NATIONAL ENVIRONMENTAL LABORATORY ACCREDITATION CONFERENCE (NELAC)

ON-SITE LABORATORY ASSESSMENT

PROTOZOA & VIRUS MICROSCOPY CHECKLIST (8 PAGES TOTAL)

LABORATORY: _____

Physical Address: _____

_____

Mailing Address: _____
(if different from above)

_____

Telephone Number: _____ Facsimile Number: _____

E-mail address: _____

INSPECTED BY: (Name) (Affiliation)

_____ _____

_____ _____

_____ _____

INSPECTION DATES: _____

LABORATORY TECHNICAL DIRECTORS AND MANAGEMENT:

(Name) (Title)

_____ _____

_____ _____

_____ _____

_____ _____

_____ _____

_____ _____

_____ _____

_____ _____

_____ _____

GENERAL INSTRUCTIONS: Before each item is a blank line and a NELAC Standard citation in **Bold Numerals**.

Place a check mark (__----) in the blank if the laboratory meets the NELAC Standard referenced.

Place an X-mark (X) in the blank if the Standard is not met and the laboratory must devise an acceptable Plan of Correction and estimated completion date. **The NELAC Standard reference must be cited in in the on-site assessment report.**

Mark **"N/A"** in the blank if the NELAC Standard is not applicable to this laboratory, either because of the nature of its business mission, because of the analytical tests it performs, or because of the situation never ever happening.

Notes:

The use of **EPA 1622 and 1623** for CWA is mandated at **40 CFR Part 136, Table 1A**

The use of **ASTM D4994-89** is mandated at **40 CFR Part 503.8**

If the laboratory appears to meet a particular NELAC Standard but does not have the documentation to back up its claim, use the following:

_____ **5.0** Does the laboratory have **all items** identified in NELAC Chapter 5 Quality Systems **available** for on-site inspection or data audit

MICROSCOPY LABORATORY TOUR

____ **5.5.5.2.1(d)** Is the following **support equipment** associated with microbiological testing checked with NIST traceable materials (where possible)

- ____ pH meter
- ____ Balance(s)
- ____ Conductivity meter
- ____ Refrigerator(s) for sample storage and/or media storage
- ____ Meter for measuring Residual Chlorine
- ____ Incubators

____ **5.5.5.2.1(d)** Is the **support equipment acceptability** for use according to the **needs of the analysis** or the application for which the equipment is being used

Viruses incubation: 36.5 +/- 1.0 C

____ **5.5.6.4(d)** Do all containers of prepared reagents & standards have a **unique identifier & expiration date** that **links** these specific containers of reagents & media to their preparation records

____ **5.5.8.3.1(a)(2)** Has the laboratory **checked samples** for **proper preservation**

COMMENTS:

____ **5.1.1** **Does the laboratory fulfill the** requirements of the following test methods **that it performs**

EPA 1622 (Cryptosporidium Oocysts) and 1623 (Giardia Cysts & Cryptosporidium Oocysts)

Polyethersulfone capsule filter media, to collect cysts & oocysts
Eluting solution (Laureth-12, 1 M pH 7.4 Tris buffer, 0.5 M pH 8.0 EDTA, Antifoam-A)
OPTIONAL COLLECTION MEDIA: **Polycarbonate capsule filter** or **Foam filter** media
Eluting solution (pH 7.4 phosphate buffered saline (w/ KCl, NaCl), Tween 20 or 80, Antifoam A)

