ALWAYS WEAR LAB COAT

Fecal Float Protocol
(To check for viable E.mac)
(Do within 1 week of obtaining samples)

1. Label each tube with the animals name
2. Obtain 2g feces- “rule of thumb” – an amount approximately the size of the 2nd half of one’s thumb
3. Add 15 ml distilled water in a Dixie cup
4. Mix feces with water by using a tongue depressor
5. Strain into a clean Dixie cup through **2** layers of cheese cloth (~100 micrometers)
6. Pinch off Dixie cup and pour into labeled 15 ml graduated centrifuge tube
7. Rinse out Dixie cup with an additional 3-5 ml and fill test tube approximately ¼ inch from the top
8. Centrifuge at 1500 rpm for 5 minutes
9. Pour off supernatant (liquid)-once pellet is disturbed pour until movement reaches the end. (Do not start, stop and restart pouring)
10. Add Sheather’s Sugar solution (spg 1.275) halfway, mix with a small wooden dowel
11. Fill Sheather’s sugar solution to the very top of the tube
12. Place a cover slip over the tube (make sure it is in contact with the solution) and centrifuge at 1500 rpm for 5 minutes
13. Place cover slip on slide and scan @ 100 magnification

Wisconsin Double-Cover Slip
(To estimate oocyst counts in samples)
(Conduct on all positive samples)

1. Label each tube with the animals name
2. Obtain 1g feces (weigh it out by tarring a dixie cup) (Can use two grams and split it between two tubes and take an average count…add 30 ml distilled water if splitting)
3. Add 15 ml distilled water in a Dixie cup
4. Mix feces with water by using a tongue depressor
5. Strain into a clean Dixie cup through **2** layers of cheese cloth (~100 micrometers)
6. Pinch off Dixie cup and pour into labeled 15 ml graduated centrifuge tube
7. Rinse out Dixie cup with an additional 3-5 ml and fill test tube approximately ¼ inch from the top
8. Centrifuge at 1500 rpm for 5 minutes
9. Pour off supernatant (liquid)-once pellet is disturbed pour until movement reaches the end. (Do not start, stop and restart pouring)
10. Add Sheather’s Sugar solution (spg 1.275) halfway, mix with a small wooden dowel

11. Fill Sheather's sugar solution to the very top of the tube
12. Place a cover slip over the tube (make sure it is in contact with the solution) and centrifuge at 1500 rpm for 5 minutes (**DO NOT discard tube)**
13. Refill tubes with Sheather's sugar and place a coverslip-let sit for 10 minutes
14. Place cover slip #1 on slide and scan @ 100 magnification.
15. Count and record number of oocysts on the first cover slip
16. After ten minutes, place cover slip #2 on slide and scan @ 100 magnification
17. Count and record number of oocysts
18. Add number from first and second coverslips for a total oocyst/gram count.

Modified Concentration Method
(To concentrate and collect E.mac for sporulation use from llama feces)
(Conduct on all samples with sufficient oocysts/gram counts ~ 25+ and large amounts of feces 500 grams +)

1. Place sample in gallon sized zip-lock bag if not already in one
2. Cover feces with warm tap water (approximately 1-1.5 liters)
3. Mix feces in bag with water until solution is homologous (there should be very few large clumps of feces left)
4. Let bag sit at room temperature for 10 minutes and remix
5. Filter feces by placing approximately ~1 cup of solution into **3** layers of cheesecloth resting in a funnel. Drain solution into a beaker to save. (When draining slows, squeeze sample through cheesecloth until no more liquid drains out. You will be left with a clay like consistency-discard)
6. Repeat step 5 with fresh cheesecloth until entire ziplock bag has been filtered into beaker
7. Rest a #80 standard sieve over a #400* standard sieve
8. Pour approximately 100 ml of filtered solution through the #80 into the #400. (Not much will be left from the #80, it only stands to remove any large debris)
9. It seems counter intuitive but the less solution poured the faster the process will go. Too much and it will not filter fast
10. *If available use a #270 filter (~55 µm) instead of the #400. Procedure will be MUCH faster
11. Once all 100 mls have filtered through the #80 remove and place to the side
12. Rotate and shake the #400 to encourage drainage (it helps to shake it back and forth and tilt it on its side) being careful not to spill any of the solution
13. Once solution has drained wash the remaining sediment into a 50 ml centrifuge tube using a squeezable water bottle and label with a number and date of sample
14. Repeat steps 7 – 12 until all solution in beaker is filtered. NOTE: the #80 filter can be cleaned and sediment discarded as needed. The sediment from the #400 or #270 filter is what we save.

