variation of the method (different volumes, filters, elution and concentration procedures, and immunomagnetic separation kits) should submit a complete set of IDC data for each variation.

   If your laboratory currently participates in the EPA PT sample program and the required IDC data have already been submitted, the data do not need to be resubmitted. Please indicate this is the case on the initial demonstration of capability summary form.

6. Table of contents from your laboratory's quality assurance plan. The quality assurance plan should specifically address the requirements of *Cryptosporidium* analysis under the Lab QA Program.

7. An example of the data reporting form used to submit *Cryptosporidium* results to your clients.

8. A statistical summary of percent recoveries for all OPR and MS samples analyzed at your laboratory for the past six months.

Application materials should be submitted to the following address:

Cryptosporidium Laboratory QA Program Coordinator
CSC Water Programs
6101 Stevenson Avenue
Alexandria, VA 22304

Send comments on the Agency's need for this information, the accuracy of the provided burden estimates, and any suggested methods for minimizing respondent burden, including through the use of automated collection techniques to the Director, Collection Strategies Division, U.S. Environmental Protection Agency (2822T), 1200 Pennsylvania Ave., NW, Washington, D.C. 20460. Include the OMB control number in any correspondence. Do not send the completed form to this address.

When your application package has been received and reviewed, you will be notified whether it is complete or has any deficiencies. After your application has been accepted, you will be notified of when you should expect your initial set of PT samples. If you have any questions about the laboratory application materials or evaluation process, please feel free to contact either me at moulton.carrie@epamail.epa.gov or Jennifer Scheller at jscheller@csc.com.

Sincerely,

Carrie Moulton
Manager, *Cryptosporidium* Laboratory Approval Program
Technical Support Center
26 West Martin Luther King Drive
Cincinnati, OH 45268

Attachments

Burden Statement: The public reporting and recordkeeping burden for this collection of information is estimated to average 18 hours per response or 72 hours per respondent annually. Burden means the total time, effort, or financial resources expended by persons to generate, maintain, retain, or disclose or provide information to or for a Federal agency. This includes the time needed to review instructions; develop, acquire, install, and utilize technology and systems for the purposes of collecting, validating, and verifying information, processing and maintaining information, and disclosing and providing information; adjust the existing ways to comply with any previously applicable instructions and requirements; train personnel to be able to respond to a collection of information; search data sources; complete and review the collection of information; and transmit or otherwise disclose the information. An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.

Appendix F

Checklist for the Laboratory Quality Assurance Evaluation Program for Analysis of *Cryptosporidium* under the Safe Drinking Water Act

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Checklist for the Laboratory Quality Assurance Evaluation Program for Analysis of *Cryptosporidium* under the Safe Drinking Water Act

Part A: Personnel, Facilities, Equipment, and Quality Assurance

Laboratory Name	Date of Evaluation	Name and Affiliation of Evaluator for Part A

Part A: Facilities, Equipment, and Quality Assurance

Item to be Evaluated		Classification	Yes, No, Unknown*, or NA
1 Laboratory Equipment and Supplies			
1.1 Reagent-grade water testing			
1.1.1	Is reagent water tested monthly for these minimum parameters: conductivity, total chlorine residual; and annually for metals-Pb, Cd, Cr, Cu, Ni, Zn?	Critical	
1.1.2	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, and each metal not greater than 0.05 mg/L and collectively not greater than 0.1 mg/L?	Critical	
1.1.3	Is reagent water tested monthly for heterotrophic plate count?	Critical	
1.1.4	Are the results for the heterotrophic plate count acceptable, < 500 CFU/mL?	Critical	
1.2 Laboratory pH meter:			
1.2.1	Accuracy ± 0.1 units, scale graduations, 0.1 units?	Critical	
1.2.2	Is a record maintained for pH measurements and calibrations used?	Critical	
1.2.3	Is pH meter standardized each use period with pH 7, 4 or 10 standard buffers (selection dependant upon desired pH)?	Critical	
1.2.4	All pH buffers are dated when received and opened and are discarded before expiration date?	Critical	
1.3 Balances (top loader or pan balance):			
1.3.1	Are balances calibrated monthly using Class S/S-1 weights, or weights traceable to Class S/S-1 weights?	Critical	
1.3.2	Is correction data available with S/S-1 weights?	Critical	
1.3.3	Is preventative maintenance conducted yearly at a minimum?	Recommendation	

