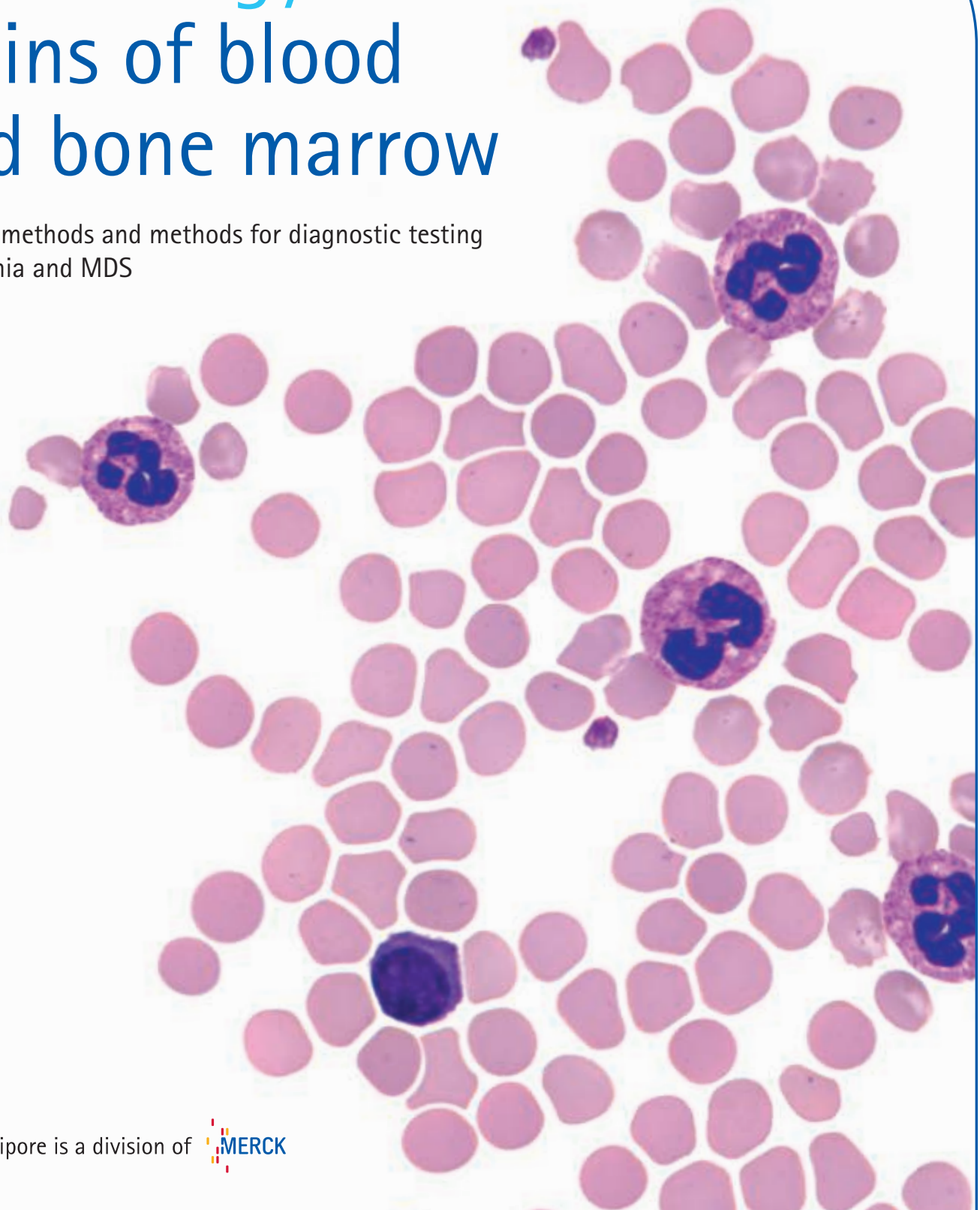
Hematology
stains of blood and bone marrow

Panoptic methods and methods for diagnostic testing of leukemia and MDS



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Content

Introduction

"Blood is a very special kind of liquid," says Mephistopheles in Goethe's "Faust" in 1808. Blood is a highly specialized fluid, a liquid organ, that is available in the whole body and will get fresh cell material from bone marrow. The specific functions of blood and bone marrow have brought these matters to the center of medical attention a long time ago. Tests in hematology are used with staining methods for a long time now. Changes in blood and bone marrow can give patients severe health issues. Blood and bone marrow stainings are proper and well-known methods for the diagnostic of hematological diseases. Nowadays flow cytometry or immunohistochemical methods, and molecular biological methods are used for research and become more and more routine use. Traditional staining methods are of interest in combination with modern methods for approval and confirmation of results.

CE certification of IVD's

The European In Vitro Medical Devices Directive (98/79/EC) of 20th October 1998 is the legislative instrument on which local IVD legislation within the EU Member States is based. It has been mandatory since December 2003 for all In Vitro Medical Devices (IDV's) that are placed on the market in the EU by manufacturers to be CE certified and bear the CE marking on product labels, instructions for use and sales packaging. All IVD's placed on the market by suppliers and used as IVD's must bear the CE marking as from December 2005.

The term In Vitro Diagnostic Devices means any medical device which is a reagent, reagent product, calibrator, control material, kit, instrument, apparatus, equipment or system, whether used alone or in combination, intended by the manufacturer to be used in vitro for the examination of specimens, including blood and tissue donations, derived from the human body, solely or principally for the purpose of providing information:

- concerning a physiological or pathological state, or
- concerning a congenital abnormality, or
- to determine the safety and compatibility with potential recipients, or
- to monitor therapeutic measures.