15. After the 50 ml tubes have time to settle, make sure one does not have way more sediment than another. Reproportion as needed (should have ~ 16 total 50 ml tubes)
16. Spin tubes at 1500 rpm for 5 minutes
17. Decant supernatant making sure not to pour off any of the pellet
18. At this point you can typically combine two tubes into one by adding water to reconstitute the pellet and pouring off into another tube and giving it a quick rinse (Make sure there is no more than 7.5 ml worth of sediment in any one tube. Also be careful not to lose any part of the pellet)
19. Fill each tube (should have ~ 8 now) with water and spin at 1500 rpm for 5 minutes
20. Decant supernatant
21. Repeat this wash until water is somewhat clear
22. The pellet (in theory) should be concentrated E.mac
23. After final wash, fill each tube with Sheather's Sugar **(With OUT Preservative)*** halfway up each tube
24. *You will have to make the Sheather's Sugar without preservative per protocol found in Lab
25. Mix the pellet with a wooden dowel
26. Fill with more Sheather's Sugar sans preservative (does not need to go all the way to the top since we are not using a coverslip)
27. Spin at 1500 rpm for 5 minutes
28. Using the "fish pump" pipetter, siphon off the top 5 mls from each tube
29. Combine two tubes into one. Should have approximately 4 50 ml tubes each with 10 mls of sheather's sugar
30. DO NOT DISCARD THE ORIGINAL "8" 50 ML CENTRIFUGE TUBES
31. Set the original 8 tubes to the side for use at a later time
32. Take the 4 50 ml tubes and fill with water and mix
33. Centrifuge at **1800** rpm for **8** minutes
34. Using the pipetter Siphon off the supernatant and **discard into a beaker to save**
35. Once all 4 50 ml tubes are siphoned off, combine all four pellets into two tubes by adding a little water to each pellet and mixing.
36. You should now have **2** 50 ml centrifuge tubes with the concentrated E.mac, **1** beaker of discarded supernatant from step #34, and the original 8 50 ml centrifuge tubes with sugar from step # 26-30
37. Fill the 8 50 ml centrifuge tubes with Sheather's sugar with no preservative (not to the top), label, date, and place into the refrigerator for 3-5 days in a holder
    a. After they have set for that time period (be careful not to shake them or disturb them) repeat procedures from step #28, only this time discard the 8 50 ml tubes when done siphoning
38. Take the discard from the beaker (from step #34) and pour it into the #400 filter
39. Filter it down and wash off sediment into one 50 ml centrifuge tube
40. Spin down tube, decant supernatant, and add pellet to the 50 ml centrifuge tube.
41. Label concentrated tube with date of sample. Fill tube containing concentrated pellet with 4% potassium dichromate and store in the refrigerator (Wear nytril gloves and goggles)

42. Prior to sporulation, vortex tube, and take 1-2 ml sample and run a Wisconsin Double Coverslip count (With some math, this will give you an estimated number of oocysts present in your tube)

Notes: You will have to make up the Sheather's Sugar solution without preservative from time to time. Directions found in lab. Make sure 4% potassium dichromate is made up prior to concentration or sporulation (takes a day to dissolve and must be made my lab techs…remember to thank them)

Protocol for Making Sheather's Sugar without preservative
(Needed for concentration method)

1. Obtain heating unit and double boiler
2. Weigh out 908 grams of cane sugar
3. Fill bottom of double boiler with water (fill to calcium desposit line)
4. Place top on double boiler and begin to heat
5. Get 710 ml of distilled water and pour into top of double boiler
6. Slowly add sugar while heating until all sugar is added and dissolved
7. Pour into a 3000 ml beaker and cover with plastic wrap
8. Let sit overnight
9. Check specific gravity and pour into labeled containers (write the date, spg and NO PRESERVATIVES) on label along with Sheather's Sugar

