4B-1. Standard Operating Procedure for Detection of *Cryptosporidium* and *Giardia* in Portland Water Bureau Scat Samples

STANDARD OPERATING PROCEDURE FOR
Detection of *Cryptosporidium* and *Giardia* in Portland Water Bureau Scat Samples

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**** All related QA/QC must be performed prior to and during performance of this method. See ASI224-1.doc for QA/QC requirements and methodologies.**

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STANDARD OPERATING PROCEDURE FOR
Detection of *Cryptosporidium* and *Giardia* in Scat Samples

1.0 APPROVALS

The signatures of the following individuals indicate that this SOP is approved and complete:

Carolyn Fogg: Carolyn M. Fogg (Signature) 12/3/10 (Date)

Colin Fricker: (Signature) 12/3/10 (Date)

Monique DeSarno: Monique DeSarno (Signature) 12/3/10 (Date)

2.0 SCOPE AND APPLICATION

2.1 This SOP outlines the procedure for determination of the concentration of *Cryptosporidium* and *Giardia with* ColorSeed™ in animal scat by immunomagnetic separation (IMS), and immunofluorescence microscopy (FA). Confirmation of *Cryptosporidium* and *Giardia* is accomplished using morphometrics, 4',6-diamidino-2-phenylindole (DAPI) vital dye staining and differential interference contrast (DIC) microscopy. The use of ColorSeed™ as internal positive control is included.

2.2 This method will not identify the species of *Cryptosporidium* or *Giardia* or the host species of origin, nor can it determine the viability or infectivity of detected oocysts and cysts.

2.3 This method is for use only by persons experienced in the determination of *Cryptosporidium* and *Giardia* by IMS, FA and trained on the detection of ColorSeed™.

2.4 The document control number is ASI224-5.doc.

3.0 SUMMARY OF METHOD

3.1 A scat sample is collected in the field and shipped to the laboratory. A predetermined amount of sample is weighed, homogenized and spiked with ColorSeed™. The sample is allowed to sit overnight refrigerated at 2 - 8°C. The following day, the sample is processed with ether extraction (if requested by the client), then sodium pyrophosphate treatment. The sample is then mixed with magnetic beads conjugated to anti-*Cryptosporidium* and anti-*Giardia* antibodies. Using a magnet, the organisms are separated from the extraneous materials in the sample. The oocysts and cysts are stained on well slides with fluorescently labeled monoclonal antibodies and examined under fluorescence and differential interference contrast (DIC) microscopy.

3.2 The organisms are identified by size, shape, and fluorescence characteristics, and quantified by counting the identified and confirmed oocysts and cysts. ColorSeed organisms are distinguished from indigenous organisms by characteristic red fluorescence when examined using epifluorescence microscopy and a Texas Red filter set.

4.0 DEFINITIONS

Please refer to SOP ASI224-6.doc

5.0 INTERFERENCES

5.1 Some samples may contain high levels of interfering organisms, substances, or materials. Some samples will not allow separation of the oocysts and cysts from the eluate, and others may contain materials that preclude or confuse microscopic examination (e.g., cross reactors). Ether extraction may be requested to aid in organism recovery.

5.2 Chemicals added to source water during treatment may interfere with the concentration, separation, and examination of the sample.

5.3 Freezing may interfere with detection and/or identification of organisms.

6.0 SAFETY

6.1 Extreme caution needs to be taken when working with DAPI. Consult the material safety data sheet (MSDS) for safe handling and disposal of DAPI.

6.2 Strict adherence to laboratory safety requirements, such as the use of personal protective equipment including gloves and lab coats, must be practiced when indicated by laboratory safety policies and procedures.

6.3 All samples should be handled as if they contain risk group 2 pathogens. As defined by ASI, based on literature from the Centers for Disease Control and Canadian Laboratory Biosafety Guidelines, risk group 2 pathogens are those that can cause human disease but under normal circumstances are unlikely to be a serious hazard to healthy laboratory workers. Personal protective equipment (PPE) such as lab coat, gloves, etc., is worn at all times, and procedures that may create hazardous or infectious aerosols or splashes are conducted in the fume hood.

6.4 Samples thought to be hazardous should be handled with personal protection equipment for chemical hazards as well as potential pathogens. Follow specific project guidelines if provided.

7.0 SAMPLE CONTAINERS, COLLECTION AND PRESERVATION

7.1 Samples are typically collected in plastic 100mL containers and shipped to the laboratory for spiking, immunomagnetic separation (IMS), staining and examination.

7.2 Store samples at 1 – 10°C between collection and shipment to the laboratory and upon receipt at the laboratory until ready for processing. Do not allow them to freeze.

