Laboratory Manager Approval: Mary K. Bowman / 08/19/2021

QA Manager Approval: Jeffrey Moore / 08/19/2021

Standard Operating Procedure for the Epifluoresence Microscope and Digital Imaging System

Access to this SOP shall be available within the laboratory for reference purposes; the official copy of this SOP resides on the official Georgia EPD website at https://epd.georgia.gov/about-us/epd-laboratory-operations. Printed copies of this SOP will contain a watermark indicating the copy is an uncontrolled copy.

1.0 Scope and Application

1.1 This SOP describes the calibration and operation procedures for the Zeiss Axioskop and the BioVID HD 1080+ camera.

2.0 Definitions

2.1 Refer to Section 3 and Section 4 of the Georgia EPD Laboratory Quality Assurance Manual for Quality Control Definitions. (See SOP reference 13.5)

3.0 Interferences

3.1 Improper settings and lens that have not been properly cleaned will interfere with proper identification of organisms.

4.0 Safety

4.1 Analysts should be aware of the biohazard risks that are associated with this method and take adequate precautions. There is a high risk of infection from oocysts and cysts because live organisms are handled. Laboratory staff should 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.

4.2 Each chemical compound should be treated as a potential health hazard. Exposure to these compounds should be reduced to the lowest possible level. Refer to material safety data, which are available on all chemicals and reagents used in the Microbiology laboratory.

4.3 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 and cysts. Do not mouth-pipette.

4.4 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.

4.5 For additional safety points, please refer to the EPD Laboratory Safety/ Chemical Hygiene Plan & Fire Safety Plan, online revision. (See SOP reference 13.6)

5.0 Apparatus and Equipment

5.1.1 Zeiss Axioskop

5.1.2 BioVID HD 1080+ Camera with Toupview imaging software

5.1.3 Lens Paper

5.1.4 Canned Air

5.1.5 Fisherbrand Lens Cleaner

5.1.6 Zeiss Immersol, Immersion oil

5.1.7 Lint-free gloves

5.1.8 Polyester tipped swabs

5.1.11 Microscope cleaning procedure

5.1.12 Use canned air to remove dust from the lenses, filters, and microscope body.

5.1.13 Use a Kimwipe dampened with a microscope cleaning solution (MCS) consisting 2 parts of 90% isopropanol and 1 part acetone to wipe down all surfaces of the microscope body. Dry off with a clean, dry Kimwipe.

5.1.14 Protocol for cleaning oculars and condenser

5.1.15 Use a new, clean Swab dampened with MCS to clean each lens. Start at the center of the lens and spiral the Swab outward using little to no pressure. Rotate the Swab head while spiraling to ensure a clean surface is always contacting the lens.

5.1.16 Repeat the procedure using a new, dry Swab.

5.1.17 Repeat Sections 5.1.15 and 5.1.16.

5.1.18 Remove the ocular and repeat the cleaning procedure on the bottom lens of the ocular.

5.1.19 Protocol for cleaning objective lenses

5.1.20 Wipe 100X oil objective with lens paper to remove the bulk of the oil from the objective.

5.1.21 Hold a new Swab dampened with MCS at a 45° angle on the objective and twirl.

5.1.22 Repeat Sections 5.1.20 and 5.1.21, with a new, dry swab.

5.1.23 Clean all objectives whether they are used or not.

5.1.24 Protocol for cleaning light source lens and filters

5.1.25 Using a Kimwipe dampened with microscope cleaning solution, wipe off the body of each lens and filter. Clean the lens surface with a dampened lens paper.

5.1.26 Repeat the procedure using a dry Kimwipe and lens paper.

5.1.27 Repeat Sections 5.1.25 and 5.1.26.

5.1.28 Protocol for cleaning microscope stage

5.1.29 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 clear fixative. Remove the stage clip if necessary to ensure that it is thoroughly cleaned.

5.1.30 Use disinfectant and a paper towel to clean the bench top surrounding the microscope.

5.1.31 Frequency

5.1.32 Perform Sections 5.1.11 through 5.1.28 and 5.1.30 after each microscope session.
5.1.33 Perform complete cleaning each week.
5.1.34 Take color photographs of *Cryptosporidium* oocysts and *Giardia* cysts FA, 4',6-diamidino-2-phenylindole (DAPI), and DIC that have been determined to be accurate and file under the sample lab number.

6.0 Reagents

6.1 Vendor Purchased Reagents
6.1.1 Acetone
6.1.2 DAPI (4'6- diamidino- 2-phenylindole)
6.1.3 Ethanol
6.1.4 Methanol, Absolute
6.1.5 Isopropyl Alcohol
6.2 Prepared Lab Reagents
Microscope Cleaning Solution: 2 parts 90% isopropanol and 1 part acetone.