Item to be Evaluated		Classification	Yes, No, Unknown*, or NA
1.4	**Autoclave:**		
1.4.1	Is unit equipped with a temperature gauge/operational safety valve?	Critical	
1.4.2	Are date, contents, sterilization time and temperature recorded for each cycle?	Critical	
1.4.3	Is a maximum registering thermometer or continuous monitoring device used during each autoclave cycle?	Critical	
1.4.4	Is automatic timing mechanism checked with stopwatch quarterly?	Critical	
1.4.5	Are spore strips or ampules used monthly to confirm sterilization?	Critical	
1.5	**Refrigerator/Freezer:**		
1.5.1	Is refrigerator able to maintain temperature of 1°C to 5°C?	Critical	
1.5.2	Is temperature recorded once daily for days in use?	Critical	
1.6	**Temperature recording device:**		
1.6.1	Are calibration of glass/mercury thermometers checked annually (dial thermometers quarterly) at the temperature used against a reference NIST thermometer or equivalent? [Section 8.1.4]	Requirement	
1.7	**Micropipetters:**		
1.7.1	Have micropipetters been calibrated within the past year? [Section 9.2.1]	Requirement	
1.8	**Centrifuge**		
1.8.1	Is a maintenance contract in place, or internal maintenance protocol available? [Section 9.1]	Critical	
1.8.2	Are RPM and RCF calibrated yearly?	Critical	
1.9	**General**		
1.9.1	Are calibration and maintenance records complete and well organized? [Section 9.1]	Recommendation	
2	**Quality Assurance**		
2.1	Does the laboratory have a formal QA laboratory plan prepared and ready for examination? [Section 9.1]	Requirement	
2.2	Are employee resumes present and complete? [Section 9.1]	Requirement	
2.3	Is a training protocol for new employees present? [Section 9.1]	Requirement	
2.4	Is the laboratory performing analyst verification of examination monthly and does the lab have corrective action procedures in place if criteria are not met? [Section 10.6]	Requirement	
2.5	Are employee training records available and up to date? [Section 9.1]	Requirement	

Note: All section references in [ ] refer to Method 1623 December 2005

Item to be Evaluated	Classification	Yes, No, Unknown*, or NA
2.5.1 Have technicians/analysts analyzed the required number of samples using Method 1622/1623? [Section 22.2]	Critical	
2.6 Are all relevant SOPs present and current?	Critical	
2.7 Are sampling instructions present for clients collecting and/or filtering samples in the field?	Critical	
2.8 Does the laboratory have criteria for sample acceptance and corrective action procedures? [Section 8.1.4]	Requirement	
2.9 Are data recording procedures present?	Critical	
2.9.1 Does the laboratory have an SOP for checking all manual calculations?	Critical	
2.10 Are corrective action contingencies present?	Requirement	
2.10.1 For OPR failures? [Section 9.7.4]	Requirement	
2.10.2 For method blank contamination? [9.6.2.2]	Requirement	
2.10.3 For positive/negative staining control failures?	Critical	
2.11 Does the quality assurance plan specifically address requirements for protozoa analysis under the programs for which the laboratory intends to analyze samples?	Critical	
2.12 Is a laboratory organization chart or other information available listing staff organization and responsibilities? Does it identify the QA manager?	Recommendation	
2.12.1 Is the QA manager separate from the lab manager?	Recommendation	
2.13 Does the laboratory have a list of preventative maintenance procedures and schedules? [Section 9.1]	Requirement	
2.14 Date range covered for quality control (QC) sample audit?		
2.15 When did the laboratory begin processing samples with the Envirochek filter?		/ /
2.16 When did the laboratory begin processing samples with the Filta-Max filter (if applicable)?		/ /
2.17 When did the laboratory begin processing samples with the CrypTest filter (if applicable)?		/ /
2.18 Approximately how many field samples were analyzed using methods 1622/1623 since the lab started using Method 1622/1623?		Field samples ___ MS_____
2.19 Have acceptable initial precision and recovery analyses been performed for each version of the method the laboratory is using? [Section 9.1.2.1.1]	Requirement	
2.20 Were method blanks run once per week or per 20 samples during this period? [Section 9.6.1]	Requirement	
2.20.1 If the answer to 2.20 is no, then at what frequency where method blanks performed?		
2.20.2 What percentage of method blanks evaluated were without contamination?		

