GIEMSA STAIN

PREANALYTICAL CONSIDERATIONS

I. Principle

Giemsa stain is used to differentiate nuclear and/or cytoplasmic morphology of platelets, RBCs, WBCs, and parasites (1,2). The most dependable stain for blood parasites, particularly in thick films, is Giemsa stain containing azure B. Liquid stock is available commercially. The stain must be diluted for use with water buffered to pH 6.8 or 7.0 to 7.2, depending on the specific technique used. Either should be tested for proper staining reaction before use. The stock is stable for years, but it must be protected from moisture because the staining reaction is oxidative. Therefore, the oxygen in water will initiate the reaction and ruin the stock stain. The aqueous working dilution of stain is good only for 1 day.

II. Specimen

The specimen usually consists of fresh whole blood collected by finger puncture or of whole blood containing EDTA (0.020 g/10 ml of blood) that was collected by venipuncture and is less than 1 h old. Heparin (2 mg/10 ml of blood) or sodium citrate (0.050 g/10 ml of blood) may be used as an anticoagulant if trypanosomes or microfilariae are suspected. If slides have been prepared, the specimen may be a thin blood film that has been fixed in absolute methanol and allowed to dry, a thick blood film that has been allowed to dry thoroughly and is not fixed, or a combination of a fixed thin film and an adequately dried thick film (not fixed). The combination thick/thin blood film is also acceptable.

III. Materials

A. Reagents

1. Giemsa stain
2. Giemsa buffer

B. Supplies

1. Glass slides (1 by 3 in., or larger if you prefer), alcohol washed
2. Glass marker
3. Blood collection supplies (if applicable)
4. Paper with newsprint-size print
5. Applicator sticks

C. Equipment

1. Microscope, binocular with mechanical stage; low (10x), high dry (40x), and oil immersion (100x) objectives; 10x oculars; calibrated ocular micrometer; light source equivalent to 20-W halogen or 100-W tungsten bulb; blue and while ground-glass diffuser filters
2. Timer, 1 h or more in 1-min increments

ANALYTICAL CONSIDERATIONS

IV. Quality Control

A. The stock buffer solutions and buffered water should be clear, with no visible contamination.

B. Check the Giemsa stain reagents, including the pH of the buffered water, before each use.

C. Prepare and stain films from “normal” blood, and microscopically evaluate the staining reactions of the RBCs, platelets, and WBCs; this assessment can also be accomplished by the examination of your patient slide. If the staining reactions are acceptable, then the QC is considered acceptable.

   a. Macroscopically, blood films appear purplish. If blue, the buffered water was too alkaline; if pink to red, the buffered water was too acid.

   b. Microscopically, RBCs appear pinkish gray, platelets appear deep pink, and WBCs have purple-blue nuclei and lighter cytoplasm. Eosinophilic granules are bright purple-red, and neutrophilic granules are purple. Basophilic stippling within uninfected RBCs is blue.

   c. Slight variation may appear in the colors described above depending on the batch of stain used and the character of the blood itself, but if the various morphological structures are distinct, the stain is satisfactory.

D. 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 required on a yearly basis.

E. Record all QC results.

V. Procedure

A. Wear gloves when performing this procedure.

B. Thin blood films (only)

1. Fix air-dried film in absolute methanol by dipping the film briefly (two dips) in a Coplin jar containing absolute methanol.
2. Remove and let air dry.
3. Stain with diluted Giemsa stain (1:20, vol/vol) for 20 min. For a 1:20 dilution, add 2 ml of stock Giemsa to 40 ml of buffered water in a Coplin jar.
4. Wash by briefly dipping the slide in and out of a Coplin jar of buffered water (one or two dips). *Note: Excessive washing will decolorize the film.*
5. Let air dry in a vertical position.

C. Thick blood films (only)

1. Allow film to air dry thoroughly for several hours or overnight. Do not dry films in an incubator or by heat, because this will fix the blood and interfere with the lysing of the RBCs. *Note: If a rapid diagnosis of malaria is needed, thick films can be made slightly thinner than usual, allowed to dry for 1 h, and then stained.*
2. DO NOT FIX.
3. Stain with diluted Giemsa stain (1:50, vol/vol) for 50 min. For a 1:50 dilution, add 1 ml of stock Giemsa to 50 ml of buffered water in a Coplin jar.
4. Wash by placing film in buffered water for 3 to 5 min.
5. Let air dry in a vertical position.