Centrifuge, to concentrate cysts & oocysts into a pellet at 1100-g
Immunomagnetic Separation of cysts & oocysts from centrifuge pellet with Dynabeads GC-Combo (attachment of magnetic beads conjugated to anti-Cryptosporidium & anti-Giardia antibodies)
Magnetic Particle Concentrators
HCl, to dissociate magnetic beads from the cysts & oocysts
Positive Control: 100-500 Giardia Lamblia Cysts and/or Cryptosporidium Parvum Oocysts (analyzed each week of method use)
Negative Control: Reagent water (analyzed each week of method use)
Diluted Primary Antibody & Labeling Reagent, to stain cysts & oocysts (1-minute contact period in well slides)
(Indirect Fluorescence Assay: fluorescein isothiocyanate (FITC)-labeled specimens)
(DAPI stain: 4',6-Diamidino-2-phenylindole)
2% DABCO (1,4-diazabicyclo[2.2.2]octane) **– Glycerol mounting medium**
Microscope with **epifluorescence** and **differential interference contrast** (or oil immersion Hoffman modulation) optics with Kohler illumination; with stage & ocular micrometers; 20X-100X objectives
Excitation/band-pass filters (Immunofluorescent assay: 450-490 nm exciter filter, 510 nm dichroic beam-splitting mirror, 515-520 nm barrier or suppression filter; DAPI: 340-380 nm exciter filter, 400 nm dichroic beam splitting mirror, 420 nm barrier or suppression filter)
Cysts (8-18 um long by 5-15 um wide) & Oocysts (4-6 um diameter) are **apple-green fluorescent round-oval** shapes
Cysts & Oocysts have **sky-blue nuclei & intense blue internal staining, or light blue internal staining** under DAPI
Giardia cyst internal morphological characteristics (D.I.C.): 1-4 nuclei, axonemes, & median bodies
Cryptosporidium oocyst internal morphological characteristics: 1-4 sporozoites

Microscope **Epifluorescent Hg Bulb adjustments** - when microscope is first used & when replacing bulbs
Transmitted Bulb adjustments – when the bulb is changed
Adjustment of **interpupillary distance & oculars** for each eye – done **each time** the analyst uses the microscope
Calibration of Ocular Micrometer – when microscope is first used & **each time** the objective is changed
Establishing **Kohler illumination (DIC)** – for **each use** of the microscope & **each time** the objective is changed

Acceptance criteria for the **4-replicate Initial Demonstration of Capability**: ~25-100%, <50%RSD each organism
Acceptance criteria for the **week-of-use Demonstration of On-going Capability**: 11-100% oocysts, 14-100% cysts
Acceptance criteria for **Matrix Spikes/Matrix Spike Duplicates**, analyzed **initially & every 20 samples per utility**: 13-111% recovery, <61%RSD Cryptosporidium oocysts; 15-118% recovery, <30%RSD Giardia cysts
Each analyst counts **DAPI-positive & DAPI-negative** cysts & oocysts from the **same prepared slide monthly**, with counts agreeing within 10%
Holding Times: 0-8 C, sample filtration & elution within 96 hours of collection; elution, pelletization, & immunomagnetic purification done the same work day; staining within 72 hours of purification; microscope examination & verification within 7 days of staining; cyst & oocyst suspensions spiked within 24 hours of enumeration.
True count of oocysts & cysts in spiking suspensions determined by either **hemacytometer or by direct analysis** on well slides or membrane filters

EPA 1601 (Male-Specific & Somatic Coliphage by 2-Step Enrichment)
EPA 1602 (Male-Specific & Somatic Coliphage by Single Agar Layer Procedure)

Log-phase host bacteria: E. coli Famp for male-specific coliphage, & E. coli CN-13 for somatic coliphage;
Store at 1-5 C after preparation up to 48 hours until ready to use;
Freeze host bacteria at –20 C to keep for 2 months, or at –70 C to keep for one year
Coliphage stock: MS2 (ATCC #15597-B1, male-specific), & Phi-X 174 (ATCC #13706-B1, somatic);
Store at 2-8 C for up to 5 years
Raw sewage must be analyzed within 24 hours of collection; at least 10 mL filtered sewage must be obtained
If filtrate stored over 24 hours, it must be re-titered before use (can hold 72 hours if titer not decreased >50%)
Media & Antibiotic stocks: must always add antibiotic to medium after autoclaving, store frozen at –20 C for 1 yr,
Thaw at 37 C & mix well prior to use
10X Tryptic Soy Broth (TSB): store at 1-5 C until used
1.5% Tryptic Soy Agar (TSA) with antibiotic: store with plates inverted at 1-5 C for 2 weeks
0.7% TSA with antibiotic: must use on day of preparation, keep at 45-48 C until used
2X TSA with antibiotic (EPA 1602): must use on day of preparation, keep at 45-48 C until used
Spot Plates: store at 1-5 C for up to 4 days
Holding Times: 48 hours from collection to incubation