Item to be Evaluated	Classification	Yes, No, Unknown*, or NA
2.20.3 Was an acceptable method blank associated with each field sample examined? [Section 9.6.2.2]	Requirement	
2.20.4 How many method blanks were evaluated?		
2.21 Were ongoing precision and recovery (OPR) samples run once per week or per 20 samples during this period? [Section 9.7]	Requirement	
2.21.1 If the answer to 2.21 is no, then at what frequency where OPR samples performed?		
2.21.2 What percentage of OPR samples evaluated met the recovery criteria? [Table 3; Section 9.7.3]		
2.21.3 Does the laboratory maintain control charts of OPR results? [Section 9.7.6]	Recommendation	
2.21.4 Was an acceptable OPR associated with each field sample examined? [Section 9.7.4.2]	Requirement	
2.21.5 How many OPR samples were evaluated?		
2.21.6 How many OPR samples were analyzed during the past six months?		
2.21.7 What is the mean and relative standard deviation (RSD) of the recoveries of the OPR samples analyzed during the past six months?		Mean_____ RSD_____
2.22 Were matrix spike (MS) samples analyzed at the method -specified frequency? [Section 9.1.8]	Requirement	
2.22.1 If the answer to 2.22 is no, then at what frequency were MS samples analyzed?		
2.22.2 How many MS samples were evaluated?		
2.22.3 How many MS samples were analyzed during the past six months?		
2.22.4 What is the mean and relative standard deviation of the MS samples analyzed during the past six months?		Mean_____ RSD_____
2.23 Were OPR and MS samples spiked with 100 - 500 organisms? [Section 9.7]	Requirement	
2.23.1 If the answer to 2.23 is no, then at what level were samples spiked?		
2.24 Are the laboratory personnel performing the QC analyses representative of the personnel seeking approval under this program?	Critical	
2.25 Does the laboratory have records of all QC checks available for inspection? [Section 9.1]	Requirement	
2.26 Does the laboratory have an adequate record system for tracking samples from collection through log-in, analysis, and data reporting?	Critical	
2.27 Are results from each sample maintained electronically?		
2.28 If data are stored electronically, are files backed up on more than one disk to ensure data are not lost in the eventuality of some hardware failure?	Critical	
2.29 If data is stored electronically, does the laboratory have an SOP for checking the accuracy of data entry into an electronic system?	Critical	

Item to be Evaluated	Classification	Yes, No, Unknown*, or NA
2.30 Is the laboratory using the December 2005 version of Method 1622/1623 for LT2 samples? [CFR 40 Part 141.704]	Requirement	
3 Data Recording Procedures		
3.1 Is shipping information complete, including the time and date of sample receipt, sample condition, and noting any discrepancies between samples on the traffic report and samples received? [Section 8.1.3]	Requirement	
3.2 Do sample numbers on the shipping forms match the sample numbers on the report forms?	Requirement	
3.3 Are current Method 1622/1623 bench sheets used to record sample processing data?	Recommendation	
3.4 Are all primary measurements during each step recorded, including all raw data used in calculations? [Section 11.0, 12.0, 13.0]	Requirement	
3.5 Name of analyst or technician performing the elution is recorded?	Critical	
3.6 Date and time of elution is recorded? [Section 12.2.6.2.1]	Requirement	
3.7 Name of analyst or technician performing the concentration is recorded?	Critical	
3.8 Date and time of concentration is recorded? [Section12.3.3.3.2]	Requirement	
3.9 Are batch and lot numbers of reagents used in the analysis of the sample recorded?	Critical	
3.10 Lot number for the IMS kit is recorded?	Critical	
3.11 Are Method 1622/1623 *Cryptosporidium* report forms used to record sample examination results? [Section 15.2]	Requirement	
3.12 Name of examining analyst is recorded? [Section 15.2.6]	Requirement	
3.13 Date and time of sample examination is recorded? [Section 15.2.4]	Requirement	
3.14 Are calculations of final concentrations and recoveries complete and correct?	Requirement	
3.15 Do values recorded on the data sheets match the reported values?	Requirement	
3.16 Are mistakes on all forms crossed out with a single line, initialed, and dated?	Critical	
3.17 Are data always recorded in pen?	Critical	
3.18 Are hardcopy records well organized, complete, and easily accessible?	Critical	
3.19 Does the laboratory include a disclaimer on the report to the client if method QC requirements were not met?	Recommendation	
3.20 Is the manually recorded data legible?	Critical	
3.21 Do records demonstrate each analyst's characterization of 3 oocysts and 3 cysts from positive control for each microscopy session? [Section 15.2.1.1]	Requirement	
3.22 Data shows that no more than 0.5 mL of pellet was used per IMS? [Section 13.2.4]	Requirement	