7.0 Sample Collection

7.1 N/A

8.0 Calibrations

8.1 Calibration of an Ocular Micrometer
8.1.1 This calibration should be done for each objective in use on the microscope.
8.1.2 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.
8.1.3 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.
8.1.4 Without changing the stage adjustment, find a point as distant as possible from the two "0" lines where two other lines are exactly superimposed.
8.1.5 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: 48 ocular micrometer spaces equal 0.6 mm.
8.1.6 Calculate the number of mm/ocular micrometer space.
For example:

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

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:

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

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

8.1.8

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 x (1000 µm/mm)) ÷ no. ocular micrometer spaces

[3]N.A. refers to numerical aperture. The numerical aperture value is engraved on the barrel of the objective

8.2 Kohler Illumination

8.2.1 Perform the following steps each day prior to the microscopic analysis of samples:

8.2.1.1 Place a prepared slide on the microscope stage, place oil on the slide, move the 100X oil objective into place and turn on the transmitter light (brightfield).

8.2.1.2 Focus the specimen (note: from this point on, do not touch the stage focus knob).

8.2.1.3 Completely open the condenser Aperture Diaphragm and completely close the Field Diaphragm in the base until the lighted field is reduced to a small opening.

8.2.1.4 Focus the edges of the Field Diaphragm by moving the condenser up or down using the condenser focus knob.

8.2.1.5 Center the Field Diaphragm using the condenser centering screws so that the Diaphragm image is exactly centered in the field of view.

8.2.1.6 Open the Field diaphragm until its shadow disappears from the field of view (note: opening the Field Diaphragm more than this amount causes extra glare and decrease image contrast).

8.2.1.7 Adjust the Aperture Diaphragm to make it compatible with the numerical aperture of the objective by performing the following:

8.2.1.7.1 Remove the right ocular

8.2.1.7.2 Look down the tube at the rear focal plane of the objective

8.2.1.7.3 Adjust the Aperture Diaphragm or Stage Diaphragm to 2/3 open

8.2.1.7.4 Replace right ocular.

9.0 Quality Control

9.0 Refer to Table 14.1 for Quality Control parameter, acceptance criteria and corrective action procedures.

9.1 Refer to SOP 8-001 Section 10.8 for Verification of Analyst Performance (See SOP reference 13.8)

10.0 Procedure

10.1 Operating the Microscope

10.1.1 Turn the microscope on by turning on the external power source and by pressing the rotating dial on the SpeedDIAL Manual Controller. Fill out the microscope log book.

10.1.2 If using a microscope with a joystick, turn on the joystick before turning on the external power source.
Note: When using the X-cite LED lightsource, adjust the light intensity by rotating the dial to the left or right to increase or decrease the brightness.

10.1.3 Lower stage by turning the coarse focus knob and place slide on stage, securing it with the stage clips.

10.1.4 Rotate the objective lenses to the lowest power (10X) using the nosepiece. The lens should 'click' when secured

10.1.5 Using x, y-axis knobs, place an edge of the slide well in the light.

10.1.6 Adjust the ocular lenses to where the interpupillary distance meets the scientist's field of vision to minimize eyestrain. Looking through the ocular lenses, slowly raise the stage using the coarse focus until the edge of the well is clear.

10.1.7 Use x, y- axis knobs to scan through the slide.

10.1.8 If the microscope has a joystick instead of x, y-axis the slide is read by pushing up and down, left or right on the joystick. Be careful not to go diagonally by pushing at an angle and to go faster press the button on the top of the joystick.

10.1.9 **Only** use coarse focus to focus on the 10X and 20X objectives. 40X and 100X (oil immersion) use the fine focus knob.

10.2 Changing the Objectives

10.2.1 When changing objectives, turn the knurled ring of the nosepiece only. Never change objectives by handling the objective.

10.3 Using immersion oil with the 100X lens

10.3.1 Lower the stage a little and rotate the nosepiece where the slide is in between the 40X and the 100X objectives.

10.3.2 Add one drop of immersion oil to the slide. (Diagram 1)

10.3.3 Viewing from the side, rotate the 100X lens into place.

10.3.4 Slowly raise the stage using the coarse focus until the immersion oil meets the objective. The oil bubble will form into a column when the objective meets it.
DO NOT get immersion oil on any other objectives and only use fine focus to focus

10.3.5 After the slide is read, lower the stage and remove slide. Wipe lens and slide off with a lens paper to remove excess oil before turning the microscope off.

10.3.6 Turn the microscope off. After the scope has been turned off clean scope and apply the dust cover.

10.3.7

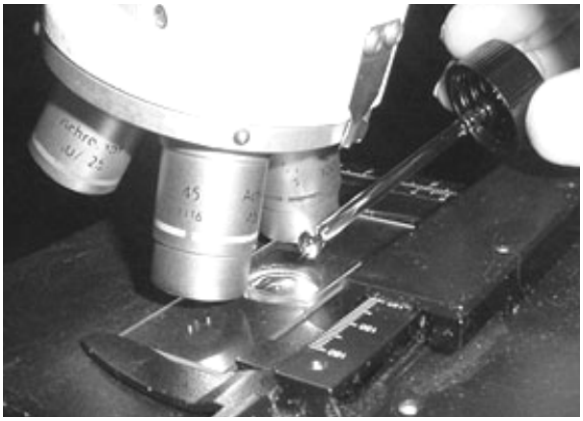


Diagram 1

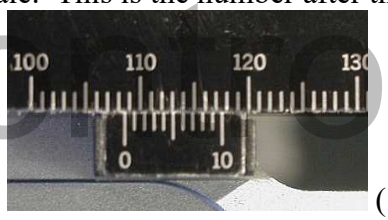
10.4 Using the Vernier Scale

10.4.1 Make sure that the slide is oriented with the writing to the left side.

10.4.2 Center target organism in the crosshairs of the scope.

10.4.3 Record the number just to the left of the "vernier zero" as the primary number.

10.4.4 Refer to the smaller scale to see which Vernier line matches up with the primary scale. This is the number after the decimal point.