7.3 Processing of samples should start within 7 days of collection (in some cases, PWB analysis only commences once PWB has reviewed the *E. coli* data). Established holding times for all method steps are presented in Attachment 1. Samples that are out of hold time may be analyzed upon direction of the client and the report will be footnoted

7.3.1 Sample temperatures are checked upon receipt. Samples with a temperature ≤20°C and with no evidence of freezing are acceptable.

8.0 APPARATUS AND MATERIALS

8.1 Refer to EPA Method 1623, December 2005, EPA 815-R-05-002, Section 6.0 (Equipment and Supplies) and Section 7.0 (Reagents and Standards). Also refer to ASI's Recipe Book (S:\Lab\Log Books\1623_MPA_FPO Reagent Recipes.doc) and the Supplemental Documents binder for manufacturer's instructions regarding storage and preparation of materials used in these procedures

8.2 ColorSeed™ is purchased from BioTechnology Frontiers (New South Wales, AU). One vial of ColorSeed™ C&G is used per sample.

8.3 2 mM Sodium Pyrophosphate

8.4 Funnel and strainer

8.5 Ether

9.0 QUALITY CONTROL

9.1 Matrix Spikes - ColorSeed™ is an internal positive control that is added to each sample. This allows determination of the recovery efficiency of spiked ColorSeed™ *Cryptosporidium* and *Giardia* from each sample. Because ColorSeed™ is used in this procedure, traditional Matrix Spikes are not performed on sediment samples.

9.2 OPRs - Solids samples spiked with ColorSeed™ are not included in standard Method 1623 Ongoing Precision and Control (OPR) batches. This is because these samples are not eluted from filters as are typical Method 1623 samples (use of a common lot of elution solution is the operational definition of an OPR batch).

9.2.1 For tracking purposes, however, solids samples are recorded in the OPR batch logbook in the margin, adjacent to the current OPR batch.

10.0 ANALYTICAL PROCEDURE

Note 1: The analysis for Giardia and Cryptosporidium is begun only if *E. coli* is detected or if stated by PWB. Refer to the attached flow chart (Attachment #3) for more information.

Note 2: The client will determine whether or not to process the sample using ether extraction. If the sample is to be processed without ether extraction, proceed to 10.2.

Note 3: If samples are being reanalyzed due to indigenous *Cryptosporidium* detection previously, do not add ColorSeed. Alternatively, weigh 2.0 grams of sample into a 50 mL centrifuge tube and using a serological pipette, add 10 mL of DI water and vortex for 1 minute +/- 10 seconds. Refrigerate overnight at 2 - 8°C. If using ether extraction proceed to 10.1.5, if not, proceed to 10.2.7.

Note 4: When preparing rabbit or rodent samples for analysis (which tend to be <1 gram in quantity), weigh the entire sample, weigh and process approximately 1/3 of the scat and store the remaining sample at 2 - 8°C. Proceed to 10.1.2 or 10.2.2, depending upon analysis requested.

10.1 Sample preparation Using Ether Extraction

10.1.1 Weigh 1.0 + 0.5 grams of scat sample. Store the remainder of the sample at 2 -8 ° C.

10.1.2 Using a micropipette, add 2.0 mL 0.05% Tween 20 to Colorseed vial. Vortex for 1 minute + 10 seconds and add to sample.

10.1.3 Rinse Colorseed vial by using a serological pipette to add 3 mL of DI water, vortex for 20 + 5 seconds and pour it into the sample. Repeat this rinse two more times.

10.1.4 Allow sample to sit refrigerated (2 - 8°C) overnight.

10.1.5 The following day, vortex the sample, then using a serological pipette, add 6.5 mL of ether and vortex for 1 minute + 10 seconds.

10.1.6 Centrifuge the sample at 2510 XG in the tabletop centrifuge for 10 minutes.

10.1.7 Aspirate ether layer and aspirate sample to 5 mL.

10.1.8 Proceed to section 10.2.9 and continue following SOP.

10.2 **Sample Preparation (Non-Ether Extraction)**

10.2.1 Weigh out 1.0 ± 0.5 grams of sample into a 50 mL centrifuge tube. Store the remainder of the scat sample at 2 – 8°C.

10.2.2 Using a serological pipette, add 10 mL of DI water and vortex to homogenize.

10.2.3 Using a micropipette, add 2 mL of 0.05% Tween 20 to a Colorseed vial.

10.2.4 Vortex Colorseed vial for 1 minute ± 10 seconds and add to the sample.

10.2.5 Rinse Colorseed vial by adding 3 mL of DI water using a serological pipette, vortex for 20 ± 5 seconds, and pouring it into the sample. Repeat this rinse two more times.