EPA 1601: Enrich water sample with MgCl2, log-phase host bacteria, & TSB; incubate overnight at xx C;
Spot samples onto lawn of host bacteria specific for each type of coliphage; incubate at xx C;
Examine for circular lysis zones, to indicate the presence of coliphage
Acceptance criteria for the **10-replicate Initial Demonstration of Capability**: spiked with enumerated sludge to 1-2 PFU per sample each coliphage type; at least 5 samples must produce positive results
Acceptance criteria for the **On-going Demonstration of Capability**: 3 LCS's at 1-2 PFU each coliphage type, every 20 samples; at least 1 out of 3 samples must produce positive results for each coliphage
Acceptance criteria for **Matrix Spikes/Matrix Spike Duplicates**, analyzed **initially & every 20 samples per utility**: 3 samples spiked at 1-2 PFU each coliphage type, at least 1 must produce positive results each type
Positive Control each spot plate: spiked with 20 PFU each coliphage type from sewage fitrate, or 60 PFU from pure coliphage stock culture
Method Blank each spot plate used

EPA 1602: Add in the following order: sample, MgCL2, host bacteria, & double-strength TSA with antibiotic;
Pour into 5-10 plates; incubate overnight at xx C;
Examine for circular lysis zones (plaques), to indicate the presence of coliphage
Acceptance criteria for the **4-replicate Initial Demonstration of Capability**: spike to ~80 PFU per sample 9-130%, <46%RSD for male-specific coliphage; 86-177%, <23% RSD for somatic coliphage
Acceptance criteria for the **On-going Precision & Recovery**: LCS at 80 PFU each batch of 20 or fewer samples; 4-135% recovery for male-specific coliphage; 79-183% recovery for somatic coliphage
Acceptance criteria for **Matrix Spikes/Matrix Spike Duplicates**, analyzed **initially & every 20 samples per utility**: 0-120%, <57% RSD for male-specific coliphage; 48-291%, <28% RSD for somatic coliphage
Method blank each batch

ASTM D4994-89 / SM9510G (Viruses)

Positively-charged **1MDS filter cartridge**, to collect viruses from water (pH of sample < 8.0)
Negative QC Sample: Sterile 1MDS filter (analyzed with each batch of samples)
Positive QC Sample: 40 L water spiked with 200 PFU attenuated poliovirus, process through 1MDS filter (analyzed with each batch of samples)
pH 9.5 **1.5% Beef Extract / 3.75% Glycine**, to elute viruses from the filter cartridges (autoclave at 121 C, 15 min) (stable for 1 week if refrigerated)
Note: Screen each new lot of Beef Extract with 200 PFU poliovirus. Use a single passage with undiluted, 1:5 diluted, & 1:25 diluted samples. Mean recovery > 50% for the 3 trials.
0.05 M Aluminum Chloride, to salt sludge samples
HCl, to **adjust eluate or sludge sample pH to 3.5 +/- 0.1** (precipitate forms in this **Organic Flocculation** step) (pH<3.4 may inactivate viruses)
Centrifuge suspension at **4 C, 2500-g, 15 min** (viruses remain in the precipitate)
pH 9.0-9.5 **0.15 M Phosphate Buffer**, to dissolve precipitate (or add buffered Beef Extract to precipitated sludge)
Centrifuge at **4 C, 4000-10000 g, 30 min** (viruses now in supernatent), then adjust pH to 7.0-7.5
0.22-um porosity membrane filter, to remove microbial interferences from supernatent
Flocculated Beef Extract, to concentrate viruses from sludge samples further
2 subsamples prepared, refrigerated at 4 C if assayed within 24 hr, frozen at –70 C otherwise