Note: All section references in [ ] refer to Method 1623 December 2005

Item to be Evaluated			Classification	Yes, No, Unknown*, or NA
4	**Holding Times**			
4.1	**Samples analyzed according to December 1999 version of Method 1622/1623**			
	4.1.1	Is time from initiation of sample collection to completion of concentration 72 hours or less?	Requirement	
	4.1.2	Concentrate is held no longer than 24 hours between IMS and staining?	Requirement	
	4.1.3	Are stained slides read and confirmed within 72 hours of staining?	Requirement	
4.2	**Samples analyzed according to the April 2001, 2003, or December 2005 version of Method 1622/1623**			
	4.2.1	Is sample elution initiated within 96 hours of sample collection or field filtration? [Section 8.2.1]	Requirement	
	4.2.2	Are sample elution, concentration, and purification steps completed in one work day? [Section 8.2.2]	Requirement	
	4.2.3	Are slides stained within 72 hours of application of the purified sample to the slide? [Section 8.2.3]	Requirement	
	4.2.4	Are stained slides read and confirmed within 7 days of staining? [Section 8.2.4]	Requirement	
5	**Spike enumeration procedures**			
5.1	What method does the laboratory currently use to estimate spike doses:(A) flow-sorted spikes, (B) well-slide-counted spikes, (C) hemacytometer-counted spikes, or (D) membrane-filter-counted spikes			Circle one: A B C D
	5.1.1	If flow-sorted spikes are used, on what date did the laboratory begin using flow-sorted spikes?		/ /
	5.1.2	If counted manually, does the laboratory follow Method 1622/1623 procedures for establishing spike level? [Section 11.3]	Requirement	
	5.1.3	What were the relative standard deviations of the last four spike enumerations?		1.
				2.
				3.
				4.
5.2	Source of oocysts for spikes			
5.3	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.			

Note: All section references in [ ] refer to Method 1623 December 2005

Part B: Sample Processing and Examination

Laboratory Name	Date of Evaluation	Name and Affiliation of Evaluator for Part B

Item to be evaluated	Classification	Yes, No, NA or Unknown
6 Laboratory Facilities and Laboratory Safety		
6.1 Are laboratory coats and gloves worn in the laboratory? [Section 5.3]	Critical	
6.2 No other safety or facility issues were observed?		
7 Sample Spiking Technician:		
7.1 What method does laboratory currently use to estimate spike doses:(A) flow-sorted spikes, (B) well-slide-counted spikes, (C) hemacytometer-counted spikes, or (D) membrane-filter-counted spikes		Circle one: A B C D
7.2 With what filter type did the laboratory demonstrate their spiking procedure?		
7.3 Is the carboy used for negative control randomly selected from carboy stock to check efficacy of cleaning system?	Critical	
7.4 If flow-sorted spikes are used, was suspension vial vortexed for 30 seconds or per manufacturer's instructions? [Section 11.4.3]	Method Procedure	
7.5 Was the suspension vial adequately rinsed? [Section 11.4.3.1]	Method Procedure	
7.6 Does the laboratory have an acceptable SOP for sample spiking?	Critical	
7.7 Other than the issues noted for items 7.2 through 7.6 (if any) was sample spiking demonstrated successfully?		
8 Envirochek (Complete Sections that apply)		
8.1 Envirochek Filtration Technician:		
8.1.1 Are all components required for sample filtration present and in good condition? [Section 6.2]	Requirement	
8.1.2 Is the filter assembly set up correctly? [Figure 3a, pg 63]	Method Procedure	
8.1.3 Is the pump adequate for needs? [Section 6.3.3]	Requirement	