10.2.6 Vortex spiked sample for 1 minute ± 10 seconds and allow sample to sit refrigerated (2-8°C) overnight.

10.2.7 The following day, centrifuge sample at 2510 x G in the tabletop centrifuge for 10 minutes.

10.2.8 Aspirate the sample down to approximately 5 mL.

10.2.9 Using a serological pipette, add 20 mL of 2 mM Sodium pyrophosphate to the tube and vortex.

10.2.10 Using a small funnel and strainer, strain the sample into a second labeled 50 mL centrifuge tube and vortex for 30 ± 10 seconds.

10.2.11 Centrifuge strained sample at 2510 x G for 10 minutes in the tabletop centrifuge.

10.2.12 Aspirate the sample to approximately 5 mL.

10.2.13 Using a serological pipette, adjust the volume to 20 mL with DI water.

10.2.13.1 If using the ether extraction, mix thoroughly and using a serological pipette, transfer 5 mL into each tube (4 tubes total).

10.2.13.2 If not using ether extraction, mix thoroughly and using a serological pipette, transfer 10 mL to one tube and the remaining 10 mL to a second tube.

10.3 IMS Procedure – Preparation of Reagents

10.3.1 To each flat-sided sample tube (125 x 16-mm Leighton tube), add 1.0 mL of 10X SL-buffer-A (supplied – not 1X SL-buffer-A). Check buffer before use to ensure no precipitate is present. Each sample will need two Leighton tubes (labeled -1 and -2) if ether extraction is not required. If it is a resample due to indigenous Cryptosporidium being found previously, or if using ether extraction, a total of 4 tubes will be needed (labeled -1, -2, -3, and -4).

10.3.2 Using a micropipette, add 1.0 mL of the 10X SL-buffer-B (supplied: magenta solution) to each sample tube containing the 10X SL-buffer-A.

10.4 IMS Procedure – Organism Capture

10.4.1 Vortex Dynabeads® GC-Combo vials for a minimum of 10 seconds to suspend the beads. Invert the vials to make sure there is no residual pellet at the bottom.

10.4.2 Using a micropipette, add 100 µL of each of the resuspended beads to the Leighton tube containing the water sample concentrate and SL-buffers. To ensure proper homogenization of the bead mixture, beads should be mixed by pipette 3 or 4 times between samples.

10.4.3 Place a new cap on the L-10 tube. Invert to mix buffers, sample and beads. Verify that there are no leaks.

10.4.4 Affix the Leighton tube to a rotating mixer flat side down, and rotate at 18 rpm (see label on rotator) for at least 1 hour with a maximum of 24 tubes.

10.4.5 Label the slides with sample ID number, date and analyst initials, and place sequentially on slide warmer. Label the microcentrifuge tubes with the sample ID number.

10.4.6 After the samples have rotated for one hour, add 1.0 mL of PBS with 2% Tween 80 using a micropipette and allow to rotate for 5 more minutes $\pm$ 1 minute.

10.4.7 Remove the first six tubes from the rotator and place in magnetic particle concentrator (MPC-6) with the flat side of the tube toward the magnet. Re-start the rotator.

10.4.8 Gently rock the tube by hand end-to-end through 90°, cap-end and base-end of the tube up and down in turn. Continue for 2 minutes $\pm$ 10 seconds at approximately 1 tilt/sec. Do not allow to stand motionless for more than 10 seconds. Action in the tube should be gentle and continuous.

10.4.9 Return the MPC-6 to an upright position. Remove and discard the cap (into trash) and decant all the supernatant from the first tube into a waste container, with the flat side of the Leighton tube facing up. Up to three adjacent sub-samples of the same sample may be decanted simultaneously. Do not shake the tube and do not remove the tube from the MPC-6 while decanting. Remove each tube from the MPC-6 after it has been decanted, and set in a rack. In between tubes, perform three 90° actions.

10.4.10 Using a micropipette, resuspend each sample in the rack in 0.5 mL of 1X SLA by rinsing the flat side of the tube.

Note: Mix gently to resuspend liquid and all material of interest (consisting of beads and sample concentrate that were attached to the magnet); do not vortex.

10.4.11 Place the micro centrifuge tube(s) in the MPC-S with the magnetic strip in the vertical position.

10.4.12 Transfer all the liquid sample from the Leighton tube to the labeled 1.5-mL microcentrifuge tube using a 5-mL pipette.

10.4.12.1 Using a serological pipette, rinse the Leighton tube with 0.5 mL 1X SLA and transfer rinsate to the microcentrifuge tube.

10.4.12.2 Repeat rinse with 0.5 mL 1X SLA. Transfer rinsate to microcentrifuge tube.

10.4.12.3 Allow each Leighton tube to sit for a minimum of 1 minute after transfer of the final rinse, then use pipette to collect any residual volume that drips down to the bottom of the tube and add to the labeled microcentrifuge tube.

10.4.12.4 Repeat steps 10.4.7 – 10.4.12.3 for remaining samples.

10.4.13 Without removing the microcentrifuge tube(s) from the MPC-S, gently rock the tube(s) 180° by hand. Continue for 1 minute $\pm$ 10 seconds (1 rock/sec). The beads should produce a distinct dot on the back of the tube.

