WRIGHT'S DIP STAT STAIN

PREANALYTICAL CONSIDERATIONS

I. Principle

Wright'sDip STAT Stain is used to differentiate nuclear and/or cytoplasmic morphology of platelets, RBCs, WBCs, and parasites (1,2). The traditional Wright's stain, an alcoholic solution of methylene blue and eosin Y, dates from the early 1890's. There have been many modifications, most of which involve oxidative demethylation of the methylene blue to improve polychroming. Modern day samples of the dye usually contain mixtures of methylene blue, azure A, and thionin (the mixture is often called "polychromed methylene blue") compounded with eosin Y. Medical Chemical's Wright's Dip Stat contains the azures and the eosin Y in separate solutions, which improves staining control and reproducibility, as well as speed. The total staining time is about thirty seconds.

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. Tinted Methanol (Wright's Dip Stat #1 Fixative)
2. Eosinate Stain (Wright's Dip Stat #2)
3. Polychromed Methylene Blue (Wright's Dip Stat #3)
4. Deionized Water

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 solutions and deionized water should be clear, with no visible contamination.

B. 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. Microscopically, RBCs appear light tan, reddish or buff, and WBCs have bright blue nuclei and lighter cytoplasm. Eosinophilic granules are bright red, and neutrophilic granules are pink or light purple.

   b. 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.

c. If malaria parasites are present, the cytoplasm stains pale blue and the nuclear material stains red. Schüffner's dots and other RBC inclusions usually do not stain or stain very pale with Wright's stain. While the sheath of microfilariae may not always stain, the nuclei within the microfilariae always stain pale to dark blue.

C. 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.

D. 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 for 30 seconds in a Coplin jar containing absolute methanol (**Wright's Dip Stat #1 Fixative**).
2. Allow the film to air dry.
3. Transfer the fixed smear to the eosinate stain (**Wright's Dip Stat #2**) for 6 seconds.
4. Rinse the slide in deionized or distilled water
5. Immerse the rinsed smear in polychrome stain (**Wright's Dip Stat #3**) for 20 seconds
6. Rinse with distilled or deionized water and air dry
7. 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. Lake the thick film by immersing in distilled or deionized water for 10 min.
3. Allow the film to air dry thoroughly.
4. Fix air-dried film in absolute methanol for 30 seconds in a Coplin jar containing absolute methanol (**Wright's Dip Stat #1 Fixative**).
5. Allow the film to air dry.
6. Transfer the fixed smear to the eosinate stain (**Wright's Dip Stat #2**) for 6 seconds.
7. Rinse the slide in deionized or distilled water
8. Immerse the rinsed smear in polychrome stain (**Wright's Dip Stat #3**) for 20 seconds
9. Rinse with distilled or deionized water and air dry
10. 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 thin 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. Lake the thick film by immersing in distilled or deionized water for 5 to 10 min.
5. Allow the film to air dry thoroughly.
6. Fix air-dried film in absolute methanol for 30 seconds in a Coplin jar containing absolute methanol (**Wright's Dip Stat #1 Fixative**).
7. Allow the film to air dry.
8. Transfer the smear to the eosinate stain (**Wright's Dip Stat #2**) for 6 seconds.
9. Rinse the slide in deionized or distilled water
10. Immerse the rinsed smear in polychrome stain (**Wright's Dip Stat #3**) for 20 seconds
11. Rinse with distilled or deionized water and air dry
12. Let air dry in a vertical position.

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 (See Appendix).
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**. If stained as a thick film, remember to lake the film, allow to air dry, and fix with methanol for 30 seconds (**Wright's Dip Stat #1 Fixative**).
9. If staining will be delayed and the smear will be stained as a thick film, lyse the RBCs on the slide by placing the slide in distilled or deionized water for 10 min, remove it from the water, and place it in a vertical position to air dry, then fix for 30 seconds in methanol (**Wright's Dip Stat #1 Fixative**).
10. Transfer the air-dried slide to the eosinate stain (**Wright's Dip Stat #2**) for 6 seconds.
11. Rinse the slide in deionized or distilled water
12. Immerse the rinsed smear in polychrome stain (**Wright's Dip Stat #3**) for 20 seconds
13. Rinse with distilled or deionized water and air dry
14. Let air dry in a vertical position.

** Staining times vary with the thickness of the blood film. Each laboratory should determine the exact staining times.*

VI. Results

A. If malaria parasites are present, the cytoplasm stains pale blue and the nuclear material stains red. Schüffner's dots and other RBC inclusions usually do not stain or stain very pale with Wright's stain. 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.

B. While the sheath of microfilariae may not always stain, the nuclei within the microfilariae always stain pale to dark blue.

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. 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.

C. Tap water is unacceptable for the rinsing solution; the chlorine may bleach the stain.

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.

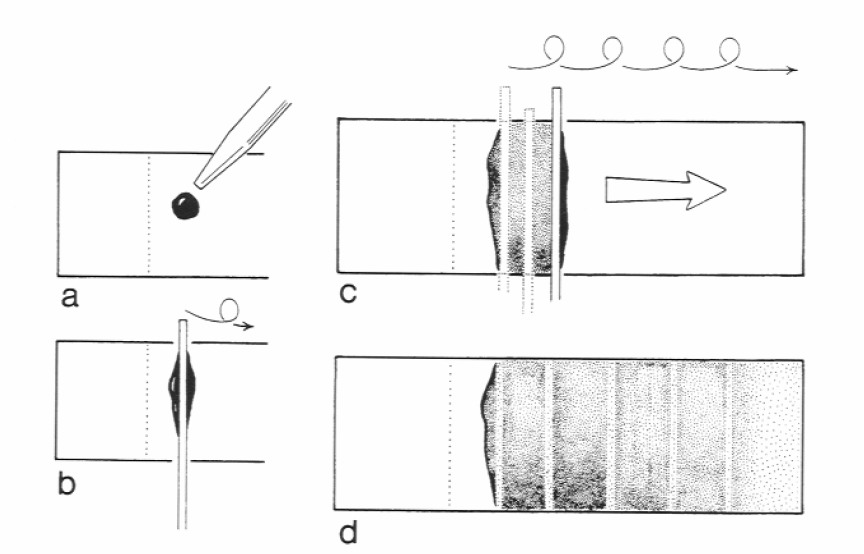
2. **NCCLS**, 2000. *Laboratory Diagnosis of Blood-Borne Parasitic Diseases*. Approved Guideline M15-A. National Committee for Clinical Laboratory Standards, Villanova, PA.

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).


a
b
c
d

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 Tinted Methanol	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 bottles 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
Deionized Water	9265B	9265B – 1 gal 1 gal 9265B – 2.5 gal 2.5 gal 9265B – 5 gal 5 gal

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