WORLD HEALTH ORGANIZATION
ORGANISATION MONDIALE DE LA SANTE

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ENGLISH ONLY

STANDARDIZED ROMANOWSKY STAINING OF BLOOD AND BONE MARROW FILMS

Prepared on behalf of the World Health Organization,

by

S. M. Lewis, M.D., FRCPath. and Ulla Rousöe, Tutor Technologist

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1. INTRODUCTION

Stained blood and bone marrow films have an essential role in the diagnosis of blood disorders. The Romanowsky stains are universally employed for the staining of these films.

The remarkable property of the Romanowsky dyes to make subtle distinctions in shades of staining between different constituents of cells, depends on two components, namely, azure B and eosin Y.

Different combinations of these stains have been used and the combinations have been given different names according to their originators, e.g. May-Grünwald, Giemsa, Leishman, and Wright. These differences cause variable staining. In addition, variation in the purity of the dyes used in any stain may cause batch-to-batch variation.

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It was appreciated that the workers in any one laboratory will be accustomed to the appearances in their own stained films, but when films are referred for review to another laboratory using a different staining method, this may give rise to confusion and even to a wrong morphological diagnosis being made. Also, reference to an atlas may cause problems because the illustrations may depict a different stain to the one normally used by the reader.

The need to standardize the staining method is important not only for routine diagnostic work, but also for research in, for example, the classification of leukaemia and other blood diseases and for interlaboratory collaborative studies.

In order to overcome the problems of variable staining and especially interlaboratory variations the International Committee for Standardization in Haematology (ICSH) established a standardized method for staining films. The ICSH Expert Panel identified three essential requirements for a consistent and reproducible Romanowsky effect:

(a) the use of azure B and eosin Y dyes of sufficient purity;

(b) the correct and appropriate combination of these dyes in the staining solution;

(c) a standardized method for using the stain with a selected buffer.

Dyes of sufficient purity are now available from several commercial companies (see Appendix).

2. METHOD OF STAINING BLOOD AND BONE MARROW FILMS

2.1 Materials

Volumetric flask 1000 ml
Conical flasks 1000 ml, 2000 ml
Measuring cylinders 100 ml, 1000 ml
Brown bottle 1000 ml
Timer
Waterbath 37°C
pH-meter
Analytical balance

2.2 Chemicals

2.2.1 Azure B

Purity: not less than 95%.
For specifications, see Appendix.
Warning: avoid skin contact and inhalation of the powder.

2.2.2 Dimethylsulphoxide (DMSO) (Mol wt. 78,13)

Purity: not less than 99%
Warning: avoid skin contact and inhalation.

2.2.3 Eosin Y

Purity: not less than 90%
For specifications, see Appendix.

2.2.4 HEPES (Mol wt. 238,3)

(N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid).

2.2.5 Methanol (Mol wt. 32,04)

Warning: inflammable and poisonous; avoid inhalation and ingestion.
Storage: protect from light. Keep in well-stoppered bottle and avoid unecessary exposure to air.

2.3 Stock solutions

2.3.1 HEPES - buffer 10 mmol/l, pH 6.6

HEPES	2,38 g
Distilled water to	1000 ml
Adjust pH with sodium hydroxide 1 mol/l	

2.3.2 HEPES - buffer 10 mmol/l, pH 6,6 with DMSO

HEPES - buffer 10 mmol/l, pH 6,6	1000 ml
DMSO	50 ml

2.3.3 Azure B - dimethylsulphoxide solution

Azure B	3 g
DMSO	400 ml

Preheat DMSO at 37°C before adding azure B

Let stand for at least 30 minutes at 37°C; during this period shake vigorously for about 30 seconds at 5 minute intervals

2.3.4 Eosin Y - methanol solution

Eosin Y	1 g
Methanol	600 ml

Mix well till complete solution is obtained.

2.3.5 Azure B - eosin Y - stock solution

Azure B - DMSO solution	400 ml
Eosin Y - methanol solution	600 ml

Mix slowly the azure B-DMSO solution with the eosin Y methanol solution over 30 minutes, using a magnetic stirrer, if available.