Item to be evaluated		Classification	Yes, No, NA or Unknown
8.1.4	Is the appropriate flow rate maintained (approximately 2 L/min)? [Section 12.2.1.2]	Method Procedure	
8.1.5	Is the volume filtered measured using a flow totalizer or calibrated carboy? [Section 12.2.4.2]	Requirement	
8.1.6	Is the system well maintained and cleaned appropriately following use?	Critical	
8.1.7	Is the system able to maintain seal during use with no leaks?	Requirement	
8.1.8	Does the laboratory have an acceptable SOP for Envirochek filtration?	Critical	
8.1.9	Other than the issues noted in items 8.1.1 through 8.1.8, was Envirochek filtration demonstrated successfully?		
8.2	**Envirochek capsule filter elution** **Technician:**		
8.2.1	Is the elution buffer prepared as per Method 1622/1623? [Section 7.4.1]	Method Procedure	
8.2.2	Is the wrist-shaker assembly set up correctly? [Section 12.2.6.1.1]	Method Procedure	
8.2.3	Does the eluting solution cover the membrane? [Section 12.2.6.2.2]	Method Procedure	
8.2.4	Are the samples shaken at an appropriate speed? [Section 12.2.6.2.3]	Method Procedure	
8.2.5	Are the samples shaken three times for 5 minutes each time, and each in a different orientation? [Section 12.2.6.2]	Method Procedure	
8.2.6	Does the laboratory have an acceptable SOP for Envirochek capsule filter elution?	Critical	
8.2.7	Other than the issues noted for items 8.2.1 through 8.2.7 (if any) was Envirochek filter elution demonstrated successfully?		
9	**This section is no longer applicable and has been deleted.**		
10	**Filta-Max**		
10.1	**Filta-Max filtration** **Technician:**		
10.1.1	Are all components required for sample filtration present and in good condition? [Section 6.2.3]	Requirement	
10.1.2	Is the filter assembly set up correctly? [Fig. 3b, pg. 64]	Method Procedure	
10.1.3	Is appropriate flow rate maintained of <4 L per minute? [Section 12.3.1.1.3}	Method Procedure	
10.1.4	Is the volume filtered measured correctly using a flow meter or calibrated carboy? [Section 12.3.1.5.2]	Requirement	
10.1.5	Is system well maintained and cleaned appropriately following use? [Section 12.3.4]	Requirement	
10.1.6	Is system able to maintain seal during use with no leaks?	Requirement	

Item to be evaluated		Classification	Yes, No, NA or Unknown
10.1.7	Does the laboratory have an acceptable SOP for Filta-Max filtration?	Critical	
10.1.8	Does the laboratory indicate on the filter housing the correct direction of flow? [Section 12.3.1.3]	Critical	
10.1.9	Other than the issues noted in items 10.1.1 through 10.1.8 (if any) was Filta-Max filtration demonstrated successfully?		
10.2 Filta-Max filter wash station elution	**Technician:**		
10.2.1	Is an automatic or manual wash station used?		
10.2.2	Is the filter wash station set up correctly? [Section 12.3.2.1]	Requirement	
10.2.3	Is PBST used to elute the filter? [Section 7.4.2.4]	Method Procedure	
10.2.4	Is an appropriate amount of PBST used for each wash? (approx. 600 mL) [Section 12.3.2.2.1]	Method Procedure	
10.2.5	During the first wash, is the plunger moved up and down 20 times? [Section 12.3.2.2.1]	Method Procedure	
10.2.6	Is the plunger moved up and down gently to avoid generating excess foam?	Method Procedure	
10.2.7	During the second wash, is the plunger moved up and down 10 times? [Section 12.3.2.2.2]	Method Procedure	
10.2.8	If the automatic washer is used, is the machine operating properly? [Section 12.3.2.1]	Requirement	
10.2.9	Is the wash station cleaned adequately between samples? [Section 12.3.4.2]	Requirement	
10.2.10	Does the laboratory have an acceptable SOP for Filta-Max elution with the wash station?	Critical	
10.2.11	Other than the issues noted for items 10.2.2 through 10.2.10 (if any) was elution of the Filta-max filter using the wash station demonstrated successfully?		
10.3 Filta-Max filter stomacher elution	**Technician:**		
10.3.1	Is PBST used to elute the filter? [Section 7.4.3.4]	Method Procedure	
10.3.2	Is an appropriate amount of PBST used for each wash? (approx. 600 mL) [Section 12.3.2.3]	Method Procedure	
10.3.3	Are two washes performed for 5 minutes each? [Section 12.3.2.3]	Method Procedure	
10.3.4	Is the stomacher in good condition and operating properly?	Requirement	
10.3.5	Does the laboratory have an acceptable SOP for Filta-Max elution using a stomacher?	Critical	
10.3.6	Other than the issues noted for items 10.3.1 through 10.3.5 (if any) was elution of the Filta-Max filter using the stomacher demonstrated successfully?		
10.4 Filta-Max filter sample concentration (as an alternative to Section 11)	**Technician:**		

