

WHEATLEY'S MODIFICATION OF THE GOMORI TRICHROME STAIN

Preanalytical Considerations

I. PRINCIPLE

It is generally recognized that stained fecal films are the single most productive means of stool examination for intestinal protozoa. The permanent stained smear facilitates detection and identification of cysts and trophozoites and provides a permanent record of the protozoa found. The trichrome technique of Wheatley for fecal specimens is a modification of Gomori's original staining procedure for tissue. It is a rapid, simple procedure that produces uniformly well-stained smears of intestinal protozoa, human cells, yeast cells, and artifact material.

II. SPECIMENS

The specimen usually consists of unconcentrated fresh stool smeared on a microscope slide and immediately fixed in Schaudinn's fixative or of polyvinyl alcohol (PVA)-preserved stool smeared on a slide and allowed to air dry. Stool preserved in sodium acetate-acetic acid-formalin (SAF) or any of the single-vial fixatives for parasitology are also acceptable.

III. MATERIALS

A. Reagents: Reagents may be purchased commercially or prepared in the laboratory.

- a. Trichrome (Wheatley's formulation)
 - i. Chromotrope 2R..... 0.6 g
 - ii. Light green SF..... 0.3 g
 - iii. Phosphotungstic acid..... 0.7 g
 - iv. Acetic acid (glacial)..... 1.0 ml
 - v. Distilled water..... 100.0 ml

Prepare the stain by adding 1.0 ml of acetic acid to the dry ingredients. Allow the mixture to stand (ripen) for 30 min at room temperature. Add 100 ml of distilled water. Properly prepared stain will be purple in color. The staining solution should be protected from light. Store in a glass or plastic bottle at room temperature. The shelf life is at least 24 months.

- b. 70% ethanol
- c. 70% ethanol plus iodine

Prepare a stock solution by adding iodine crystals to 70% ethanol until you have a dark solution (1-2 g/100 ml). To use, dilute the stock solution with 70% alcohol until a dark reddish brown (strong tea color) is obtained. As long as the color is acceptable, new working solution does not have to be replaced. Replacement time will depend on the number of smears stained and the size of the container (1 to several weeks).

- d. 90% ethanol, acidified

90% ethanol	99.5 ml
acetic acid (glacial)	0.5 ml

Prepare by combining.

- e. 100% ethanol
- f. Xylene (or xylene substitute)

B. Supplies

- a. Glass slides (25 by 75 mm), frosted ends desirable
- b. Coverslips (22 by 22 mm; no. 1)
- c. Glass or plastic pipettes
- d. Coplin jars or other suitable staining containers
- e. Immersion oil

C. Equipment: Optional materials, depending on specimen source of laboratory protocol

- a. Binocular microscope with 10X, 40X, and 100X objectives (or the approximate magnifications for low power, high dry power, and oil immersion examination). A 50X or 60X oil immersion objective is also very helpful in screening stained smears.
- b. Oculars should be 10X. Some workers prefer 5X; however, overall smaller magnification may make final organism identifications more difficult.
- c. Fume hood to contain staining setup (optional)

Analytical Considerations

IV. QUALITY CONTROL

- A. Stool samples used for QC can be either fixed stool specimens known to contain protozoa or PVA-preserved negative stools to which buffy coat cells have been added. Use a QC smear prepared from a positive PVA specimen or PVA containing buffy coat cells when new stain is prepared or at least once each week. Cultured protozoa can also be used; however, very few laboratories provide intestinal protozoan culture methods.
- B. Include a QC slide when you use a new lot number of reagents, when you add new reagents after cleaning the dishes, and at least weekly.
- C. If the xylene becomes cloudy or has an accumulation of water in the bottom of the staining dish, use fresh 100% ethanol and xylene.
- D. Cover all staining dishes to prevent evaporation of reagents
- E. Depending on the volume of slides stained, change staining solutions on an as-needed basis.
- F. When the smear is thoroughly fixed and the stain is performed correctly, the cytoplasm of protozoan trophozoites will be blue-green, with sometimes a tinge of purple. Cysts tend to be slightly more purple. Nuclei and inclusions (chromatoid bodies, RBCs, bacteria, and Charcot-Leyden crystals) are red, sometimes tinged with purple. The

background material usually stains green, providing a nice color contrast with the protozoa. This contrast is more distinct than that obtained with the hematoxylin stain.

- G. The specimen is also checked for adherence to the slide (macroscopically).
- H. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted on the body of the microscope). Although recalibration every 12 months may not be necessary, this will vary from laboratory to laboratory, depending on equipment care and use. Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not recommended on a yearly basis.
- I. Known positive microscope slides, Kodachrome 2 x 2 projection slides, and photographs (reference books) should be available at the work station.
- J. Record all QC results, including a description of QC specimens tested; the laboratory should also have an action plan for "out of control" results.

