Title:	Stool Processing and Cryptosporidium/Giardia Detection Standard Operating Procedure		
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Revision History	2.0	Applied CLSI formatting, updated reference, removed instructions for MERIFLUOR® C/G kit and provided link to manufacturer's SOP instead.

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1 Purpose

The purpose of this SOP is to document the procedures for processing stools for ova and parasites and for Cryptosporidium/Giardia detection in stool specimens by Ziehl-Neelson (Z-N) staining.

2 Scope

Users of the ACTG/IMPAACT Laboratory Manual

3 Background

3.1 Parasitic diseases of humans are endemic to all regions of the world, Cryptosporidium parvum (C. parvum) is a significant cause of diarrhea and wasting in persons with AIDS. Acute cryptosporidiosis resolves spontaneously when it occurs in HIV-infected adults with relatively intact immune systems and higher CD4 counts. The severity of cryptosporidiosis increases with declining Immune status such that fulminant disease occurs in adults when CD4 counts decline to < 50/mm^3. The detection and identification of C. parvum in stool specimens of HIV infected individuals is therefore important in those individuals with diminished immune capacity.

3.2 Studies of anti-parasitic interventions such as Nitazoxonide (NTZ) require that patients collect stool culture at defined intervals to assess therapeutic response. To accomplish this stools collected in ParapakTM preservatives are screened for ova and parasites in local laboratories using standard procedures capable of identifying commonly encountered protozoa and helminths. Ova and parasites are typically identified by microscopic examination of properly preserved stool concentrates.

3.3 A modified acid-fast staining method, also known as the Ziehl-Neelson (Z-N) method is often the assay of choice for determination of C. parvum in the stool samples. However, the MERIFLUOR® Cryptosporidium/Giardia (C/G) direct immunofluorescent procedure is also widely used for detection of Cryptosporidium oocysts and Giardia cysts. An SOP for the MERIFLUOR® C/G kit can be found on the Meridian Bioscience website at http://www.meridianbioscience.com/diagnostic-products/cryptosporidium-and-giardia/merifluor/merifluor-cryptosporidium-and-giardia.aspx.

4 Authority and Responsibility

4.1 The Network Laboratory Directors (or his/her designee) have the authority to establish, review and update this procedure.

4.2 The ACTG/IMPAACT Laboratory Technologist Committee (LTC) is responsible for the maintenance and control of SOP documentation.

4.3 The Laboratory Director is responsible for the implementation of this LTC SOP or laboratory-specific SOP and for ensuring that all appropriate personnel are trained. A laboratory-specific SOP must:

4.3.1 Include, without procedural modification, the portions of the current version of the LTC SOP that are used within the network site-affiliated laboratory;

4.3.2 Reference the current version of the LTC SOP.

4.4 All technicians are responsible for reading and understanding this SOP prior to performing the procedures described.

4.5 The site PI and designees are responsible for understanding and adhering to the patient preparation and specimen collection components.

5 Equipment, Consumables and PPE

5.1 Collection Procedures:

5.1.1 Parapaks™ (MERIFLUOR®Diagnostics)

5.2 Extraction Procedures:

5.2.1 15 mL glass or polypropylene conical tubes

5.2.2 10% Formalin

5.2.3 Ethyl acetate

5.2.4 Centrifuge with 15 mL tube carriers

5.2.5 Applicator sticks

5.2.6 Pasteur pipettes

5.2.7 Cotton Swabs

5.2.8 4x4 inch gauze pads

5.2.9 Disposable conical paper cups

5.3 Microscopic Examination

5.3.1 Light microscope with 20 and 100X objectives

5.3.2 Fluorescence microscope with filters for fluorescein isothiocyanate (FITC) examinations

5.3.3 Slides

Slide options include:

5.3.3.1 Multiwell printed microscope slides (Example: #101007 Carlson Scientific, Peotone, IL)

5.3.3.2 Standard glass slides

5.3.3.3 Frosted glass slides

5.3.4 Plastic humidor

5.3.5 Squeeze bottle

5.3.6 Staining trays

5.4 Personal Protective Equipment

5.4.1 Lab coat

5.4.2 Gloves

5.4.3 Goggles or eye protection

6 Safety

6.1 Some reagents in MERIFLUOR® C/G kit contain sodium azide, which is a skin irritant. Avoid skin contact with reagents.

6.2 Disposal of reagents containing sodium azide into lead or copper plumbing can result in the formation of explosive metal azides. This can be avoided by flushing with a large volume of water during such disposal.

6.3 Para-Pak reagents are toxic and must be handled with care. Patients or guardians using Para-Paks for home sample collection must be informed of the proper handling and storage of collection devices. Note that handling instructions and safety measures are addressed in the product ParaPak product insert for English speaking users.

7 Reagents and Reagent Preparation

Note: See Versalovic et.al. for detailed reagent preparation instructions.

7.1 Carbol fuchsin

7.2 Acid alcohol (HCl with 95% alcohol)

7.3 Methylene blue

8 Procedure: Stool Collection

Note: Freshly collected stool samples should be added to SAF and PVA within 1 hour for routine ova and parasite examination and processed for Immunofluorescence Assay (IFA) as described below.

