APPENDICES

I. Buffered Neutral Formalin Solutions

1. 10% formalin, buffered

Na_2HPO_4	10.7 g
NaH_2PO_4	0.23 g
Formalin (commercial)	1200 ml
Water	10,800ml

Mix the formalin with water and then add the buffer salts.
Mix throughly before dispensing.
Smaller quantities can be prepared but this solution is very stable.
The pH should be approximately 7.0.
Label as "10% formalin, buffered" and date.

2. 5% formalin, buffered

Na_2HPO_4	10.7 g
NaH_2PO_4	0.23 g
Formalin	600.00 ml
Water	10,800.00 ml

Prepare as for 10% buffered formalin.
Label as "5% formalin, buffered" and date.
It is suggested that each buffer salt is weighed as listed above and mix the two thoroughly.
Store the mixture in a tightly closed bottle.
Prepare one litre of either 10% or 5% formalin and add 0.8 g of the buffer salt mixture.
Phosphate salts are purchased from VWR.

II. Phosphate Bufer

Na_2HPO_4	10.7 g
NaH_2PO_4	0.23 g
Water	11,200.00 ml

Label as "Phosphate buffer" and date.
It is suggested that each buffer salt is weighed as listed above and mix the two thoroughly.
Store the mixture in a tightly closed bottle.
Phosphate salts are purchased from VWR.

III. Lugols Solution

Iodine	5.00 g
Potassium iodide	10.00 g
dissolve in distilled water	100.00 ml

Label as "Lugols Solution" and date.

IV. Modified Schaudinn's Fixative (working solution)

Glacial acetic acid	5.00 ml
Glycerol	1.50 ml
Schaudinn's stock solution (two parts saturated mercuric chloride solution and one part 95% Ethanol)	93.50 ml

label as "Modified Schaudinn's Fixative" and date.

V. SAF Fixative

Sodium acetate	1.50 g (1.5%)
Acetic acid, glacial	2.00 ml (2.0%)
Formaldehyde, 40% commercial solution	4.00 ml (1.6%)
Water	92.50 ml (94.9%)

Label as "SAF Fixative" and date.

Remarks
As 40% formaldehyde is used, to calculate the percentage in the final solution, one has to divide by 2.5.

VI. Iodine Alcohol

Prepare a stock solution by adding enough iodine crystals to 70% alcohol to make a dark, concentrated solution. Label as "Iodine Alcohol" and date. For use, dilute some of the stock with 70% alcohol until a strong coloured solution like "tea" is obtained.

VII. Haematoxylin

Stock Solution 'A'

Haematoxylin crystals	10. g
95% ethyl alcohol	1000. ml

Label as “Haematoxylin Stock A” and date.
Allow to ripen in the light for one week (the solution may require filtration).
Solution A is purchased from VWR (R03439).

Stock Solution 'B' - (Mordant)

Ferrous ammonium sulfate	10. g
Ferric ammonium sulfate	10. g
Concentrated hydrochloric acid	10. ml
Distilled water	1000. ml

Label as “Haematoxylin Stock B (Mordant)” and date.
Solution B is purchased from VWR (R03439)

Working Solution

Solution 'A'	25. ml
Solution 'B'	25. ml

This should be prepared at least 3-4 hours before staining commences.

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VIII. Picric Acid Solution

Saturated aqueous picric acid	25. ml
Distilled water	25. ml

Label as “Picric Acid Solution” and date. Make sure bottle is sealed.
Saturated aqueous picric acid is purchased from VWR (3347-1).

IX. Carboxylol

Phenol, liquified in a warm water bath	250. ml
Xylol	750. ml

Label as “Carboxylol” and date.
Add the phenol to the xylol, keeping the phenol warm so that it does not start to crystallize.
Phenol (B29477-34) and Xylol (B30575-86) are purchased from VWR

X. Kato Thick Smear - Cellophane Solution

Glycerin	100. ml
Distilled water	100. ml
3% aqueous malachite green	1. ml

Label as "Kato Thick Smear- Cellophane Soln" and date. Soak the cellophane for at least 24 hours in this solution.
Malachite Green is purchased from VWR (Gurr B34045).

XI. Field's Stain

Solution 1

Methylene blue	0.80 g
Azure B	0.50 g
Disodium hydrogen phosphate (anhydrous)	5.00 g
Potassium dihydrogen phosphate (anhydrous)	6.25 g
Distilled water	500.00 ml

Label as "Field's Stain- Soln 1" and date.