Storage: keep in a dark cupboard at room temperature in a tightly stoppered brown bottle.

Stability: 3 months.

2.3.6 Azure B - eosin Y staining solution

Azure B - eosin Y stock solution	1 volume
HEPES - buffer pH 6,6 with DMSO	14 volumes

The solution has to be prepared freshly.

Stability: 4 hours.

2.4 Staining jar procedure

2.4.1 Materials

Staining solution
Staining jars
Holders for slides
Timer
Blotting paper

2.4.2 Staining technique

Place 3 staining jars in a row and fill them as fo

Jar No.	Reagent
1	Methanol
2	Azure B - eosin Y staining solution
3	Demineralized water

Insert the slides into the holder and place sequen the following times:

Jar No.	Time
1	10 minutes
2	25 minutes for blood; 35 minutes for
3	a few seconds

When rinsing in demineralized water move the holde

Dry the slides in air, placing the holder on a she

Change the solutions in the jars as follows:

Jar No.	
1	after 4 hours use
2	after 4 hours use, or after having stained
3	when coloured

2.5 "Dropper" procedure

2.5.1 Materials

Staining solution
Glass rods
Glass, plastic or metal tray
Pasteur pipettes and teats (or drop bottles)
Blotting paper
250-500 ml beaker

2.5.2 Staining technique

1. Fix 2 glass rods parallel to each other, about
2. Place the slides on the rods. With a Pasteur methanol and leave for 10 minutes to fix.
3. Pour off the methanol and with another pipett demineralized water for a few seconds.
4. Pour off the water and with a third pipette f eosin Y staining solution.
5. Leave to stain for 25 minutes for blood; 35
6. Pour off the stain and rinse each slide by ag beaker of demineralized water. Change the wa
7. Dry the slides in the air, placing them with sheet of blotting paper.

2.6 Rapid stain for emergency use

1. Fix the slide in methanol for 2-3 minutes. Then p a freshly made stain solution consisting of 1 volu HEPES buffer pH 6,6.
2. Stain for 3 minutes. Pour off stain, rinse in dem described in 2.5.2.

2.7 Malaria parasites

Malaria parasites, especially Schüffner's dots, are best stained at pH 7.2.

If the stain is to be used specifically for the detection and identification of malaria parasites, prepare the working solution of stain with HEPES buffer at pH 7.2. Adjust the buffer to this pH by adding an appropriate amount of sodium hydroxide 1 mol/l.

2.8 Coverglass

It is essential to be able to screen the smear under a low-power magnification before using an oil-immersion lens. Thus, when the slides are completely dry add a drop of immersion oil and cover the smeared area (including edges and tail) with a rectangular coverglass. The same coverglass can be used for successive slides by carefully removing it onto the next slide before using the oil immersion lens. For a permanent use a drop of neutral mountant instead of oil.

3. ROMANOWSKY STAIN EFFECT

There is a complex mechanism by which some components of a cell stain with a dye and other components fail to do so, although staining with other dyes. This phenomenon depends on differences in binding of dyes to chemical structures and interactions between the dye molecules. With Romanowsky dyes, the acidic groupings of the nucleic acids and the cell nuclei and cytoplasm determine their uptake of the basic dye azure B. Conversely, the presence of basic groupings on the haemoglobin molecule results in an affinity for acidic dyes and staining by eosin. The granules in the cytoplasm of neutrophil leucocytes are weakly stained by the azure complexes. Eosinophilic granules contain a spermine derivative with an alkaline grouping which stains strongly with the acidic component of the dye, whereas basophilic granules contain heparin which has an affinity for the basic component of the dye.

General scheme of colour reactions

Nuclear chromatin	purple
Nucleoli	light blue
Basophilic cytoplasm	blue
Basophilic granules	purple-black
Eosinophilic granules	red-orange
Neutrophilic granules	purple
Toxic granules	black
Platelet granules	purple
Haemoglobinized erythrocytes	pink-orange
Reticulocytes	grey-blue
Auer rods	purple
Doehle bodies	bright blue
Howell-Jolly bodies	purple

4. QUALITY ASSURANCE

4.1 Quality control

1. Regular checking of stained films by supervisor.
2. The exchange of slides with other laboratories.
3. Including, at intervals, slides from normal controls and where possible from patients with known abnormalities of red cells (e.g. hypochromasia, polychromasia) and leucocytes (e.g. toxic granulation of neutrophils).