D. Thin and thick blood films on the same slide

1. Allow the thick film to air dry thoroughly
2. Fix air-dried film in absolute methanol by dipping the film briefly (two dips) in a Coplin jar containing absolute methanol. Be sure not to get the alcohol or its fumes on the thick film by slightly tilting the slide.
3. Remove and let air dry with the *thick film up*. Be sure slide is thoroughly dry before staining. Introducing even a minute amount of methyl alcohol into the stain dilution will interfere with the lysing of the RBCs in the thick films.
4. Stain the entire slide with diluted Giemsa stain (1:50, vol/vol) for 50 min. For a 1:50 dilution, add 2 ml of stock Giemsa to 40 ml of buffered water in a Coplin jar. Place the slide in the stain, *thick film dow*n to prevent the debris caused by dehemoglobinization from falling onto the thin film.
5. Rinse the thin film by briefly dipping the film in and out of a Coplin jar of buffered water (one or two dips). Wash the thick film for 3 to 5 min. Be sure that the thick film is immersed but *do not allow the water to cover any part of the thin film.*
6. Let air dry in a vertical position with the *thick film down*.

E. Combination thin and thick blood films on the same slide (see protocol) (Figure 1)

1. Place a clean 1- by 3-in. glass microscope slide on a horizontal surface.
2. Place a drop (30 to 40 µl) of blood onto one end of the slide about 0.5 in. from the end
3. Using an applicator stick lying across the glass slide and keeping the applicator in contact with the blood and glass, rotate (do not “roll”) the stick in a circular motion while moving the stick down the glass slide to the opposite end.
4. The appearance of the blood smear should be alternate thick and thin areas of blood that cover the entire slide.
5. Immediately place the film over some small print and be sure that the print is just

barely readable.

6. Allow the film to air dry horizontally and protected from dust for at least 30 min to 1 h. Do not attempt to speed the drying process by applying any type of heat, because the heat will fix the RBCs and they subsequently will not lyse in the staining process.
7. **This slide can be stained as either a thick or thin blood film**.
8. Label the slide appropriately.
9. If staining with Giemsa (as a thick film) will be delayed for more than 3 days or if the film will be stained with Wright's stain, lyse the RBCs on the thick film by placing the slide in buffered water (pH 7.0 to 7.2) for 10 min, remove it from the water, and place it in a vertical position to air dry.
10. If staining with Giemsa (as a thin film), after the film is completely dry, fix it by dipping the slide into absolute methanol, and allow the film to air dry in a vertical position. If the film will be stained with Wright's stain, it does not need to be fixed. Wright's stain contains the fixative and stain in one solution.

VI. Results

A. If *Plasmodium* organisms are present, the cytoplasm stains blue and the nuclear material stains red to purple.
B. Schüffner's stippling and other inclusions in the RBCs infected by *Plasmodium* spp. stain red.
C. Nuclear and Cytoplasmic colors that are seen in the malarial parasites will also be seen in the trypanosomes and any intracellular leishmaniae that are present.
D. The sheath of microfilariae may or may not stain with Giemsa, while the body will usually appear blue to purple.
E.

POSTANALYTICAL CONSIDERATIONS

VII. Reporting Results

A. Report any parasite, including the stage(s) seen (do not use abbreviations).
Examples*:* *Plasmodium falciparum* rings and gametocytes, rings only
Plasmodium vivax rings, trophozoites, schizonts, and gametocytes
Wuchereria bancrofti microfilariae
Trypanosoma brucei gambiense/rhodesiense trypomastigotes
Trypanosoma cruzi trypomastigotes
Leishmania donovani amastigotes
B. Any laboratory providing malaria diagnoses should be able to identify *Plasmodium vivax* and *Plasmodium ovale*, even in the absence of Schüffner's stippling.

VIII. Procedure Notes

A. Blood films prepared from venipuncture blood when an anticoagulant is used must be prepared within 1 h of collection. Otherwise, certain morphological characteristics of both parasites and infected RBCs may be atypical. Also, thick blood films may wash off the slide during the staining procedure.
B. The correct pH for all buffered-water and staining solutions is also important. Solutions with the incorrect pH will prevent certain morphological characteristics (stippling) from being visible and will not give typical nuclear and cytoplasmic colors on the stained film.
C. Stain a QC slide each time patient blood films are stained (the patient slide can actually be used for the QC slide. If a separate QC slide is used and several patient specimens are stained on the same day (using the same reagents), only one control slide need be stained and examined.

IX. Limitations of the Procedure

A. *Finding no parasites in one set of blood films does not rule out a parasitic infection*.
B. *Examine a minimum of 300 oil immersion (x 1,000) fields before reporting no parasites found*.
C. Examine the entire smear under low power (100x) for the presence of microfilariae. Remember that the sheath may not be visible stained with Giemsa (*W. bancrofti*).
D. If a tube of blood containing EDTA cools to room temperature and the cap has been removed, several parasite changes can occur. The parasites within the RBCs with respond as if they were now in the mosquito after being taken in with a blood meal.