Item to be evaluated		Classification	Yes, No, NA or Unknown
10.4.1	Is concentrator set up correctly? [Section 12.3.3.2.1 b.]	Requirement	
10.4.2	Is the force of the vacuum maintained below 30 cm Hg? [note, pg. 43]	Method Procedure	
10.4.3	Is concentration performed after each of the washes?	Method Procedure	
10.4.4	Is the concentrate from the first wash added to the 600 mL of eluate from the second wash?	Method Procedure	
10.4.5	Is the sample concentrated so that some liquid remains above the filter (enough to cover the stirbar about half-way)? [Section 12.3.3.2.1]	Method Procedure	
10.4.6	Are the stir bar and concentration tube rinsed after each concentration and the liquid added to the concentrate? [Section 12.3.3.2.1 c.]	Requirement	
10.4.7	Was the filter membrane washed twice? [Section 12.3.3.2.3]	Method Procedure	
10.4.8	Was 5 mL of PBST used each time? [Section 12.3.3.2.3]	Method Procedure	
10.4.9	Is the membrane adequately washed to remove oocysts from filter?	Method Procedure	
10.4.10	Is the pellet volume determined? [Section 12.3.3.3]	Requirement	
10.4.11	Is there a set of standards for comparison of pellet size?	Recommendation	
10.4.12	Does the laboratory have an acceptable SOP for concentration using the Filta-Max concentrator?	Critical	
10.4.13	Other than the issues noted in items 10.4.1 through 10.4.12 (if any) was sample concentration using the Filta-Max concentrator demonstrated successfully?		
11 Concentration			
11.1 Envirochek, CrypTest, and Filta-Max filter sample centrifugation		**Technician:**	
11.1.1	Is the sample centrifuged at 1500 x G using a swinging bucket rotor? [Section 13.2.1]	Method Procedure	
11.1.2	Are the centrifuge tubes properly balanced prior to centrifugation?	Critical	
11.1.3	Is the sample centrifuged for 15 minutes? [Section 13.2.1]	Method Procedure	
11.1.4	Is the centrifuge slowly decelerated at the end without the brake? [Section 13.2.1]	Method Procedure	
11.1.5	Is the pellet volume determined? [Section 13.2.1]	Requirement	
11.1.6	Is there a set of standards for comparison of pellet size?	Recommendation	
11.1.7	Does the laboratory have an acceptable SOP for sample concentration?	Critical	
11.1.8	Is residual suspension rinsed from all containers and gloves?	Critical	

Item to be evaluated		Classification	Yes, No, NA or Unknown
11.1.9	Other than the issues noted in items 11.1.1 through 11.1.8 (if any) was sample concentration demonstrated successfully?		
12 Reagents, equipment and clean-up			
12.1 Source for reagent-grade water:			
12.1.1	Is still or DI unit maintained according to manufacturer's instructions?	Critical	
12.1.2	Is reagent grade water used to prepare all media and reagents? [Section 7.3]	Requirement	
12.2 Centrifuge:			
12.2.1	Does centrifuge have a swinging bucket rotor? [Section 6.8.1]	Requirement	
12.2.2	Does lab have easily accessible method for determining relative centrifugal force of centrifuges?	Critical	
12.3 SOP's for Reagents			
12.3.1	Are SOP's available for the preparation of all essential chemicals and reagents?	Critical	
12.3.2	Are SOP's posted or easily accessible at the bench?	Recommendation	
12.3.3	Are all reagents clearly labeled with date of preparation, technician initials, and expiration date?	Critical	
12.4 Clean-up			
12.4.1	Is all glassware and plasticware washed well and stored appropriately between uses?	Critical	
12.4.2	Is distilled or deionized water used for final rinse?	Critical	
12.4.3	Is an SOP available for glassware washing?	Critical	
13 Purification and Slide Preparation	**Technician:**		
13.1	What IMS kit/manufacturer is used?		
13.2	Is the supernatant from the centrifuged sample aspirated no lower than 5 mL above the pellet? [Section 13.2.2]	Requirement	
13.3	Is the pellet vortexed a sufficient time for resuspension? [Section 13.2.3]	Method Procedure	
13.4	Does the lab have an appropriate SOP for dividing pellets greater than 0.5 mL into subsamples and analyzing?	Critical	
13.5	Is no more than 0.5 mL of pellet used per IMS? [Section 13.2.4]	Method Procedure	
13.6	Is the resuspended pellet volume quantitatively transferred to the Leighton tube (2 rinses)? [Section 13.3.2.1]	Method Procedure	