ICR MICROBIAL MANUAL, SECTION VIII; ASTM D4994-89 / SM9510G (continued)

Buffalo Green Monkey (BGM) cell line, from African green monkey kidney cells
Note: For ICR (SDWA) only passages between 117-250 may be used
Negative Assay Control: BGM cell monolayer inoculated with pH 7.0-7.5 phosphate buffer
Analyzed with each group of subsamples inoculated onto cell cultures
Recommended criteria for batch acceptance: No cytopathic effects (CPE) observed
Positive Assay Control: BGM cell monolayer inoculated with pH 7.0-7.5 phosphate buffer containing 20 PFU (Plaque Forming Units) attenuated poliovirus type 3
Analyzed with each group of subsamples inoculated into cell cultures
Recommended criteria for batch acceptance: CPE developed
For each sample, subsample #1 inoculated onto 10 BGM cell cultures, incubated for 80-120 min to permit viruses to infect cells (warm subsamples to room temperature before inoculation)
Add **maintenance medium** (MEM/L-15 medium with 2% or 5% calf serum, 0.1% penicillin-streptomycin, 0.05% tetracycline, 0.02% fungizone), **incubate at 36.5 +/- 1 C**
If cytotoxicity not evident & > 3 cultures negative for CPE, inoculate subsample 2 into 10 additional BGM cell monolayers
If cytotoxicity not evident & > 7 cultures positive for CPE, inoculate 10 BGM cell monolayers each with undiluted, 1:5, & 1:25 dilutions of subsample 2 (same for Positive QC Samples & PT samples)
If all (30 total) inoculated cultures turn out positive (for CPE), inoculate 10 BGM cell monolayers each with 1:125, 1:625, & 1:3125 dilutions
Assay higher dilutions until at least 1 test vessel at the highest dilution is negative for CPE
If cytotoxicity is evident in subsample 1, omit the 80-120 min infection period when inoculating subsample 2 but rinse BGM cell monolayer with **washing solution** (2% calf serum in 0.85% salt solution)
Examine cultures microscopically for CPE daily for first 3 days, then every couple days for 14 days (CPE identified as **cell disintegration or changes in cell morphology**)
Freeze positive cultures at –70 C when > 75% of monolayer shows signs of CPE
Freeze negative cultures after 14 days incubation

Confirmation of all results from previous cell passage:

Thaw culture, filter 10% of medium through 0.22-um filters, & inoculate another BGM cell monolayer
Add maintenance medium, incubate, examine microscopically as above
Score cultures with **CPE in both first & second passages as confirmed positives**
Viruses quantitated as **Most Probable Number**

BGM Cell Culture Maintenance:

Test **each lot** of calf serum for **cell growth & toxicity**

Pass stock BGM cell cultures **every 7 days** with **growth medium** (MEM/L-15 with 10% calf serum, 0.1% penicillin-streptomycin, 0.05% tetracycline, 0.02% fungizone)

Discard maintenance medium, dislodge monolayer with **EDTA-trypsin** reagent (<5 min)

Centrifuge at <1000-g for 10 min to pellet cells

Suspend cells in growth medium, incubate at 36.5 +/- 1 C in air-tight culture vessels

Replace growth medium with maintenance medium when cell monolayers are 95-100% confluent

BGM cell line splits at 1:2 ratio for passages 117-150, at 1:3 ratio for passages 151-250

Determine **viable cell counts** each time BGM cells are passed or prepared for storage

Add **0.5% trypan blue** to cell suspension

Count cells in hemocytometer (in 4 large corner sections & center section, count clear cells , (not blue cells), count cells on the top & left lines of each section but not on the bottom & right lines)