Solution 2

Eosin (yellow, water soluble)	1.00 g
Disodium hydrogen phosphate (anhydrous)	5.00 g
Potassium dihydrogen phosphate (anhydrous)	6.25 g
Distilled water	500.00 ml

Label as "Field's Stain- Soln 2" and date.

Note:
Solutions 1 and 2 are purchased commercially from VWR (35056 3E & 35057 3G).
The stains may be kept for several weeks and used over and over, if they are in covered taining jars. When the eosin solution turns greenish, the stains should be renewed.

XII. Giemsa Stain

Giemsa stain powder	600. mg
Methyl alcohol (acetone free, neutral)	50. ml
Glycerin (neutral, from freshly opened bottle)	50. ml

Label as "Giemsa Stain" and date.
Note: Giemsa stain is purchased commercially from VWR (GURR R66 35086).

XIII. Robinson's Medium for *E. histolytica* Culture

Materials

1) Saline agar slope. Prepare 1.5% agar in 0.7% NaCl, distribute in 2.5 ml lots in quarter-ounce screw capped glass bottles, autoclave and slope.

2) Erythromycin solution. Prepare 20% in 70% ethanol in a sterile tube and leave for 2 hours at room temperature. Further dilute to 0.5% in sterile distilled water (0.5 ml of 20% + 19.5 ml distilled water).

3) Bactopeptone. 10 g in 50 ml distilled water, autoclave and aliquot in 5 ml lot.

4) Rice starch (BDM brand).

5) "R" medium for growing *Escherichia coli*, strain B
Stock solution

NaCl	25.00 g
Citric acid, monohydrate	10.00 g
Pot. dihydrogen phosphate	2.50 g
Magnesium sulfate heptahydrate	0.25 g
Lactic acid (BDH 90.08%)	20.00 ml
Distilled water	500.00 ml

Working Solution

Stock solution	100.00 ml
40% NaOH	7.50 ml
0.04% bromothymol blue solution	2.50 ml
Distilled water	1,000.00 ml

Adjust pH to 7.0 and autoclave.

6) Pot phthalate (0.5 M) dissolve 20.4 g in 10 ml of 40% NaOH and add distilled water to 200 ml. Adjust pH to 6.3 and autoclave.

Working Solution
Dilute the 0.5 M phthalate to 0.05 M in sterile distilled water and adjust pH to 6.5.

7) Basal amoebic medium ("BR"). *E. coli* is grown for two days at 37°C in shallow layers of "R" medium in sealed flat bottles and then store at room temperature up to two months. pH should not exceed 7.3.

8) Horse serum. Heat inactivate at 56°C for 30 minutes each on successive days and store at 4°C.

9) Complete medium ("BRH"). Equal volume of horse serum and "BR" are mixed, incubated 24-48 hours at 37°C and stored at room temperature up to one month.

XIV. NNN Medium (Novy-MacNeal-Nicolle)

Bacto-agar	7. g
Sodium chloride	3. g
Distilled water	450. ml
Rabbit, defibrinated blood	150. ml

1) Add the Bacto-agar and sodium chloride to the distilled water in a flask and bring to the boil.

2) Autoclave at 12 lb. pressure for 15 minutes.

3) Cool the mixture to 52°C, add 150 ml rabbit blood and mix.

4) Pipette 5 ml amounts into sterile screw-capped test tubes and slant to produce a long slant. Label as “NNN Medium” and date.

5) Incubate tubes at 37°C for 24 hours to test for sterility. Antibiotics can be added here if necessary.

Preparation of Agar Plates

1. Remove the plates from the refrigerator and place in a 37°C incubator for 30 minutes.

2. Add 0.5 ml of Page's medium to a slant culture of *E. coli* or *E. aerogenes*. Gently scrape the surface of the slant with a sterile bacteriological loop (do not break the agar surface). Using a sterile Pasteur pipette, uniformly suspend the bacteria by gentle pipetting. Add two to three drops of this suspension to the middle of a

warmed agar plate and spread the bacteria over the surface of the agar with a bacteriological loop.