10.4.14 If no debris is present in the bottom of the tube, using a long Pasteur pipette and squeeze bulb, immediately aspirate the supernatant and debris from each tube and cap held in the MPC-S, and put the supernatant in the waste container. If there is more than one sample, conduct three 90° 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. If there is visible debris in the bottom of any microcentrifuge tube, the following steps may be performed as an additional clean-up:

10.4.14.1 Using the long Pasteur pipette, immediately aspirate the supernatant and debris from each tube and cap held in the MPC-S to just below the level of the beads.

10.4.14.2 Discard the contents of the pipette.

10.4.14.3 Using a clean long Pasteur pipette, resuspend the debris in the remaining volume, being careful not to disturb the beads.

10.4.14.4 Aspirate and discard the contents of the pipette.

10.4.14.5 If debris is still present in the tube, continue with the following additional rinse. Otherwise, continue to step 10.4.15.

10.4.14.6 Using a micropipette, gently add 1 mL of reagent water or PBS to the bead suspension.

10.4.14.7 Gently invert the tube 5-10 times to completely resuspend the bead pellet.

10.4.14.8 Replace the magnetic strip in the MPC®-S and repeat Sections 10.4.13 through 10.4.14.2.

10.4.15 After all the microcentrifuge tubes have been aspirated, pour waste liquid down drain in sink. Rinse beaker and place it in the dish room. Note on the IMS Log book any samples that received additional clean-up procedures.

10.5 IMS Procedure – Dissociation of Beads/(Oo)cyst Complex

Note: Additional tips for minimizing carry over of debris onto microscope slides are described in section 13.3.4 of the Method (EPA, Dec. 2005).

10.5.1 Remove the magnetic strip from the MPC-S.

10.5.2 Using a micropipette, add 50 µL of 0.1 N HCl to each microcentrifuge tube, then remove tubes from the MPC-S and place in vortex tool. Vortex for 50 ± 10 seconds. Vortex should be on its highest setting.

10.5.3 Allow tubes to stand in a vertical position for 10 ± 1 minutes at room temperature.

10.5.4 While dissociation occurs, get ready for staining by preparing positive and negative staining controls and preparing slides.

10.5.4.1 For the positive control, vortex positive antigen for at least 15 seconds, and then micropipette 50 µL of positive antigen into the center of the well. The positive antigen is provided in the *EasyStain* kit, and contains approximately 200-400 intact (oo)cysts per 50 µL.

10.5.4.2 For the negative control, micropipette 50 µL of 1X SLA into center of well.

Note: Positive and negative controls do not need to be performed if the only samples being processed are an OPR and Blank. The OPR will serve as its own positive control, and the Blank will serve as the negative control.

10.5.4.3 Using a micropipette, add 10 µL of 1.0 N NaOH to each slide except the staining controls.

10.5.5 After the 10 ± 1-minute period, vortex the microcentrifuge tubes vigorously for at least 30 seconds to ensure that the entire sample is at the base of the tube.

10.5.6 Switch the magnetic strip in the MPC-S to the tilted position, and replace the microcentrifuge tubes in the MPC-S. Allow to stand undisturbed for at least 10 seconds.

10.5.7 Do not remove the micro centrifuge tube from the MPC-S. Assess the clarity of the purified sample concentrate and determine whether the two dissociations will be combined on one slide (standard procedure) or whether the concentrate is turbid enough to warrant two slides (one slide for each dissociation). If one slide is adequate, skip to 10.15.8. If two or more slides are needed, follow the directions in the sub-section below.

10.5.7.1 Label a second slide with the appropriate sample ID number, date and analyst initials, and using a micropipette, add 5 µL of 1.0 N NaOH to the sample well on slide #2.

10.5.7.2 Using a micropipette, add 50 µL of straight 0.1 N HCl to slide #1 for this sample.

10.5.8 Transfer the entire sample concentrate from the microcentrifuge tube in the MPC-S to the well of the appropriately labeled slide. When pipetting, take care not disturb the beads at the back wall of the tube.

10.5.8.1 After the first dissociation has been completed, turn the slide warmer on to 37 – 42°C.

10.5.8.2 Repeat steps 10.5.1 through 10.5.3, 10.5.5, 10.5.6, and 10.5.8, applying the second dissociation to the same slide as the first dissociation (except as noted in 10.5.7.1 above).

10.5.9 Record sample application time, date and analyst's initials in the IMS logbook. Dry the samples on the slide warmer (37-42°C) and record the slide warmer temperature in the IMS logbook. Leave slides on warmer for approximately 10 minutes after all slides are visibly dry. Place slides on tray and place in incubator at 41 ± 1°C for 15 ± 1 minute.