V. PROCEDURE

- A. Wear gloves when performing this procedure
- B. Slide preparation
 - 1. Fresh Fecal specimens
 - a. When the specimen arrives, prepare two slides with applicator sticks and immediately (without drying) place them in Schaudinn's fixative. Allow the specimen to fix for a minimum of 30 min; overnight fixation is also acceptable. The stool smeared on the slide should be thin enough that newsprint can be read through the smear. Proceed with the trichrome staining smear.
 - b. If the fresh specimen is liquid, place 3 or 4 drops of PVA on the slide, mix several drops of fecal material with the PVA, spread the mixture, and allow it to dry for several hours (1 hour minimum) in a 35° - 37°C incubator or overnight at room temperature.¹ Proceed with the trichrome staining procedure by placing the slides in iodine-alcohol.
 - 2. PVA-preserved fecal specimen (mercuric chloride base)

¹ Remember that this approach is reserved for liquid stools only; do not use this approach with semi-formed or formed stool. There will not be enough contact between the fixative and stool to preserve any organisms that might be present.

- a. Allow the stool specimens that are preserved in PVA to fix for at least 30 min. Thoroughly mix the contents of the PVA bottle with two applicator sticks.
 - b. Pour some of the PVA-stool mixture onto a paper towel, and allow it to stand for 3 min to absorb the PVA. Do not eliminate this step.²
 - c. With an applicator stick, apply some of the stool material from the paper towel to two slides, and allow them to dry for several hours (minimum 1 hour) in a 35° - 37°C incubator or overnight at room temperature.
 - d. Place the dry slides into iodine-alcohol
 - e. If the stool was not thoroughly mixed with PVA by the patient, apply some stool material to two slides and immediately immerse in Schaudinn's fixative for a minimum of 30 min; then proceed with the trichrome method.³
3. Modified PVA-preserved fecal specimens (copper or zinc base, single-vial systems)
- a. Allow the stool specimens that are preserved in PVA or other fixative to fix for at least 30 min. Thoroughly mix the contents of the fixative vial with two applicator sticks.
 - b. Pour some of the fixative-stool mixture onto a paper towel, and allow it to stand for 3 min to absorb the PVA. Do not eliminate this step if the fixative contains PVA.
 - c. With an applicator stick, apply some of the stool material from the paper towel to two slides, and allow them to dry for several hours (minimum of 1 hour) in a 35° - 37°C incubator or overnight at room temperature.
 - d. Begin the trichrome staining process by placing the slides into a dish of 70% alcohol then trichrome stain, or the slides can be placed directly into the trichrome stain step (iodine-alcohol step can be eliminated).
4. SAF-preserved fecal specimens
- a. Thoroughly mix the SAF-stool mixture, and strain through gauze into a 15-ml centrifuge tube.
 - b. After centrifugation (10 min at 500 Xg), decant the supernatant fluid. The final sediment should be about 0.5 to 1.0 ml. If necessary, adjust by repeating step "a" (if too little sediment is present) or by suspending the sediment in saline (0.85% NaCl) and removing part of the suspension (if too much sediment is present).

² If you take the specimen directly from the specimen, you may get too much PVA and not enough stool; the amount of PVA required to "glue" the specimen onto the slide is very minimal. Too much PVA on the slide may cause the material to fall off during processing.

³ If the lag time between specimen passage and fixation is too long, regardless of the extra fixation step in Schaudinn's fixative, the overall organism morphology may be marginal. You can also scrape some stool from that portion that has been in contact with the fixative and use that for smear preparation.

- c. Prepare a smear from the sediment for later staining.⁴
 - d. After drying, place the smear in 70% alcohol (iodine-alcohol step can be eliminated).
- C. Staining smears.
1. Remove slide from Schaudinn's fixative, and place slide in 70% ethanol for 5 min.
 2. Place slide in 70% ethanol plus iodine for 1 min for fresh specimens or 5 to 10 min for PVA-preserved air-dried smears.⁵
 3. Place slide in 70% ethanol for 5 min*
 4. Place in 70% ethanol again for 3 min.*⁶
 5. Place in trichrome stain for 10 min.⁷
 6. Place in 90% ethanol plus acetic acid for 1 to 3s. Immediately drain the rack, and proceed to the next step. Do not allow slides to remain in this solution.
 7. Dip several times in 100% ethanol. Use this step as a rinse.
 8. Place in two changes of 100% ethanol for 3 min each.*
 9. Place in xylene for 5 to 10 min.*
 10. Place in xylene again for 5 to 10 min.*
 11. Mount with coverslip (no. 1 thickness) by using mounting medium (e.g., Permount).
 12. Allow the smear to dry overnight or after 1 h at 37°C.
 13. Examine the smear microscopically with the 100X objective. Examine at least 200 to 300 oil immersion fields.⁸

* Slides may be held for up to 24 h in these solutions without harming the quality of the smear or the stainability of organisms.