XV. Page's Medium for Free-Living Amoebae

Sodium chloride (NaCl)	120. mg
Magnesium sulfate ($MgSO_4 . 7 H_2O$)	4. mg
Calcium chloride ($CaCl_2 . 2 H_2O$)	4. mg
Disodium hydrogen phosphate (Na_2HPO_4)	142. mg
Potassium dihydrogen phosphate (KH_2PO_4)	36. mg
De-ionized water	1000. ml

Label as "Page's Medium" and date.
This is dissolved and autoclaved at 15 lb./in.^{2} for 15 minutes. The solution may be stored in the refrigerator for up to six months.

XVI. Non-Nutrient Agar Plates

Agar	1.5 g
Page's amoebae medium	100. ml

Dissolve agar in the saline with heat and sterilize by autoclaving at 15 lb./in.^{2} for 15 minutes. Cool to 60°C and aseptically pour into plastic petri dishes: 20 ml for 100 x 15 mm dish, 5 ml for 16 x 15 mm dish. Label and date plates. Plates may be kept in canisters for about three months when stored at 4°C. The plates may be kept at 4°C for about three months.

XVII. Carbol Fuchsin Stain Solution

Basic fuchsin	21.0 g
Liquid phenol (85%)	85.2 ml
95% ethyl alcohol	150.0 ml
10% Tween 80	7.5 ml

Label as "Carbol Fuchsin Stain" and date.
Heat mixture in 45°C incubator for 24 hours to dissolve the crystals. Add distilled water to bring volume up to 1,800 ml.

XVIII. Malachite green

Malachite green	0.5 g
Distilled H_2O	100.0 ml

Label as "Malachite Green Stain" and date.

XIX. Chromotrope stain

Chromotrope 2R	6.00 gm
Fast green	0.15 gm
Phosphotungstic acid	0.70 gm
Glacial acetic acid	3.00 ml

Label as "Chromotrope Stain" and date.
Mix ingredients and allow to stand for 30 minutes. Then add 100 ml distilled water.

XX. Acid alcohol for Chromotrope 2R Stain

90% ethyl alcohol or 95% methyl ethanol	995.5 ml
Glacial acetic acid	4.5 ml

Label as "Acid-Alcohol Soln." and date.

XXI. Proper Use of a Microscope

1. Place the microscope on a firm bench so that it does not vibrate. Do not place it in direct sunlight.

2. Switch on the source of light.

3. Place the specimen on the stage.

4. Select the objective to be used. The 10x objective can be used for adjusting the illumination.

5. Fully rack up condenser with top lens swung in.

6. Focus on specimen with objective. For this, rack the objective carefully downwards, while looking at it from the side, until the lens is near the specimen but not touching it. Then, while looking through the eyepiece, rack the objective slowly upwards until the image comes into view and is sharply focused.

7. Choose down lamp field stop in microscope base (Köhler illuminating base).

8. Focus the condenser by slightly lowering condenser until stop image is in optimum focus.

9. Use the two condenser centering screws to center field stop in field of view.

10. Open up field stop far enough to clear entire field of view. The higher objectives require the field stop to be opened more widely than 10x.

11. Adjust the aperture of the condenser iris to control image contrast. For this, remove eyepiece and look through the tube. Adjust the aperture of the iris so that it fills about three quarters of the lens with the light.

12. Adjust image brightness by means of filter or by varying lamp voltage.

13. After changing objectives, just adopt lamp field stop to size of visual field and condenser diaphragm to objective aperture.

A. <u>Calibration of the Microscope</u>

1. Remove one eyepiece from the microscope and unscrew the top eye-lens. Place the micrometer disc on the diaphragm without the ocular so that the <u>engraved side is underneath</u>. Screw back the eye-lens and insert the ocular in the microscope. Be careful to keep both micrometer and lens clean.

2. Place the stage micrometer on the stage and focus on some portion of the scale.

3. Looking through the microscope, examine the ruling of the stage micrometer so that you can distinguish between the large (0.1 mm) and the small (0.01 mm) divisions.

4. Adjust the field so that the 0 line on the ocular micrometer is exactly <u>superimposed</u> upon the 0 line on the stage micrometer.

5. Without moving the stage micrometer, find another point at the extreme right where two other lines are exactly superimposed. This second set of superimposed lines should be as far as possible from the 0 line, but the distance will vary with the objective and microscope.

6. Knowing that each of the large divisions of the stage micrometer equals 0.1 mm, determine the total distance (in millimeters) between the two points of superimposition and the number of small ocular units necessary to cover the same distance.