10.5.10 If the slides are not stained on the same day the sample is applied to the slide, store the slides in a protected environment overnight. Place a wire rack inside of a clean container with Drierite covering the bottom. Place the slides on top of the wire rack. Close the lid on the container and leave at room temperature.

10.6 Sample Staining

Note: The sample(s) must be stained within 72 hours of application of purified sample to slide. The procedure below is based on the manufacturer's recommendations for use of *EasyStain*™ (BTF, Sydney, Australia).

10.6.1 Using a micropipette, add 50 µL of DAPI stock (2 mg/mL) into 50 mL 1X PBS to make a working-strength solution of DAPI.

10.6.2 Using a micropipette, add 50 µL of the working-strength DAPI solution to the slide well, and leave it for at least two (2) minutes.

10.6.3 Use a Pasteur pipette to carefully remove the DAPI. Ensure that you do not touch the surface of the well slide with the Pasteur pipette. Wipe the end of the pipette with alcohol between each slide.

10.6.4 Using a micropipette, add 50 µL of reagent water to the well and allow to stand for at least one (1) minute.

10.6.5 Use a Pasteur pipette to carefully remove the reagent water. Ensure that you do not touch the surface of the well slide with the Pasteur pipette. Wipe the end of the pipette with alcohol between each slide.

10.6.6 Using a micropipette, add 50 µL *EasyStain* (antibody solution) to the well and incubate at room temperature in a humidified chamber for at least 30 minutes.

10.6.7 Use a Pasteur pipette to carefully remove the antibody. Ensure that you do not touch the surface of the well slide with the Pasteur pipette. Wipe the end of the pipette with alcohol between each slide.

10.6.8 Using a micropipette, slowly add 300 µL of Fixing Buffer to the well and allow to stand at room temperature for at least two (2) minutes. The Fixing Buffer should flow over the edges of the well slide. This will help to reduce background staining around the edges of the slide.

10.6.9 Gently aspirate the excess Fixing Buffer from below the well using a clean Pasteur pipette. Wipe the end of the pipette with alcohol between each slide.

10.6.10 Using a micropipette, add an appropriate amount of Mounting Medium (5 – 15 ul) to the well and apply a coverslip. Seal the coverslip around all edges with clear nail polish.

10.6.11 Record the date and time that staining was completed on the bench sheet. Slides may be stored in the dark at 2°C to 8°C until ready for examination.

10.7 Slide Examination

Note: Slide examination must be completed within 144 hours (6 days) of sample staining.

10.7.1 Scanning technique: Scan each well in a systematic fashion. An up-and-down or a side-to-side scanning pattern may be used.

10.7.2 Slides are examined by scanning each field under 200x using FITC and then Texas Red. After each field the results are recorded. If any oocysts or cysts are detected that do no fluoresce under Texas Red, they are confirmed using FA, DAPI staining characteristics, and DIC microscopy. Record results on the report form. All observed oocysts and cysts must be reported.

10.7.3 Positive and negative staining control:

10.7.3.1 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 must be conducted at a minimum of 1000X. Size, shape, and DIC and DAPI characteristics of the three *Cryptosporidium* oocysts and *Giardia* cysts must be recorded by the analyst on a microscope log. The analyst also must indicate on each sample report form whether the positive staining control was acceptable.

10.7.3.2 Examine the negative staining control to confirm that it does not contain any oocysts or cysts. Indicate on each sample report form whether the negative staining control was acceptable.

10.7.3.3 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, proceed to sample examination.

10.7.4 Slide Examination by Field

10.7.4.1 Under 200X total magnification, use epifluorescence to scan the field then record the results.

10.7.4.2 Switch to the Texas Red filters and re-scan the field of view. Record the results.

10.7.4.2.1 If the organism fluoresces green with the FITC filter set and red with the Texas Red filter set (and meets other protozoa criteria), then it is a ColorSeed organism

10.7.4.2.2 If an organism fluoresces only with the FITC filter set and meets all other protozoa criteria, then it is considered an indigenous organism. Proceed to characterization.

10.7.4.2.3 If no suspect protozoa are observed, proceed to 10.7.4.4.

10.7.4.3 Characterization –

10.7.4.3.1 If there is an (oo)cyst-like organism that is detected under FITC that does not fluoresce under Texas Red, then it may be indigenous protozoa and must be characterized. Proceed to 10.7.4.3.3.

10.7.4.3.2 The first three ColorSeed™ oocysts and first three ColorSeed™ cysts observed must also be characterized. Proceed to 10.7.4.3.3

10.7.4.3.3 Switch magnification to 1000X total magnification and use DAPI and DIC for confirmation (as described in detail in 10.7.5 and 10.7.6) and record the results.