Item to be evaluated		Classification	Yes, No, NA or Unknown
13.7	Are the IMS beads thoroughly resuspended prior to addition to the Leighton tube? [Section 13.3.2.2]	Method Procedure	
13.8	Is the leighton tube rotated at 18 rpm for 1 hour at room temperature? [Section 13.3.2.6]	Method Procedure	
13.9	Is Leighton tube correctly placed in magnet and rocked through 90 degrees about once per second? [Section 13.3.2.9]	Method Procedure	
13.10	Is all the liquid removed when decanting is performed with the magnet up? [Section 13.3.2.11]	Method Procedure	
13.11	Is the sample quantitatively transferred from the Leighton tube to the microcentrifuge tube (2 rinses)? [Section 13.3.2.13]	Method Procedure	
13.12	Are extra rinses to minimize debris performed appropriately when needed? Does the laboratory rinse A) IMS beads in the Leighton tube prior to transfer, B) Leighton tube, not IMS beads, prior to transfer, C) IMS beads in microcentrifuge tube prior to dissociation?	Method Procedure	Circle one: A B C
13.13	Is standard NaOH (5 μL, 1N) and standard HCl (50 μL, 0.1N) used? [See note on pg 49]	Requirement	
13.14	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? [Section 13.3.3]	Method Procedure	
13.15	Is a second dissociation performed? [Section 13.3.3.10]	Method Procedure	
13.16	When the second dissociation is performed, does the laboratory: (A) use a second slide, or (B) add the additional volume to the original slide?		Circle one: A B
13.17	Are the slides clearly labeled so they can be associated with the correct sample? [Section 13.3.3.7]	Requirement	
13.18	What type of slides are used?		
13.19	Is slide dried at a) room temperature or b) 35 to 42 C? [Section 13.3.3.12]		Circle one: A B
13.20	If the slide is warmed, is incubator or slide tray calibrated and labeled?	Critical	
13.21	Does the laboratory have an acceptable SOP for sample purification?	Critical	
13.22	Other than the issues noted in items 13.1 through 13.21 (if any) were sample purification and slide preparation performed successfully?		
14 Sample staining		**Technician:**	
14.1	What staining kit/manufacturer is used? [Section 14.2]		
14.2	Is FITC stain applied according to manufacturer's directions? [Section 14.2]	Method Procedure	
14.3	Are positive and negative staining controls performed? [Section 14.1]	Requirement	

Item to be evaluated		Classification	Yes, No, NA or Unknown
14.4	Are the slides incubated in a humid chamber in the dark at room temperature for approximately 30 minutes or per manufacturer's directions? [Section 14.3]	Method Procedure	
14.5	Are the labeling reagents rinsed away properly after incubation, without disturbing the sample? [Section 14.5]	Method Procedure	
14.6	Was the working DAPI stain prepared the day it was used? [Section 7.7.2]	Method Procedure	
14.7	Is stock DAPI stored at 1 to 10^{o}C in the dark? [Section 7.7.2]	Method Procedure	
14.8	Is the DAPI stain applied properly and allowed to stand for a minimum of 1 minute? [Section 14.6]	Method Procedure	
14.9	Is the DAPI stain rinsed away properly without disturbing the sample? [Section 14.7]	Method Procedure	
14.10	Is the mounting media applied properly?	Method Procedure	
	14.10.1 What type of mounting media is used?		
	14.10.2 Are all the edges of the cover slip sealed well with clear fingernail polish, unless Elvenol is used? [Section 14.9]	Method Procedure	
14.11	Are the finished slides stored in a humid chamber in the dark at 1 to 10^{o}C (humid chamber not required for Evenol)? [Section 14.10]	Method Procedure	
14.12	Does the laboratory have an acceptable SOP for sample staining?	Critical	
14.13	Other than the issues noted in items 14.2 through 14.13 (if any) was sample staining demonstrated successfully?		
15 Microscope and Examination			
15.1	Is microscope equipped with appropriate excitation and band pass filters for examining FITC labeled specimens? (Exciter filter - 450-490 nm, dichroic beam-splitting mirror - 510 nm, barrier or suppression filter: 515-520 nm)? [Section 6.9.2]	Requirement	
15.2	Is microscope is equipped with appropriate excitation and band pass filters for examining DAPI labeled specimens? (Exciter filter - 340-380 nm, dichroic beam-splitting mirror - 400 nm, barrier or suppression filter - 420 nm) [Section 6.9.3]	Requirement	
15.3	Does the microscope have HMO or DIC, objectives? [Section 6.9.1]	Requirement	
15.4	Is microscope operation easily changed from epifluorescence to DIC/HMO?	Recommendation	
15.5	Does the microscope have a 20 X scanning objective? [Section 6.9.1]	Requirement	
15.6	Does the microscope have a 100 X oil immersion objective? [Section 6.9.1]	Requirement	
15.7	Is the microscope equipped with an ocular micrometer? [Section 6.9.1]	Requirement	
15.8	Is a stage micrometer available to laboratory? [Section 10.3.5]	Requirement	