VI. RESULTS

- A. Protozoan trophozoites and cysts will be readily seen.
- B. Helminth eggs and larvae may not be easily identified; therefore, examine wet mounts of concentrates.

⁴ Many people prefer to coat the slide with albumin prior to smearing the stool onto the slide (in order to glue the material onto the slide).

⁵ All slides exposed to mercuric-chloride-based fixatives must be placed in the iodine dish to remove the mercury. The subsequent rinses in ethanol remove the iodine. At the point the slide is placed into trichrome stain, both the mercury and iodine have been removed from the fecal smear.

⁶ Fecal smears prepared from SAF-preserved stool material do not require the iodine step and can be placed in this alcohol dish before trichrome staining.

⁷ Fecal smears prepared from modified PVA-fixed material (copper or zinc base) do not require the iodine step or subsequent alcohol rinses, but can be placed directly into the trichrome stain. One alcohol rinse may be used before this trichrome step; some labs prefer this approach. This approach is also acceptable for the single-vial systems that do not contain any mercury.

⁸ A 50X or 60X oil immersion objective can be used for screening; however, some of the small protozoa may be missed without using the 100x oil immersion objective prior to indicating no parasites were seen.

- C. Yeast and human cells can be identified. Human cells include macrophages, PMNs, and RBCs. Yeasts include single and budding cells and pseudohyphae.

Postanalytical Considerations

VII. REPORTING RESULTS

- A. Report the organism and stage (do not use abbreviations)
Example: *Entamoeba histolytica*/*E. dispar* trophozoites present
- B. Quantitate the number of *Blastocystis hominis* seen (rare, few, moderate, many, packed). Do not quantitate other protozoa
- C. Note and quantitate the presence of human cells.
Example: Moderate WBCs, many RBCs, few macrophages, rare Charcot-Leyden crystals:
- D. Report and quantitate yeast cells.⁹
Example: Moderate budding yeast cells and few pseudohyphae
- E. Save positive slides for future reference. Record information prior to storage (name, patient number, organisms present).
- F. Quantitation of parasites, cells, yeast cells, and artifacts.
- Few = 2 per 10 oil immersion fields (x 1,000)
 - Moderate = 3 to 9 per 10 oil immersion fields (x 1,000)
 - Many = 10 per 10 oil immersion fields (x 1,000)

VIII. PROCEDURE NOTES

- A. Fixation of specimens is important. Improperly fixed specimens will result in protozoan forms that are nonstaining or predominantly red.
- B. Spread the PVA-stool mixture to the edges of the glass slide; this will cause the film to adhere to the slide during staining. It is also important to thoroughly dry the slides to prevent the material from washing off during staining.
- C. Always drain slides between solutions. Touch the end of the slide to a paper towel for 2 s to remove excess fluid before proceeding to the next step.
- D. Incomplete removal of mercuric chloride (Schaudinn's fixative and PVA) may cause the smear to contain highly refractive granules that may prevent finding or identifying any organisms present. Since the 70% ethanol-iodine solution removes the mercury complex, it should be changed at least weekly to maintain the strong tea color.
- E. To restore weakened trichrome stain, remove cap and allow the ethanol to evaporate (ethanol carried over on staining rack from

⁹ It is important to know if the stool was immediately fixed after passage; if not, yeast results may be misleading since they will continue to multiply and often form pseudohyphae if the specimen sits around at room temperature and there is a lag time between specimen passage and fixation. If this is the case, do not report yeast results. However, if the specimen is loaded with yeast, budding cells, and/or pseudohyphae, this finding should be discussed with the physician.