10.7.4.3.4 When characterization is complete, or if no cyst or oocysts are observed, proceed to 10.7.4.4.

10.7.4.4 Move to the next field of view and repeat steps 10.7.4.1 through 10.7.4.3.4 until the entire well has been examined. When completed, proceed to 10.7.7.

10.7.5 Characterization of *Cryptosporidium* oocysts - When brilliant, apple-green fluorescing, ovoid or spherical objects 4 to 6 µm in diameter are

observed with brightly highlighted edges, switch the microscope to the UV filter block for DAPI, then to DIC.

10.7.5.1 Using the UV filter block for DAPI at not less than 400X total magnification, the object will exhibit one or more of the following characteristics:

(a) up to four distinct, sky-blue nuclei;
(b) intense blue internal staining;
(c) no blue or light blue internal staining (no distinct nuclei).

(a) and (b) are recorded as DAPI +; (c) is recorded as DAPI–.

10.7.5.2 Using DIC at not less than 1000X total magnification, look for external or internal morphological characteristics atypical of *Cryptosporidium* oocysts (e.g., spikes, spores, pores). 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

Record the shape and measurements to the nearest 0.5 µm at 1000X for each such object. For each oocyst, record the number of sporozoites observed. Record the date and time that sample examination and confirmation was completed on the report form along with second analyst confirmation (if performed).

10.7.6 Characterization of *Giardia* cysts - When brilliant, apple-green fluorescing, round to oval objects (8 – 18 µm long by 5 – 15 µm wide) are observed, switch the microscope to the UV filter block for DAPI, then to DIC.

10.7.6.1 Using the UV filter block for DAPI at not less than 400X total magnification, the object will exhibit one or more of the following characteristics:

(a) two to four sky-blue nuclei;
(b) intense blue internal staining;
(c) no blue or light blue internal staining (no distinct nuclei).

(a) and (b) are recorded as DAPI +; (c) is recorded as DAPI–.

10.7.6.2 Using DIC at not less than 1000X total magnification, look for external or internal morphological characteristics atypical of *Giardia* cysts (e.g., spikes, stalks, appendages, pores, one of two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.). If atypical structures are not observed, then identify each fluorescing object of the aforementioned size and shape 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 internal structures for each apple-green fluorescing object meeting the size and shape characteristics. On the report form, record the date and time that sample examination and confirmation was completed along with second analyst confirmation (if performed).

10.7.7 Report indigenous and ColorSeed *Cryptosporidium* and *Giardia* concentrations as oocysts/L and cysts/L.

10.7.8 Photograph any indigenous oocysts, when possible.

10.7.9 If appropriate (project dependent), prepare and analyze one or more additional aliquots (without Colorseed) for genotyping of indigenous *Cryptosporidium.*

11.0 RECORDS MANAGEMENT

11.1 Laboratory personnel are responsible for maintenance of the following logbooks:

a. 1623 Immunomagnetic Separation/Staining Logbook
b. 1623 Slide Examination Logbook

11.2 Administrative personnel maintain results records.

12.0 SUPPLEMENTAL DOCUMENTS

1. BTF *EasyStain*™ Instructions

2. PT Spiking Instructions

13.0 ATTACHMENTS

Attachment 1: Method Holding Times

Attachment 2: USEPA Method 1623 ColorSeed Data Worksheet, Slide Counting Sheet

Attachment 3: Scat Analysis Procedure Flow Chart for Portland Water Bureau Samples

14.0 SUBSTANTIVE REVISIONS

14.1 The following changes were made in November 2010:

14.1.1 Sample Preparation using Ether Extraction option (Section 10.1) was added.

14.1.2 A note to describe rabbit and rodent scat processing was added (Note 4 below Section 10.0)

15.0 REFERENCES

1. ASI 224.doc SOP for Method 1623 - Cryptosporidium and Giardia in Water by Filtration-IMS-FA.

2. ASI 224-1.doc SOP for Method 1623 Quality Assurance/Quality Control

3. EPA Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA. EPA 815-R-05-002. December 2005.

4. *ICR Microbial Laboratory Manual*, EPA/600/R-95/178, National Exposure Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, 26 Martin Luther King Drive, Cincinnati, OH 45268.

5. "The Envirochek™ HV Capsule: Recovering *Cryptosporidium* from High Volume Source and Finished Drinking Water Samples," PN 33249, Gelman Sciences, 600 South Wagner Road, Ann Arbor, MI 48103-9019 (October 2001).

6. "Dynabeads® GC-Combo," Dynal Microbiology R&D, P.O. Box 8146 Dep. 0212 Oslo, Norway (September 1998, Revision No. 01).