4.1.2 Sources of Error

1. Fixation

Inadequate fixation may affect subsequent staining and morphological appearances. This is especially likely to occur if the methanol has absorbed water, e.g. from a humid atmosphere, or from nearby indoor plants. Films should be fixed as soon as possible after they have been completely air-dried. After being fixed they may be stored overnight before being stained. If staining is further delayed the films should be refixed before staining. Older preparations, even if stored for only a few days, may not stain as well as freshly made and fixed films, but they are usually satisfactory.

2. Stain response

If the cells stain too blue or too red the staining time and/or pH of the buffer require adjustment.

If the nuclei stain blue instead of purple this is due to precipitation of eosin Y by azure B. If this occurs the staining solution must be replaced by a fresh solution.

4.2 Storage of azure B - eosin Y stock solution

The stock solution must be stored in the dark. If left exposed to light on the bench it will rapidly deteriorate. It will also deteriorate slowly with time.

If staining becomes unsatisfactory a new stock solution should be prepared; before adding the HEPES buffer, the pH of the buffer must be checked.

4.3 Film preparation

Smears which are too thick or too thin will affect the staining as well as distorting the morphological appearances.

4.4 Clean slides

If the slides are not free from dust, fat and finger smears, the staining may be affected. It is desirable to store the slides in a jar of alcohol; they should be removed and wiped with a lint-free cloth just prior to use.

4.5 Drying of stained films

The films must be dried in the air, if possible for at least 60 minutes, and this process can be assisted by exposing them to a current of air produced by a fan. The slides must not be dried by heating. Excess moisture can be eliminated by blotting the reverse side of the slides but the smears themselves must not be blotted.

APPENDIX

SPECIFICATIONS FOR AZURE B AND EOSIN Y

The quality of dyes used should comply with the physico-chemical characteristics which guarantee sufficient chemical purity as defined by the International Committee for Standardization in Haematology (ICSH).

1. Azure B (colour index no. 52010)

This specific dye must be the major fraction. It should comprise not less than 95% of the total dye content, as demonstrated by a chromatographic procedure and by spectrophotometry. The molar extinction E of azure B measured at the absorption peak of 642 nm is 85000 - 94000. This dye is produced in the form of several salts, i.e. thiocyanate, perchlorate, tetrafluroborate.[1]

The molecular weight of the dye cation is 270.2.

2. Eosin Y (colour index no. 45380)

This dye must be the major fraction and it should comprise not less than 90% of the total dye content as demonstrated by a chromatographic procedure and by spectrophotometry. The molar extinction E of eosin Y measured at the absorption peak of 517 nm is 85000 - 105000. This dye is produced as a disodium salt and an eosinic acid.[2] The eosinic acid is the recommended form.

The molecular weight of the disodium salt is 647.9.

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[1] Available from various commercial sources, for example:
R. Heyl: Goerzallée 253, West Berlin, Federal Republic of Germany;
E. Merck: Frankfurter Strasse 250, D-6100 Darmstadt 1, Federal Republic of Germany;
B.D.H. Diagnostics Limited: Broom Road, Poole, Dorset BH12 4NN, United Kingdom;
Aldrich Chemical Corporation: 940 West St Paul Avenue, PO Box 355, Milwaukie, Wisconsin 53201, United States of America.

[2] Available from various commercial sources, for example:
Koch Light Laboratories: 37 Hollands Road, Haverhill, Suffolk CB9 8PU, United Kingdom;
E. Merck: Frankfurter Strasse 250, D-6100 Darmstadt 1, Federal Republic of Germany;
B.D.H. Diagnostics Limited: Broom Road, Poole, Dorset, BH12 4NN, United Kingdom;
Aldrich Chemical Corporation: 940 West St Paul Avenue, PO Box 355, Milwaukie, Wisconsin 53201, United States of America.

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