The morphology of these changes in the life cycle and within the RBCs can cause confusion when examining blood films prepared from this blood.

a. Stippling (Schüffner's dots) may not be visible.

b. The male gametocyte (if present) may exflagellate.

c. **The ookinetes of *Plasmodium* species other than *P. falciparum* may develop as if they were in the mosquito and may mimic the crescent-shaped gametocytes of *P. falciparum.***

E. Identification to species, particularly between *P. ovale* and *P. vivax* and between the ring forms of *P. falciparum* and *Babesia* spp., may be impossible without examining one of the slides stained as a thin blood film. Also, *Trypanosoma cruzi* trypomastigotes are frequently distorted in thick films.

F. Excess stain deposition on the film may be confusing and make the detection of organisms difficult.

REFERENCES

1. **Garcia, L.S.** 2001. *Diagnostic Medical Parasitology*, ed. 4, ASM Press, Washington, D.C.

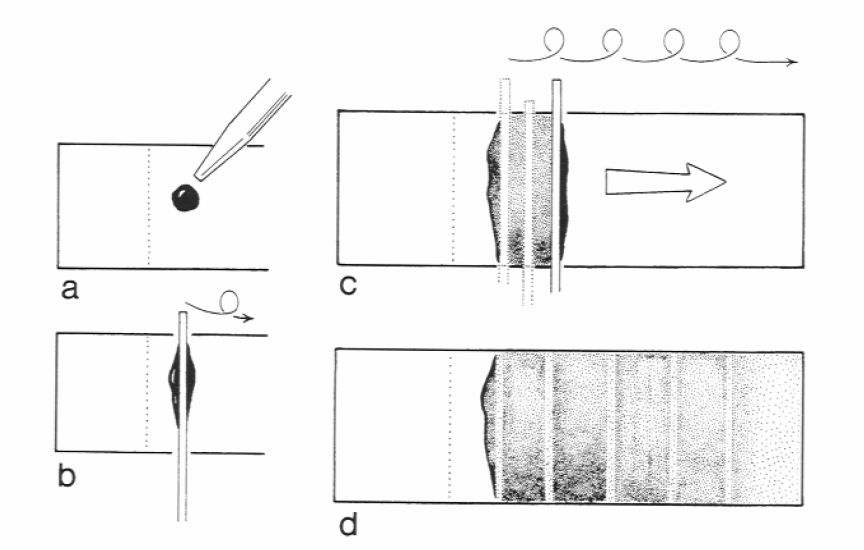
2. **NCCLS**, 2000. *Laboratory Diagnosis of Blood-Borne Parasitic Diseases*. Approved Guideline M15-A. National Committee for Clinical Laboratory Standards, Villanova, PA.

SUPPLEMENTAL READING

1. **Garcia, L.S.** 1999. Practical Guide to Diagnostic Parasitology, ASM Press, Washington, D.C.

APPENDIX

Figure 1: Method of thick-thin combination blood film preparation. (a) Position of drop of EDTA blood; (b) position of applicator stick in contact with blood and glass slide; (c) rotation of applicator stick; and (d) completed thick-thin combination blood film prior to staining. (Illustration by Sharon Belkin)(From reference 1, with permission).



APPENDIX

Blood stain reagents available from Medical Chemical Corporation are as follows:

REAGENT	CATALOG NUMBER	SIZE AND CATALOG NUMBER
Giemsa stain	591A	591A-16 oz 16 oz
Giemsa buffer, pH 6.8*	592A	592A-32 oz 32 oz
Methanol	107B	107B-16 oz 16 oz 107B-1 gal 1 gal 107B-5 gal 5 gal
Wright's Dip Stat #1 Fixative	301	301- 16 oz 16 oz 301 – 1 gal 1 gal
Wright's Dip Stat #2 Fixative (Eosinate Stain)	302	302-16 oz 16 oz 302-1 gal 1 gal
Wright's Dip Stat #3 Fixative (Polychrome Stain)	303	303-16 oz 16 oz 303-1 gal 1 gal
Wright's Dip Stat Stain Kit (Fixative, Eosinate, Polychrome, and Rinse Solutions)	300K	4 x 8 oz Kit
Wright's stain (requires buffer 593A)	926A	926A – 32 oz 32 oz 926A – 1 gal 1 gal
Wright's buffer	593A	593A – 32 oz 32 oz 593A – 1 gal 1 gal
Wright's stain, one step	929A	929A – 32 oz 32 oz 929A – 1 gal 1 gal

*Check Web site for Giemsa Buffers at pH 7.0 and 7.2.