(In this ex. the reading is 108.6)

10.4.5 Record an x-axis value first, followed by the y-axis values and the magnification of the objective being used.

10.4.6 Using Fluorescence Microscopy

10.4.7 Turn on the X-cite LED power source.

10.4.8 Ensure that the slider is positioned to one of the open rings so as not to obstruct the light from the LED bulb.

10.4.9 Make sure that the light for the transmitted light is in the off position.

10.4.10 Push the reflector slider all the way in to the 1st position where the FITC filter is located.

10.4.11 Pull the reflector slider out to the 2nd stop position (middle) in order to view the organism for DAPI.

10.4.12 Using Nomarski (DIC)

10.4.13 Block the light from the LED bulb with the slider.

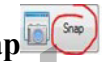
10.4.14 Turn on the transmitted light bulb.

10.4.15 Set condenser to the DIC position.

10.4.16 Have the reflector slider in the 2nd stop position (the filter interferes with DIC and will present green if in the 1st position).

10.4.17 Push in the DIC slide analyzer located on the nosepiece.

10.4.18 To improve contrast, adjust the potentiometer, condenser diaphragm and DIC prism.
10.4.19 Microscope Storage
10.4.20 Once usage is over, record the time and turn off the X-cite LED power supply.
10.4.21 Put the dust cover over the microscope.

10.5 TAKING PICTURE WITH BioVID HD CAMERA
10.5.1 Select the specimen to be photographed using 100X magnification, and set up the microscope for Kohler illumination.
10.5.2 Turn on the camera power supply button located on top of the camera.
10.5.3 Double click on the **ToupView icon**  from the desktop to open the program.
10.5.4 Click on (**XCAM1080PHA**) under the "Camera List" to show the live image of the organism.
10.5.5 Set the type of image to be taken on the microscope (**FITC, DAPI OR DIC**).
Note: All organisms must be photographed using all three illuminations.
10.5.6 Make sure the **Auto Exposure** and **Global Auto** icons are checked for auto image adjustment. Uncheck the box to make the sliders active for manual adjustment of exposure and white balance.
10.5.7 Click the **Snap**  button to capture the organism
10.5.8 Save the image to M: drive using the SAMPLE ID number.
10.5.9 To take another Image Click on **Video[XCAM1080PHA]** to show the live image of the organism.
10.5.10 Write the organism coordinates in the Microscopy Log Book.
10.5.11 Click on the program's **CLOSE** button.

11.0 Calculations
11.1 N/A

12.0 Waste Management
12.1 See GA EPD Laboratory SOP – EPD Laboratory Waste Management Standard Operating Procedures, SOP 6-015, online revision.

13.0 References
13.1 Zeiss Axioskop Operating Manual.
13.2 BioVid HD 1080+ Camera Instruction Manual.
13.3 EPA Method 1623: Cryptosporidium and Giardia in Water by Filtration/IMS/FA, December 2005.
13.4 Supplement 2 to the Fifth Edition of the Manual for the Certification of Laboratories Analyzing Drinking Water, EPA 815-F-12-006, November 2012.
13.5 GA EPD Laboratory Quality Assurance Plan, online revision.
13.6 GA EPD Laboratory Safety/Chemical Hygiene Plan & Fire Safety Plan, online revision.

13.7 GA EPD Laboratory SOPs – Initial Demonstration of Capability SOP 6-001, online revision and/or Continuing Demonstration of Capability SOP 6-002, online revision.

13.8 EPD Laboratory SOP – Standard Operating Procedure for the Detection of Cryptosporidium and Giardia in Water by Filtration, IMS, and FA (Based on EPA Method 1623, December 2005), SOP 8-001, online revision.

14.0 Quality Control parameter, acceptance criteria and corrective action procedures.

Table 14.1 Summary of Quality Control Procedures for Microscope Maintenance

Method	Applicable Parameter	QC Check	Min. Freq.	Accept. Criteria	Corrective Action	Flag Criteria
Microscope		Performance Verification Criteria	Performed monthly.	Analyst must come within 10% of confirmation analyst on both FITC and DAPI readings.	Identify source of variability, prepare new slide and retry until all pass.	
		Negative and Positive Staining Control Slides.	One of each per batch of samples stained.	Negative slide must be free of target organisms. Positive slides must have target organisms stained for both FITC and DAPI and intact organelles for DIC.	If control slides fail due to stain malfunction, prepare new slides using another box and/or lot number of stain. If failure is due to organisms, prepare new slides using new organisms.	Control slides must pass in order to read slides.

Updates to Previous Version:
Updated for online revision