7. USEPA Method 1622/1623 Microscopy Module on *Giardia* and *Cryptosporidium*. http://www.epa.gov/safewater/lt2/training/modules.html

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ATTACHMENT 1:

Method Holding Times

FOR ASI224-5

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

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ATTACHMENT 2:

USEPA METHOD 1623 ColorSeed™ DATA WORKSHEET
Slide Counting Sheet

ASI Sample ID Number:_____ **Total Volume Filtered:** _____

Slide Sub-Sample	Organism	# Indigenous organisms detected	# ColorSeed™ organisms detected
A	*Giardia*		
	Cryptosporidium		
B	*Giardia*		
	Cryptosporidium		
C	*Giardia*		
	Cryptosporidium		
D	*Giardia*		
	Cryptosporidium		
TOTAL Organisms Detected	*Giardia*		
	Cryptosporidium		
Organisms Detected PER LITER	*Giardia*		
	Cryptosporidium		

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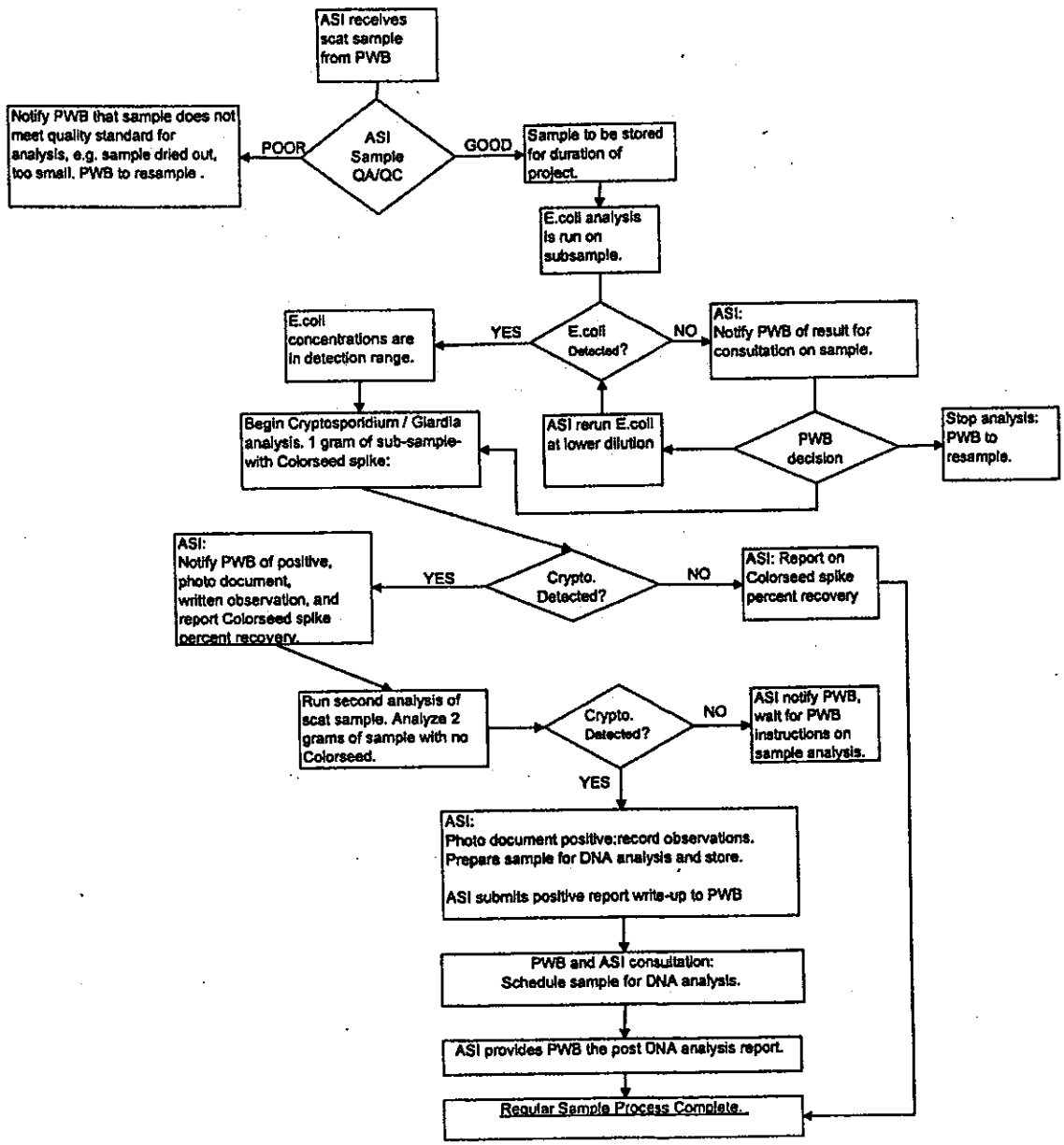
ATTACHMENT 3:

SCAT ANALYSIS PROCEDURE FLOW CHART FOR PORTLAND WATER BUREAU SAMPLES

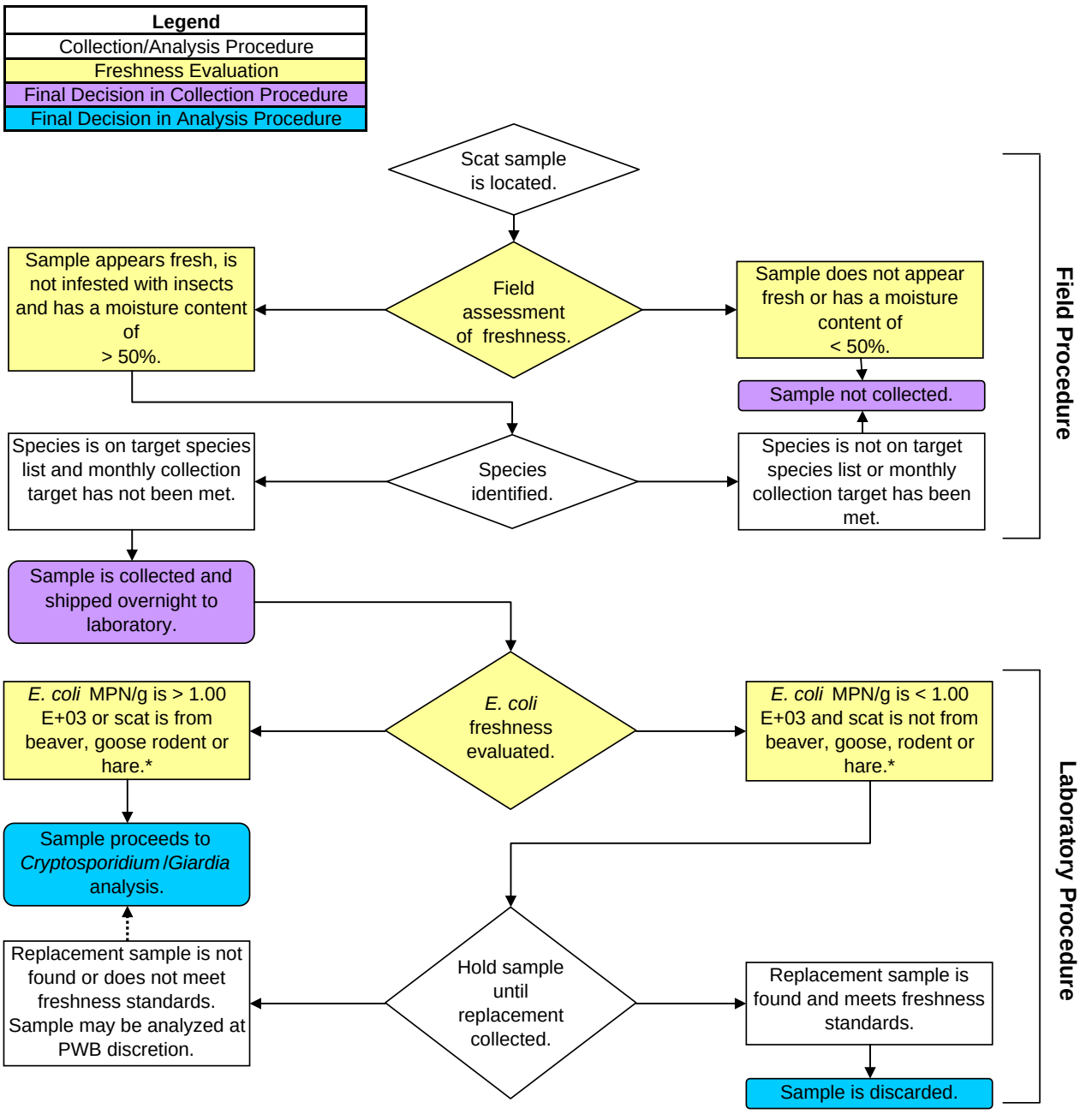
Scat analysis proceedure flow chart

For: Portland Water Bureau data collection efforts for the Pathogen Catchment Budget model as part of the LT2 Variance project.
Portland Water Bureau: Ryon Edwards Analytical Services Inc:
Revision date:12-31-09



4C-1. Portland Water Bureau Protocol for Scat Sample Freshness

Portland Water Bureau Protocol for Scat SampleFreshness



* *E. coli* based freshness standards are not taken into consideration during the winter when low temperatures cause *E. coli* counts to be low in fresh scat samples. Freshness standards are not evaluated based on *E. coli* for beaver and goose due to naturally low levels of *E. coli* found in fresh fecal material. Scat from baited/trapped animals are known to be fresh and therefore are not subject to *E. coli* freshness standards. Samples for these species are analyzed for *Cryptosporidium* and *Giardia* based on field evaluation.