	Item to be evaluated	Classification	Yes, No, NA or Unknown
15.9	Is a calibration table for each objective located close to the microscope(s)? [Section 10.3.5]	Requirement	
15.10	Has the mercury bulb been used less than the maximum hours recommended by the manufacturer? [Section 10.3.2.11]	Recommendation	
15.11	Does the positive control contain *Cryptosporidium* oocysts at the appropriate fluorescence intensity for both FITC and DAPI? [Section 15.2.1.3]	Requirement	
15.12	Does the laboratory have an acceptable SOP for sample examination?	Requirement	
15.13	Other than the issues noted for items 15.1 through 15.13 (if any) were other microscope or examination issues acceptable?		

16 Evaluation of sample processing				
Method Step (filtration, elution, concentration, purification or staining)	**Name**	**Position**	**Demonstrated Technique Successfully** yes/no	**Recorded data as sample was analyzed** yes/no

17 Was analyst microscope examination acceptable? (yes/no)									
Classification	**Requirement**	**Requirement [Section 10.3.4]**	**Requirement [Section 10.3.4.2]**	**Requirement [Section 10.3.6]**	**Requirement [Section 15.2.1.1]**	**Requirement [Section 10.6]**	**Requirement [Section 15.2.1.1]**	**Requirement [Section 15.2.1.1]**	**Recommendation**
Name	**Position**	**Adjust Interpupillary Distance**	**Focus both eyepieces**	**Establish Kohler Illumination**	**Examine and Record Characteristics of Three Oocysts**	**Crypto Count**	**Measurement with 1000X objective**	**Demonstrated Internal Structures**	**Examines Slides < 4 hrs per day**

Comments:

Appendix G

Method 1622/1623 Bench Sheet

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Laboratory Name:	Laboratory ID:

Method 1622/1623 Bench Sheet

Sample Identification Information

* Lab Sample ID:	Person Receiving Sample:
* PWS ID:	*Sample collection date and time:
* Facility ID:	Turbidity (NTU):
* Sample Collection Point ID:	Temperature, date and time @ 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:
Spiking suspension ID:			Spiking analyst:

Sample Filtration

Filter type (circle one): Envirochek Envirochek HV Filta-Max CrypTest Other (specify)			
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:	

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

Elution procedure (circle one): Wrist shaker Filta-Max wash station Stomacher Backflush/sonication	
Type of Elution buffer:	Elution time:
Elution buffer lot number:	Elution date:
Elution buffer 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 and 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

Appendix H

Method 1622/1623 Slide Examination Form

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Laboratory name:	Laboratory ID:

Method 1622/1623 Slide Examination Form

Sample ID:	Analyst:
Examination/verification completion date and time : (must be completed within 168 hours (7 days) of staining)	Slide number: Total number of slides for this sample:
Positive staining control acceptable ☐ YES ☐ NO	Negative staining control acceptable ☐ YES ☐ NO
FITC, Size, Shape, DIC and DAPI Characteristics of 3 Oocysts Recorded ☐ 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 (A)	Intense blue internal staining (B)	Number of nuclei stained sky blue (C)	Empty oocysts (D)	Oocysts with amorphous structure (E)	Oocysts with internal structure (F) Number of sporozoites
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								

Total FA number from this slide:	D.I.C. - Total number of empty oocysts (D):
DAPI -: Total number (A):	D.I.C. - Total number of oocysts with amorphous structure (E):
DAPI +: Total number (B):	D.I.C. - Total number of oocysts with internal structure (F):
DAPI +: Total number (C):	Total count DAPI + (C) that show structure by D.I.C. (F):

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 (A)	Intense blue internal staining (B)	Number of nuclei stained sky blue (C)	Empty cysts (D)	Cysts with amorphous structure (E)	Cysts with internal structure (F) Number of nuclei	Median body	Axonemes
1										
2										
3										
4										
5										
6										
7										
8										
9										
10										

Total FA number from this slide:	D.I.C.: Total number of empty cysts (D):
DAPI-: Total number (A):	D.I.C.: Total number of cysts with amorphous structure (E):
DAPI+: Total number (B):	D.I.C.: Total number of cysts with one internal structure (F):
DAPI+: Total number (C):	D.I.C.: Total number of cysts with >one internal structure (F):
	Total number DAPI + (C) that show structure by D.I.C. (F):