Sporulation Protocol
(To sporulate concentrated E.mac)

10. Get large beaker with escape valve, cork with a hole, piping, and fish pump
11. Put on Nytril gloves and goggles
12. Take concentrated samples from previous step, vortex and dump into beaker. Rinse with small amount of water to ensure all pellet is out of tube.
13. After concentrated tubes are placed in beaker, fill with 4% potassium dichromate (400 ml total)
14. Place beaker on glass dish to prevent tipping and place in incubator ~ 81 ºF
15. Place tube through cork so it will be covered by solution, and put cork in beaker
16. Plug in fish pump (double check to make sure air is flowing and bubbling in the liquid
17. Be sure to check progress of E.mac every 2-3 days
18. Should take approximately 17-21 days for complete sporulation

Sporulation Protocol
(To sporulate concentrated E.mac)

1. Put on Nytril gloves and goggles
2. Disconnect set-up and unplug
3. Carefully carry beaker over to hood and turn on blower
4. Pipette 2-3 mls (and sediment) into a 15 ml centrifuge tube
5. Refill beaker with water to the 400 ml mark to prevent drying from evaporation
6. Place back in the incubator and reattach set up…plug in.
7. Fill tube halfway with Sheather's sugar solution and mix with dowel
8. Fill tube to top with Sheather's sugar solution
9. Place coverslip on tube and centrifuge at 1500 rpm for 5 minutes
10. Check coverslip under 100 magnification (remember to remove gloves)
11. Meanwhile, refill tube with sheather's sugar and place another cover slip
12. Let sit for ~ 10 minutes while you check the first slide (Similar to the Wisconsin double cover slip method…allows you to be sure you see all E.mac)
13. Observe the oocysts. Count and note which stage of sporulation they are in
14. Photograph as needed

Washing sporulated E.mac
(To wash potassium dichromate for ingestion)

1. Put on Nytril gloves and goggles
2. Pour contents of beaker into #400 filter
3. Rinse sediment into a 50 ml centrifuge tube with water
4. Spin tube at 1500 rpm for 5 minutes
5. Decant supernatant
6. Fill with water and repeat wash until no color is present
7. Sporulated E.mac should now be suspended in water for ingestion

Storing Sporulated E.mac
(To re-concentrate and store sporulated E.mac)

1. Put on Nytril gloves and goggles
2. Pour contents of beaker into #400 filter
3. Rinse sediment into a 50 ml centrifuge tube with water
4. Spin tube at 1500 rpm for 5 minutes
5. Decant supernatant
6. Reconstitute pellet with fresh 4% potassium dichromate for storage
7. Store in refrigerator for later use

Vero Cell Culture Protocol
(Have someone demonstrate this protocol prior)

Materials
- Vero cells grown to 70-80% confluency
- Extra 25 cm^2 cell culture flask
- Medium- RPMI 1640 10% Fetal Bovine Serum, 1% antibiotics
- Isopropyl alcohol
- 10 ml pipette
- centrifuge
- Trypsin (5 ml in tube)

Procedure *MUST BE FOLLOWED WITHOUT CONTAMINATION*

1. Spray down entire hood with 70% isopropyl alcohol
2. Pour off medium from current cell culture into waste bucket
3. Pour 5 ml Trypsin into cell culture flask, place in incubator and let sit for 10-15 minutes with the occasional swirl
4. Once cells have been removed (will cloud up the trypsin), pour the solution into the original tube the trypsin was in
5. Centrifuge at 1000 rpm for 2.5-3 minutes
6. Pour off Trypsin without disturbing the pellet
7. Reconstitute pellet with 5 ml sterile medium to disperse cells
8. Add a few drops – 2 .5 ml (depending on desired confluency) into the original cell culture flask and into a new one. (Started with one…will have two now)
9. Add an additional 5 ml of sterile medium to each. Loosen cap and place back in incubator