   For example: Suppose 27 ocular units (small) equal 2 stage units (large) or 0.2 mm.

7. Calculate the number of millimeters that is measured by one small ocular unit.

   Example: 27 ocular units = 0.2 mm
   1 ocular unit = 0.2 mm/27 or 0.0074 mm

8. Since measurements of parasites are usually given in microns, the above determination must be converted to microns:

   0.0074 mm x 1000 = 7.4 µm

9. Record the calibrations with each of the objectives. With high and oil magnifications, the thinner ocular micrometer line must be centered on the broader stage micrometer line for more accurate measurement.

10. Keep the calibrations handy so that the size of any organism can be quickly determined by measuring it with the ocular micrometer and noting the particular lens combinations.

B. Use of Verniers and Scales

Most mechanical stages are fitted with two scales and verniers - one running north and south (Y-axis) and the other east and west (X-axis). [Note that these are NOT for measurements.] They are to be used as a position finding device.

Suppose that a particular organism in a slide is of such interest that you may want to re-examine it or show it to someone else at a later date. For locating and relocating the organism, the verniers and scales are very useful. The main scale has a series of lines at 1 mm intervals and a short vernier with 10 lines corresponding to 9 divisions of the main scale.

For finding the coordinates of an organism in a slide, proceed as follows:

1. Make sure the slide is seated properly in the specimen holder on the microscope stage and no longer moves when the slide is gently tapped from the sides.

2. If the slide was not seated properly, relocate the organism or structure.

3. Determine the position of the 0 line of the vernier with respect to the main scale -- for example, the 0 line likes between 93 and 94 on the main scale (in other words, the value for the x-coordinate is more than 93, but less than 94). The first part of the x-coordinate therefore is 93.····. To determine the decimal figure for the x-coordinate, examine the vernier scale. The number of the vernier line which is in closest coincidence with one of the lines of the main scale (all other vernier lines will be more separated from the main scale lines) gives the decimal. For example, if the vernier line happens to be 7, then the location of the organism along the x-axis is 93.7.

4. In the same way, determine the position along the Y-axis (north-south). For example: 12.3.

5. The complete coordinates of that particular organism are therefore: 93.7/12.3.

6. Record the orientation of the slide (label to the right or left).

For relocating that same organism, the following steps apply.

1. The same microscope used originally must be used (since vernier scales differ between microscopes, even for the same model).

2. Orient the slide in the same way as it was when coordinates were originally determined (label to the right or left).

3. Seat the slide properly.

4. Seat both scales and verniers to the recorded figures (e.g. 93.7 on the X-axis; 12.4 on the Y-axis).

5. The organism should now be in the field. If not, check orientation of the slide, seating of the slide and proper setting of the recorded figures.

Since the recorded figures determined above are applicable and usable only to one particular microscope, **further information is required if the organism is to be relocated on another microscope** (e.g. if the slide is to be sent to a reference laboratory). For this, proceed as follows:

1. Seat the slide tightly in the specimen holder.

2. Record whether the label end of the slide is to the right or to the left.

3. Move the slide until the upper right hand corner of the cover glass (or another easily identifiable mark such as a chip, etc.) is in the exact center of the field. Determine the readings of verniers and scales, A_1/B_1.

4. Move the slide until the lower left hand corner (or an easily identifiable mark) is in the exact center of the field. Again, determine the readings of scales and verniers, A_2/B_2.

With this information (orientation of slide, readings of upper and lower corners and readings of the organism), the readings of scales and verniers of the organism can theoretically be recalculated for any other microscopes equipped with scales and verniers. Since "play" between the scales, slightly inaccurate readings, etc. may result in the recalculated readings being slightly "off", each submission should be accompanied by as complete a description as possible to ensure that the same organism is being examined.

For calculating the scale and vernier readings applicable to another microscope, proceed as follows:

1. Orient the slide as usual.
2. Set the slide tightly.
3. Determine the readings for upper right hand corner or for the mark supplied with the slide; A_1/B_1.
4. Determine the readings for the lower left hand corner, A_2/B_2.
5. On graph paper, determine the "A values - microscope 1" along either the vertical or the horizontal axis, and the "A values - microscope 2" along the other axis. (Please note: the larger the scale on the graph paper, the more accurate determinations become.)
6. Then plot the values corresponding to A_1, A_2 and A_2 A_1. (These are the readings of the corners.)
7. On the same or separate sheet of graph paper, plot the B_1 B_2 and B_2 B_1 values (i.e. the Y readings of the corners).
8. After drawing lines through the corresponding points thus located, the readings for any point applicable to the second microscope can be determined from these lines.