8.1 Collect fresh stool sample in a clean plastic, glass or paper container

8.2 Use Para-Paks (Meridian Bioscience, Inc.) for the collection of fresh stool samples.

8.3 Collect both sodium acetate-acetic acid-formalin (SAF) and polyvinyl-alcohol (PVA) tubes per product insert, which is included with each pair of preservative tubes. Fill the SAF tube first when stool volumes are small.

8.4 Place diarrheal stool in vial of SAF until fluid line on vial is reached (approximately 2.0mL).

8.5 Let the stool-preservative mixture stand for a minimum of 30 minutes at 15° to 25°C to ensure adequate fixation.

8.6 Hold Para-Pak preserved samples at 15° to 25°C until they are returned to the clinic or lab.

Note: Whenever insufficient specimen is available, record the approximate amount of stool used (as a proportion of what is required to reach the line), in order to correct the final organism count for semi-quantitative preparations. This step is critical for the assessment of cryptosporidium oocysts loads.

9 Procedure: Stool Concentration

Note: The procedure listed here is acceptable for Cryptosporidium and many other parasites but may need modification if investigators are targeting other fecal parasites.

9.1 Thoroughly mix the SAF tube.

9.2 Strain stool suspension through gauze in conical paper cup with tip removed into a 15 mL conical centrifuge tube.

9.3 Add tap water or saline if needed to adjust volume to 15 mL.

9.4 Centrifuge at 650 x g for 1 minute. The resulting stool pellet should be about 1 mL. If not, add more stool suspension and centrifuge again. If pellet is excessive then remove some sedimented material, resuspend and centrifuge again as above.

9.5 Decant supernatant and wash again with tap water.

9.6 Add 10% formalin to the 10 mL mark after decanting the second water supernatant. Mix thoroughly.

9.7 Add 4 mL of ethyl acetate to the tube. Cap and shake vigorously in the inverted position for 30 seconds. Carefully remove cap to release vapor pressure.

9.8 Centrifuge the mix at 500 x g for 1 minute. Four layers will result; ethyl acetate, plug of debris, formalin, and concentrated sediment containing ova.

9.9 Gently insert a applicator stick between the plug and the tube walls to release the plug. Carefully decant the upper three layers into a biohazard waste container. Remove residual material clinging to the inner walls of the centrifuge tube with a cotton swab.

Note: This is a critical step, unwanted lipids from your sample will affect microscopic exams.

9.10 Resuspend the final sediment in a drop of formalin and prepared slides for Ziehl-Neelson and/or MERIFLUOR® C/G exams. Wet mounts can also be prepared from this sediment when examining for other ova.

10 Procedure: Modified Acid-Fast Staining Method (Z-N Method)

Note: See Versalovic et.al. for detailed reagent preparation and alternate concentration methods

10.1 Use stool sample from SAF vials.

10.2 Concentrate and extract the stool sample by standard methods.

10.3 Mix sediment from concentration method, and then smear 10 µL on slide within etched circle.

10.4 Air dry for at least 1 hour (overnight recommended).

10.5 Fix in methanol for 3 to 5 minutes.

10.6 Stain with carbol fuchsin for 20 minutes

10.7 Rinse with tap-water for 4 minutes

10.8 Decolorize with acid alcohol (HCl with 95% alcohol)

10.9 Rinse with tap water for 2 minutes

10.10 Counter stain with 3% methylene blue for 30 seconds

10.11 Rinse with tap water for 2 minutes

10.12 Air dry

10.13 Examine by standard light microscopy.

10.14 By Z-N method, Cryptosporidium sp., Isospora belli and Cyclospora sp. oocysts stain red against a blue-green background. Cryptosporidium is round and approximately 4 µm in diameter. Cyclospora is also round but it is larger than Cryptosporidium (approximately 8-10 µm diameter). Isospora is elliptical and approximately 23-33 µm by 10-15 µm.

11 Control Procedures

Run positive and negative controls with each subject run.

12 Literature References

Alles, A.J., Waldron, M.A., Sierra, L.S., and Mattia, A.R. 1995. Prospective comparison of direct immunofluorescence and conventional staining methods for detection of Giardia and Cryptosporidium spp. in human fecal specimens. J. Clin. Microbiol. 33(6): 1632-4.

Garcia, L.S., Shum, A.C. and Bruckner, D.A. 1992. A new monoclonal antibody combination reagent for fluorescence detection of Giardia cysts and *Cryptosporidium* oocysts in human fecal specimens. J.Clin. Microbiol. 30: 3255-3257.

Versalovic, J., Carroll, K.C., Funke, G., Jorgensen, J.H., Landry, M.L., Warnock, D.W. (ed.) 2011. Manual of Clinical Microbiology, 10th ed. American Society for Microbiology, Washington DC.

13 Appendix: Grade Scale for Cryptosporidium Oocyst Quantitation

Grade	Density	Cryptosporidium or Giardia Distribution
0	None	No organism per circular treated slide area
1	Rare	2-5 organisms per circular treated slide area
2	Few	One organism per 5-10 high power fields
3	Moderate	From 1-2 organisms per high power field to as few as 1 organism per 2-3 fields
4	Many	More than 2 organisms in every high power field