Cell concentration must be **greater than 200000 cells per mL**

Prepare cells for **storage** by dislodging cell monolayer with EDTA-trypsin, centrifuging, & suspending in **storage medium** (10% Dimethyl Sulfoxide in growth medium)
(still must do viable cell count test)

Refrigerate cells at 4 C for 30 min, then –20 C for 30 min, then frozen at –70 C

Thaw frozen cells rapidly at 36.5 +/- 1.0 C, sterilize outside vial surface with 0.5% iodine / 70% ethanol

Add growth medium & incubate for 18-24 hr, replace growth medium & incubate 5 more days

Pass and maintain new cultures as described above

Sterilization requirements for virus monitoring:

Autoclave at 121 C:

> 15 min: Solutions, buffers, disinfectants, media

> 30 min: Glassware/plasticware/equipment/steel vessels that come into contact with test waters, Contaminated test materials, & 1MDS filter cartridges

0.1% Chlorine in pH 6-7: pumps, filter housings, tubing

95% Ethanol lamp: scissors, forceps, other instruments

Verify sterility of liquids by adding Thioglycollate broth, incubate at 36.5 +/- 1 C for 7 days, discard liquid if growth is observed

Verify sterility of media by incubating 5% of volume to be used at 36.5 +/- 1 C for 7 days before use, discard any media that loses clarity as cloudiness indicates contamination

EPA 600/1-87-014 (Helminth Ova)

Suspend compost sample in **pH 6.7-7.7 phosphate-buffered water with 0.1% Tween 80**

Filter sample through **48-mesh Tyler sieve**, to remove large particles

Let sample settle overnight, then siphon out supernatent

Centrifuge at 400-g (1200 RPM) for 3 min

Discard supernatent & resuspend pellet in **Zinc Sulfate** (specific gravity 1.20); centrifuge at 400-g for 3 min (**density gradient centrifugation**)

Dilute ZnSO4 supernatent, allow settling for 3 hr

Aspirate supernatent, then **resuspend sediment with water**

Centrifuge at 480-g (1400 RPM) for 3 min

Resuspend pellet in **0.1 N Sulfuric Acid / 35% Ethanol**, to remove proteinaceous material

Centrifuge at 660-g (1800 RPM) for 3 min

Resuspend pellet in **0.1 N Sulfuric Acid**

Incubate at **26 C for 3-4 weeks**

Positive Control (incubated with each sample batch): Control ova dissected from adult **Ascaris lumbricoides**

Examine samples after majority of control ova are embryonated, with **Sedgwick-Rafter cell** to count ova

Note **viability** based on embryonated ova whose larval forms can be induced to move when light intensity increases

Report as # Ova per gram dry weight

Analyze **every tenth sample in duplicate,** precision criteria < 0.5702 ova/g

MICROSCOPY TEST METHODS

____ **5.5.4.1.2(a)** Does the laboratory have an **in-house methods manual** for each accredited **analyte** or **method**
Note: This manual may consist of copies of published or referenced test methods

____ **5.5.4.1.2(b)** Does the laboratory **clearly indicate** in its methods manual **any modifications** made to the referenced test method and **describe any changes or clarifications** where the referenced test method is ambiguous or provides insufficient detail

Does each test method in the in-house methods manual include or reference, where applicable:

____ **5.5.4.1.2(b)(1)** **Identification** of the test method
____ **5.5.4.1.2(b)(2)** Applicable **matrix or matrices**
____ **5.5.4.1.2(b)(3)** **Method Detection Limit**
____ **5.5.4.1.2(b)(4)** **Scope & application**, including components to be analyzed
____ **5.5.4.1.2(b)(5)** **Summary** of the test method
____ **5.5.4.1.2(b)(6)** **Definitions**
____ **5.5.4.1.2(b)(7)** **Interferences**
____ **5.5.4.1.2(b)(8)** **Safety**
____ **5.5.4.1.2(b)(9)** **Equipment & supplies**
____ **5.5.4.1.2(b)(10)** **Reagents & standards**
____ **5.5.4.1.2(b)(11)** Sample **collection, preservation, shipment, & storage**
____ **5.5.4.1.2(b)(12)** **Quality control**
____ **5.5.4.1.2(b)(13)** **Calibration & standardization**
____ **5.5.4.1.2(b)(14)** **Procedure**
____ **5.5.4.1.2(b)(15)** **Calculations**
____ **5.5.4.1.2(b)(16)** Method **performance**
____ **5.5.4.1.2(b)(17)** Pollution **prevention**
____ **5.5.4.1.2(b)(18)** **Data assessment & acceptance criteria** for quality control measures
____ **5.5.4.1.2(b)(19)** **Corrective actions** for out-of-control data
____ **5.5.4.1.2(b)(20)** Contingencies for **handling out-of-control or unacceptable data**
____ **5.5.4.1.2(b)(21)** **Waste management**
____ **5.5.4.1.2(b)(22)** **References**
____ **5.5.4.1.2(b)(23)** **Tables, diagrams, flowcharts, validation data**

____ **D** Does the laboratory ensure that the **essential standards** outlined in Appendix D are incorporated into the method manuals and/or Quality Manual

COMMENTS:

MICROSCOPY TEST METHODS ASSESSED: _____

____ **5.5.4.2.2(a) C.1** Has the laboratory performed a **satisfactory demonstration of method capability** prior to the acceptance & institution of this test method

____ **C.1** Does the laboratory **document** in its Quality Manual **other adequate approaches** to **Demonstration of Capability** if the procedure below is **not required** by the mandated test method or regulation and if the laboratory **elects not to perform** this procedure

____ **5.5.4.2.2(d) C.2** Does the laboratory use the **NELAC-specified certification statement** to document the **completion of each Demonstration of Capability** (initial & continuing)

____ **C.2** Are copies of these certification statements retained in the **personnel records** of each **employee performing the test method**

____ **5.5.2.6(c)(3)** Does each Analyst have **documentation** of **continued proficiency** by at least **one of the following once per year**:

- Acceptable performance of a **blind sample** (single blind to the analyst)
- Another **demonstration of capability**
- Successful performance of a blind performance sample on a **similar test method** using the **same technology**
- At least **4 consecutive** laboratory **control samples** with **acceptable levels** of precision & accuracy
- Analysis of **authentic samples** that have been analyzed by **another trained analyst** with **statistically identical results**

____ **5.5.4.2.2(d)** Does the laboratory **retain all associated supporting data** necessary to **reproduce the analytical results** summarized in the appropriate certification statement

____ **5.5.4.2.2(e) C.1** Does the laboratory **complete a demonstration of capability each time** there is a **change** in **instrument type, personnel, or test method**

____ **5.5.4.2.2(f)** Does the laboratory **fully document** the achievement of **demonstration of capability requirements** for each **specialized work cell**

Note: A work cell is defined as a group of analysts with specifically defined tasks that together perform the test method

____ **5.5.4.2.2(g)** Does the laboratory demonstrate & document acceptable performance through **acceptable continuing performance checks** (e.g laboratory control samples) **each time** that **membership** in a work cell **changes**

____ **5.5.4.2.2(g)** Do the **new members** of the work cell **work with experienced analysts** in the specialty area

____ **5.5.4.2.2(g)** Does the laboratory **repeat a Demonstration of Capability** with the new work cell if the **first 4 continuing performance checks** following the change in personnel **produce a failure** in any sample batch acceptance criteria

____ **5.5.4.2.2(g)** Does the **Demonstration of Capability repeated** if the entire **work cell is changed or replaced**

____ **5.5.4.2.2(h)** Is the **performance of the work cell** as a group **linked to the training records** of the **individual members** of the work cell

COMMENTS: If applicable, list all test species & test methods where the above Standards are not being met.