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Section: <b>Parasitology Manual</b>	Subject Title: <b>Laboratory Procedures for Stool Examination</b>	
Issued by: <b>LABORATORY MANAGER</b>	Original Date: March 13, 2000	
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## Hematoxylin Stain

### PRINCIPLE

Hematoxylin is a natural dye and when used in combination with aluminum and iron is a powerful nuclear stain and a chromatin stain for intestinal parasites. It has polychrome properties. The active colouring agent, hematin, is formed by the oxidation of hematoxylin. This process, “ripening” is achieved by exposure of prepared solutions to the air for several days or weeks preferably in the sunlight. The method employed is regressive staining which is accomplished by overstaining of hematoxylin then removing the stain from other constituents by molecular bombardment with a differentiating agent, picric acid. The following method is quick and reliable.

### SPECIMEN

- Properly prepared smears from SAF preserved stool  
Fresh unpreserved stool. (Prepare a smear and immediately fix in Schaudinn’s fixative. It is important that smears to not dry before fixation. Leave in fixative for 30 minutes. After fixation, place slide in 70% alcohol to which sufficient iodine has been added to give the alcohol a deep brown color).

### MATERIALS

#### Reagents:

- Ethyl alcohol solutions - 50%, 70%, 95% and 100% (absolute).
- Hematoxylin stock solution A and stock solution B (Commercial product).
- Prepare Hematoxylin working solution:  
Mix equal parts of stock solutions A and B  
Let stand at room temperature for at least 3 to 4 hours before staining commences. (See Appendix).
- NH<sub>4</sub>OH (commercial product)
- Picric acid (saturated aqueous approximately 1.2%w/v).  
Working solution is equal volumes of distilled water and Picric acid solution.  
**Important Note:** solid Picric acid should not be stored dry since it is explosive (see Appendix).
- Mayer’s albumin (commercial product).
- xylol (commercial product)
- carboxylol (see appendix)

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Equipment:

Microscope with ocular micrometer and set for Kohler illumination.

Glass microscope slides

Pasteur pipets

Glass cylinders

Staining containers

Covers slips 22 X 40 mm

Slide rack

Sharps container

## QUALITY CONTROL

1. Positive control slide of SAF preserved stool containing *Dientamoeba fragilis* and/or *Entamoeba histolytica* trophozoites stained with every rack of slides.
2. All QC results are recorded. All “out-of-control” results are referred to the Lab Director for action.
3. Ensure all reagents and chemicals used are in date.
4. The Hematoxylin working solution should be produced at least 2 to 3 hours before use.
5. If stain is to be reused, it should be checked at regular intervals by adding a few drops of used stain to tap water. If the drops remain blue or blue black that mixture is useable. If it turns brown it should be replaced with fresh stain before proceeding.
6. The microscope should be calibrated (within the last 12 months or anytime the optics are altered).
7. When the stain is thoroughly mixed and staining performed correctly, the cytoplasm of Protozoan trophozoites will be blue gray. Cysts tend to be darker. Nuclei and inclusions (chromatoid bodies red blood cells, bacteria etc. are dark gray blue - almost black).
8. All staining dishes should be covered to prevent evaporation of reagents ( use a screw top, or jars with glass lids). If the xylol becomes cloudy or if there is water at the bottom of the dish discard, and replace with fresh reagents.

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## PROCEDURE

1. Prepare the hematoxylin working solution at least two to three hours before use.
2. Prepare other required reagents and store in appropriately labeled containers and document all changes on the QC stain control sheet.
3. Place the prepared smears in a slide rack and then into the following solutions for the time indicated.
4. alcohol 70% 10 minutes
5. running tap water 10 minutes
6. hematoxylin stain 10 minutes
7. distilled water 1 minute
8. picric acid 10 minutes
9. running tap water 20 minutes
10. alcohol (70%) with drops of ammonia sufficient to achieve a pH of approximately 8 10 minutes
11. alcohol 95% 10 minutes
12. alcohol 95% 10 minutes
13. carboxylol 10 minutes
14. xylol 10 minutes
15. xylol 10 minutes
16. Using Entellan (Commercial product) mount slides, do not allow the slides to dry before mounting.
17. Store the slides in slide tray until read.

## EXAMINATION OF STAINED SMEARS

1. When mounting is dry, examine the control smear first before proceeding to patient samples.
2. Examine the smears microscopically with overlapping fields.
3. Using the low power objective (10X) examine vertically the thick areas of the smear for helminth ova and larva for at least 5 minutes. Scanning is then done with a low power oil objective (~50X) or with a high dry objective (40X) if a low power oil is not available. Switch to the 100X for more detailed study. Using the 50X oil objective, screen horizontally the whole slide for at least 15 minutes. Using the 100x oil objective, examine between two thick bands for small protozoa for at least 5 minutes.
4. Use "*Bench Aids in the Diagnosis of Intestinal Parasites*" or other manual to aid in a speciation of the observed parasites.

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## PROCEDURE NOTES

1. This stain gives the best morphological details. Organisms can be missed under bright light, therefore lower light observations may be preferable.
2. The most important step in the preparation of a well stained permanent smear is good fixation. If good fixation has not been successful the protozoa will be distorted or may stain poorly.
3. Once the staining process has started the smear should not be allowed to dry at any of the subsequent steps until they are cover-slipped.
4. Slides should always be drained thoroughly between solutions, touch the end of the slide with a paper towel to remove excess fluid.
5. In the final stages of dehydration the 100% alcohol and xylol should be kept as free from water as possible. Containers must have tight fitting caps to prevent evaporation or absorption of moisture. If xylol becomes cloudy replace with fresh stock.
6. If smears peel or flake off, the specimen may have been inadequately dried on the slide or the smear may be too thick, repeat the smears.
7. 1-2 drops of ammonia are added to bring the alcohol to ~pH 8.
8. Patients' stained slides must be allowed to dry thoroughly, (eg. overnight) before examination.
9. Vernier measurements should be taken for the location of any parasite with unusual morphology so that confirmation can be obtained.

## REPORTING

1. Report all parasites found.
2. Protozoan trophozoites and cysts will be readily seen. Report the organism and stage. Do not use abbreviations. eg. *Entamoeba histolytica* cysts, *Dientamoeba fragilis* trophozoites.
3. Do not quantitate protozoa or organisms.
4. Report but do not quantitate the presence of human cells or products. eg. Leukocytes, red blood cells and Charcot-Leyden crystals.

## LIMITATIONS OF PROCEDURE

1. The permanent stain smear is primarily for the identification and confirmation of protozoa. It is not recommended for the detection and identification of helminth eggs or larva since they often retain too much stain or are distorted. However they may be occasionally recognized and identified.

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2. Delayed fixation during this stain preparation may show unusual protozoal characteristics.
3. Coccidial oocysts will not be identified on an iron hemotoxylin stain. This requires a modified acid fast stain.

## REFERENCES

NCCLS Procedures for the Recovery and Identification of Parasites from the Intestinal Tract; Approved Guideline M28-A 17(23) December 1997

LPTP Broadsheet #5: Prevalence of Parasitic Infections Vol 2 section 4 August 18 1978  
 Garcia LS, Bruckner DA. Diagnostic Medical Parasitology. 3rd Edition. ASM Press, Washington DC. 1997 pp609-653