

# Romanowsky-Giemsa staining

WOLF D. KUHLMANN, M.D.

*Division of Radiooncology, Deutsches Krebsforschungszentrum, 69120 Heidelberg, Germany*

---

The Romanowsky-type stain variants such as those originally described or modified by numerous authors (the famous EHRLICH, CHENZINSKY, PLEHN, MALACHOWSKI, ROMANOWSKY, NOCHT, JENNER, MAY, GRÜNWARD, LEISHMAN, WRIGHT, GIEMSA etc. who laid the foundation for modern hematology) are routinely used for the staining of blood and bone marrow films. Today, we know that a two-component stain is required to produce the so-called Romanowsky-Giemsa effect. Hence, all these stains contain a mixture of methylene blue and related thiazin dyes and eosin. The thiazin component must be azure B according to G GIEMSA, A PAPPENHEIM and R LILLIE, and the eosin component may be eosin Y or one of the corresponding erythrosins. Azure B (methylene azure B) is formed by the oxidation of methylene blue.

The typical color of cell nuclei (i.e. purple), is due to molecular interaction between eosin and the azure B-DNA complex. On the molecular nature, however, there exist many theories. The intensity of staining depends on the azure B content and on the ratio azure B / eosin Y. The staining result can be influenced by several factors such as pH of the buffer, buffer substances, fixation and staining time. Minor modifications of working concentrations and staining time have been made over the years.

GIEMSA's stain is a member of the Romanowsky group of stains. Because these stains are very tedious to prepare, they are best purchased as pre-made stock stains. The staining methods are originally developed for blood films and bone marrow films, but cell smears, cellular imprints, cytopsin preparations of different origin and thin tissue sections work equally well. While PAPPENHEIM's staining, i.e. the staining by use of a combination of MAY-GRÜNWARD and GIEMSA solutions, is now the standard method in haematology, GIEMSA staining is the preferred procedure for the other histologies. The staining procedure may vary according to the material under study, particularly for tissue sections. Even if the various techniques may lead to some differences in staining, the basic mechanisms and effects, however, are almost the same. The staining in tissue sections is more variable than in blood films because (and not only due to tissue fixation) tissue sections contain more stainable components. Hence, the protocols used require additional steps such as differentiation, dehydration and embedding).

Staining must be long enough to achieve differential staining. Best staining is obtained by use of a more dilute staining solution with a longer incubation time. Differentiation can be achieved in two steps: (a) with dilute acetic acid to remove excess blue staining, and (b) 95% ethanol to remove excess eosin. This is difficult to standardize, and sections must be processed under microscopic control.\*

---

\* Dyes and other chemicals in histological staining can be toxic. They must be handled with care

## Giemsa staining (cell smears, cytopins, frozen sections)

Chemicals	Chemical solution
Azure B-Eosin stock solution (Merck), Giemsa's azure eosin methylene blue solution containing Azure B (C.I. 52010) <i>and</i> Methylene blue (C.I. 52015) <i>and</i> Eosin Y (C.I. 45380)  HEPES, free acid HEPES, sodium salt Methanol Distilled water	<ul style="list-style-type: none"> <li>• HEPES stock A: 0.1 M HEPES (free acid) in distilled water</li> <li>• HEPES stock B: 0.1 M HEPES (sodium salt) in distilled water</li> <li>• HEPES buffer: 900.0 mL HEPES stock A <i>plus</i> 100.0 mL HEPES stock B if necessary, adjust pH to 6.5 (buffer is stable for months at 4°C)</li> <li>• HEPES buffer working: 300.0 mL HEPES buffer <i>plus</i> 700.0 mL distilled water (corresponds to 0.03 mol/L HEPES)</li> <li>• GIEMSA diluted dye solution: 1.0 mL Azure B-Eosin stock solution <i>plus</i> 25.0 mL HEPES buffer working solution</li> </ul>
<b>Staining procedure</b> Air-dried specimens are fixed and stained: <ul style="list-style-type: none"> <li>– methanol 3-5 min</li> <li>– GIEMSA diluted dye solution 5-10 min</li> <li>– HEPES working buffer 2 x 1 min</li> <li>– distilled water 1 x rinse</li> </ul> Slides are air-dried in vertical position	

## Giemsa staining (paraffin sections)

Giemsa's stain is principally used for blood and bone marrow films but can be also applied to tissue sections; cut thin (2 µm) paraffin sections, tonsils may serve as control material.

Chemicals	Chemical solution
GIEMSA stock solution (Merck), (Azure eosin methylene blue solution) containing Azure B (C.I. 52010) <i>and</i> Methylene blue (C.I. 52015) <i>and</i> Eosin Y (C.I. 45380)  Sodium acetate Glacial acetic acid n-Butanol	<ul style="list-style-type: none"> <li>• Acetate buffer stock solution: 0.2 M acetate buffers with different pH (in the range of pH 3.5 to 7.0) are prepared and examined in preliminary experiments because pH optimum for the study material should be determined previously)</li> <li>• GIEMSA diluted dye solution: 1.0 mL Giemsa stock solution <i>plus</i> 50.0 mL 0.02 M acetate buffer solution (1:10 dilution of 0.2 M acetate buffer, the</li> </ul>

Distilled water	right pH is adjusted with 5% acetic acid) <ul style="list-style-type: none"> <li>Acetate wash buffer: 10.0 mL 0.2 M acetate buffer <i>plus</i> 90.0 mL distilled water pH is adjusted with 5% acetic acid</li> <li>0.01% acetic acid</li> </ul>
<p><b>Staining procedure</b></p> <p>Sections are passed through distilled water and stained:</p> <ul style="list-style-type: none"> <li>GIEMSA diluted dye solution                            1-2 hours</li> <li>acetate wash buffer    3 dips</li> <li>blot dry and control with the microscope; if “blue” predominates, differentiate in acetic acid</li> <li>differentiate in 0.01% acetic acid                    optional     under microscopic control *</li> <li>acetate wash buffer    2-3 dips</li> <li>blot dry</li> <li>n-butanol     2 x 3 min</li> </ul> <p>Slides are cleared in xylene or xylene substitute and mounted in resinous medium under coverglass</p> <p>* Fixation influences the color balance of Giemsa staining, and best results are obtained with appropriate pH of the staining solution. Adequate pH is determined by trial with Giemsa staining solutions prepared with acetate buffers in the range from pH 3.5-7.0. If “blue” predominates, then the pH is too high, and if “red” predominates, a higher pH must be chosen</p>	

## References for further readings

Ehrlich P (1879)  
Nocht B (1898)  
Romanowsky D (1891)  
Unna P (1891)  
Pappenheim A (1899)  
Ewing J (1900)  
Giemsa G (1902)  
Wright J (1902)  
Giemsa G (1903)  
Giemsa G (1904)  
Wilson TM (1907)  
Pappenheim A (1908)  
Lillie R (1944)  
Marshall PN *et al.* (1975)  
Lillie RD and Fullmer HM (1976)  
Marshall PN *et al.* (1978)  
Wittekind DH (1983)  
Schulte E (1987)

Wittekind D (1983)  
Kamino H and Tam ST (1991)  
Woronzoff-Dashkoff KP (1993)  
Kligora CJ *et al.* (1999)  
Horobin RW and Kiernan AJ (Conn's Biological Stains, 2002)

© *Prof. Dr. Wolf D. Kuhlmann, Heidelberg*

*10.09.2006*