- previous dish). After a few hours, add fresh stain to restore lost volume. Older, more-concentrated stain produces more-intense colors and may require slightly longer destaining times (an extra dip).
- F. Smears that are predominantly green may be due to the inadequate removal of iodine by the 70% ethanol. Lengthening the time of these steps or changing the 70% ethanol more frequently will help.
 - G. In the final stages of dehydration, keep the 100% ethanol and the xylenes as free from water as possible. Coplin jars must have tight-fitting caps to prevent both evaporation of reagents and absorption of moisture. If the xylene becomes cloudy after addition of slides from the 100% ethanol, return the slides to fresh 100% ethanol, and replace the xylene.
 - H. If the smears peel off, the specimen might have been inadequately dried on the slide (in the case of PVA-fixed specimens) or the slides might have been greasy. However, slides do not have to be cleaned with alcohol prior to use.
 - I. If the stain appears unsatisfactory and it is not possible to obtain another slide to stain, restain the unsatisfactory slide. Place the slide in xylene to remove the coverslip, and reverse the dehydration steps by adding 50% ethanol as the last step. Destain the slide in 10% acetic acid for several hours, and then wash it thoroughly first in water, then in 50% ethanol, and then in 70% ethanol. Place the slide in the trichrome stain for 8 min, and complete the staining procedure

IX. LIMITATIONS OF THE PROCEDURE

- A. The permanent stained smear is not recommended for staining helminth eggs or larvae. However, occasionally they may be recognized and identified.
- B. Examine the smear under the oil immersion lens (100X) for the identification of protozoa, human cells, Charcot-Leyden crystals, yeast cells, and artifact material.
- C. This high-magnification examination is recommended for protozoa; although the smears can be screened using the 50X or 60X oil immersion objectives, some of the smaller organisms could be missed without use of the 100X oil immersion objective.
- D. Screening the smear under the low-magnification lens (10X) might reveal eggs or larvae, but this is not recommended as a routine approach.
- E. Helminth eggs and larvae and *Isospora belli* oocysts are best seen in wet preparations.
- F. *Cryptosporidium parvum* and *Cyclospora cayetanensis* are generally not seen on a trichrome-stained smear (modified acid-fast stains or immunoassays are recommended).
- G. Microsporidia spores will not be seen on a trichrome-stained smear (modified trichrome stains are recommended).

SUPPLEMENTAL READING

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3. **Gomori, G.** 1950. A rapid one-step trichrome stain. *Am. J. Clin. Pathol.* **20**:661-663.
4. **Markell, E.K., D.T. John, and W.A. Krotoski.** 1999. *Markell and Voge's Medical Parasitology*, 8th ed., W.B. Saunders Co., Philadelphia.
5. **Melvin, D. M., and M. M. Brooke.** 1985. *Laboratory Procedures for the Diagnosis of Intestinal Parasites*, p. 163-189. U.S. Department of Health, Education, and Welfare publication no. (CDC) 85-8282. U.S. Government Printing Office, Washington, D.C.
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7. **Smith, J.W., and M.S. Bartlett.** 1991. Diagnostic parasitology: introduction and methods, p 701-716. *In* A. Balows, W.J. Hausler, Jr., K.L. Herrmann, H.D. Isenberg, and H.J. Shadomy (ed.), *Manual of Clinical Microbiology*, 5th ed. American Society for Microbiology, Washington, D.C.
8. **Wheatley, W.** 1951. A rapid staining procedure for intestinal amoebae and flagellates. *Am. J. Clin. Pathol.* **21**:990-991.

APPENDIX I

The Wheatley's (modification of the Gomori Trichrome) stain reagents available from Medical Chemical Corporation are as follows:

REAGENT	CATALOG NUMBER	SIZE AND CATALOG NUMBER	
Gomori's Trichrome (Wheatley's Mod)	602A	602A	16 oz
D'Antoni's Iodine	628A	628A	16 oz
95% Reagent Alcohol	3719A	3719A	1 gal
Reagent Alcohol 90% ethyl alcohol 5% methyl alcohol 5% isopropyl alcohol	374B	374B-16 oz 374B-1gal	16 oz 1 gal
Trichrome Decolorizer	3720A	3720A-16 oz 3720A-1 gal	16 oz 1 gal
Xylene	134B	134B-16 oz 134B-1 gal	16 oz 1 gal
Xylene Substitute (d-limonene)	930E	930E	1 gal

APPENDIX II

As an alternative to using mounting fluid on every slide, the following method can be used. This approach saves time (drying the slides after being mounted) and eliminates the need for routine use of mounting fluids.

- A. Remove the stained slides from the last dehydrating dish.
- B. Allow the slide to air dry (minimum of 30 minutes, especially if using xylene-substitutes).
- C. Place a drop of immersion oil directly onto the dry stool smear.
- D. Allow the oil to "sink in" for a minimum of ~15 min.
- E. Place a number 1 coverslip onto the oil-covered stool smear.

- F. Add 1 drop of immersion oil onto the coverslip and proceed to examine the smear using the 100 X oil immersion objective. A 50 X or 60 X oil immersion objective can be used for screening.
- G. *Do not use this approach unless you add the coverslip before examination of the smear. The dry stool material may be quite hard; the objective lens could accidentally be scratched if the stool smear is not covered before reading.*