XXII. Parasite Kits: Specimen Collection Instructions for Patients

1. The fluid in this container is a poison. Make sure the lid is on tightly and do not drink it.

2. Do not use laxatives, anti-diarrheal drugs, mineral oil, barium, bismuth or antibiotics within one week prior to collecting sample.

3. First pass urine into the toilet.

4. Pass the stool on a dry clean surface, such as a bed pan, plastic cup or onto saran wrap placed just under the toilet seat.

5. Place 1 ounce (walnut-sized) portion of stool into the container with the fluid fixative using one of the applicators provided.

6. Break up the stool into the fluid. MIX WELL.

7. Screw the cap on tightly. Make sure it does not leak.

8. Return the bottle to the zip lock bag.

9. Wash your hands immediately.

10. Label with your name, sex and the date. Return the specimen container with the complete information to the laboratory as soon as possible.

11. Store at room temperature. DO NOT FREEZE.

XXIII. Isolate Codes
The following SOFT computer codes are to be used to correctly report a parasitic finding on the Isolate Screen in SOFT.

Acanthamoeba species	acaspp
Amoeba species	amospp
Ancylostoma duodenale	ancduo
Ascaris lumbricoides	asclum
Babesia species	babspp
Blastocystis hominis	blahom
Brugia malayi	brumal
Chilomastix mesnili	chimes
Clonorchis sinensis	closin
Cryptosporidium parvum	crypar
Cryptosporidium species	cryspp
Cyclospora cayetanensis	cyccay
Cyclospora species	cycspp
Dicrocoelium dendriticum	dicden
Dientamoeba fragilis	diefra
Diphyllobothrium species	diphyo
Diphyllobothrium latum	diplat
Dipylidium caninum	dipcan
Echinostoma species	echisp
Echinococcus species	echspp
Encephalitozoon species	encapp
Endolimax nana	endnan
Entamoeba species	entasp
Entamoeba coli	entcol
Enterocytozoon species	entcyt
Entamoeba hartmanni	enthar
Entamoeba histolytica	enthis
Entamoeba histolytica/dispar	enthd
Enteromonas hominis	enthom
Entamoeba polecki	entpol
Enterobius vermicularis	entveo

Fasciolopsis buski	fasbus
Fasciola hepatica	fashep
Fasciola hepatica/Fasciolopsis buski	fashbu
Flagellate species	flagsp
Fluke species	fluspp
Giardia lamblia	gialam
Heterophyes heterophyes	hethet
Hookworm species	hooksp
Hymenolepis diminuta	hymdim
Hymenolepis nana	hymnan
Iodamoeba buetschlii	iodbut
Isospora belli	isobel
Leishmania species	leispp
Loa loa	loaloa
Mansonella ozzardi	manozz
Mansonella perstans	manper
Mansonella streptocerca	manstr
Metagonimus yokogawai	metyok
Metorchis conjunctus	metcon
Microfilaria species	micspe
Microsporidium species	mirspp
Necator americanus	necame
Nematode species	nemspp
Onchocerca volvulus	oncvol
Paragonimus westermani	parwes
Parasite(s) seen – referred for definitive identification	parref

Plasmodium falciparum	plafal
Plasmodium malariae	plamal
Plasmodium ovale	plaova
Plasmodium species	plaspp
Plasmodium vivax	plaviv
Retortamonas intestinalis	retoin
Sarcocystis sui/hominis	sarsh
Schistosoma haematobium	schhae
Schistosoma japonicum	schjap
Schistosoma mansoni	schman
Small flagellate	smafla
Strongyloides stercoralis	strstl
Taenia saginata	taesag
Taenia solium	taesol
Taenia species	taespo
Toxoplasma gondii	toxgon
Trematode species	trespp
Trichomonas hominis	trihom
Trichinella spiralis	trihot
Trichostrongylus species	trispp
Trichuris trichiura	tritro
Trichomonas vaginalis	trivag
Trypanosoma cruzi	trycru
Trypanosoma gambiense or *rhodesiense*	trygrh
Trypanosoma species	tryspp
Unidentified microfilaria species	unmisp
Wuchereria bancrofti	wucban