The Directive groups IVD's into categories as follows:

Class 2	IVD's in Annex II List A (which includes test kits for HIV and some blood grouping products) and List B (which includes test kits for various antibodies and viruses and test kits for blood glucose).
Class 1a	IVD's for self testing, other than those in Class 2
Class 1	all IVD's other than those covered by Annex II and IVDs for self testing

Merck Millipore's Products for Microscopy belong to Class 1.

CE certification is a self-certification process in which product details are submitted according to the valid EDMA (European Diagnostic Manufacturers Association) code based on the – in Germany – VDGH (Verband der Diagnostika-Industrie e.V.) classification.

Merck Millipore successfully completed the CE certification process in December 2003 for all Products for Microscopy at the time that were used as IVD's and belonged to the low-risk group. New products are developed, manufactured, quality-tested and documented in accordance with the regulations, and are registered with the Competent Authority before being placed on the market. The CE certification process also covers the conducting of regular in-house and external quality management system audits in accordance with ISO EN 13485.

Panoptic methods

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Panoptic methods

The combination of May-Grünwald's and Giemsa's methods for blood smears is described as panoptic staining and was developed in 1912. Nuclei are stained reddish purple, plasma of lymphocytes and monocytes bluish, and plasma of the granulocytes light pink. Panoptic staining can be used for sections and for staining of Spirochetes. Beside the panoptic method, there are other standard staining methods, which will be presented here as well.

The typical purple color of cell nuclei, is due to molecular interaction between eosin Y and an azure B-DNA complex. Both dyes build up the complex. The intensity of the 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 the pH of the solutions and buffer solution, buffer substances, fixation, and staining time.

The standard hematological staining solution and dye mixtures all contain eosin Y and a mixture of methylene blue and the oxidation products. The method characteristical composition makes the difference in the staining result. The fact is that all available methods will visualize the sample material in a comparable style. The different methods are suitable for all types of blood and bone marrow samples. It is more the experience or the tradition which gives the focus to one specific method.

Sample material

Fresh and if possible native sample material should be used for the preparation of blood and bone marrow smears. That is the starting material for all types of staining. The use of anticoagulant as EDTA should be reduced to a minimum. Anticoagulants can reduce the stainability of blood and bone marrow samples and it could be critical especially when the material is used for enzyme cytochemical methods. Blood and bone marrow smears must be dried in the air for at least 30 min and fixed then with the relevant fixative and according to the instruction. Hematological staining methods are applicable for clinical specimens in cytology as well. Specimen as urine sediment, sputum, fine needle aspiration biopsies (FNAB), imprints, lavages can be processed with these methods in a very good way and belong to the standard application.

Fixation

Methanol is the standard fixative for blood and bone marrow samples. Methanol is a solvent with a long tradition as fixative in hematology. It reacts fast and is inert e.g. no changes of fine structures will be recognized when the fixation is done with sufficient air dried material. The used methanol should have a concentration of 100 %. A sufficient quality grade should be used to prevent problems with the stainability and the quality of the sample material. Methanol is the gold standard as hematological fixative but it is under discussion because of the strong hazardous classification of the solvent. Methanol has to be used in well ventilated and air-conditioned working places. Safety protection for the staff and the working place must be organized according to the safety instructions. The valid safety data sheet is available in the internet under: **www.merckmillipore.com**

Physicochemical properties of methanol

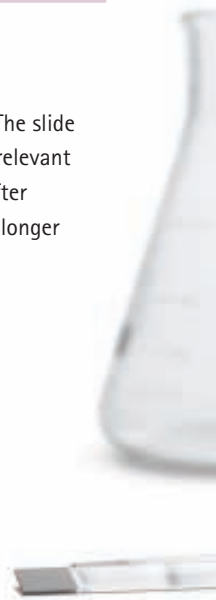
Chemical and physical data	
Ignition temperature	455°C (DIN 51794)
Solubility in water	(20°C) soluble
Melting point	-98°C
Molar mass	32.04 g/mol
Density (20°C)	0.7902 g/cm^3
pH	(H_2O) no data available
Boiling point	64.5°C (1013 hPa)
Vapor pressure	128 hPa (20°C)
Explosive limits	5.5 – 44 % (V)
Flash point	10°C
Refractive index	1.33

Ordering information

Product	Package size	Cat. No.
Methanol	1 L	**1.06009.1000**
for analysis EMSURE®	2.5 L	**1.06009.2500**
ACS, ISO, Reag. Ph Eur		

Method

Fix the air dried slides for 3 min in methanol. The slide can be used directly after the fixation for the relevant staining or the slide can be stored for hours after the fixation. Material which have to be stored longer should placed in a refrigerator.



Water quality for wash, rinse and dilution steps

Especially hematological staining methods react very sensitive and visible of changes in the quality and pH range of used rinsing solution. **Tap water or distilled water** is used normally for wash/rinse steps and for dilution of the staining solutions. Tap water has a pH of around 7 and distilled water has a pH < 7, distilled water which is stored for a while react more acid, the pH is lower. **Acid water** – Eosin Y, an acid dye, reacts more intense when the used water has an acid pH. The staining result is more reddish. **Alkaline water** – methylene blue and the oxidation products give a reinforcement and more intense azure metachromasy and simultaneously an extenuation of the eosin effect, the bluish-grey shade prevails.

Weise phosphate buffer solution

The pH of the water should be in the range of 6.8 to 7.2. Optimal and reproducible staining results are accomplished by the use of buffered solution. The phosphate buffer tablets according to Weise are especially prepared for the buffering of water for wash, rinse and dilution steps in hematological staining methods. According to the desired staining result, the pH of the used buffer solution is to choose. In the portfolio are offered buffer tablets according to Weise with pH 7.2 and 6.8. We have an extra available – buffer tablet according to Weise with pH 6.4 is used for sample material where a bright orange staining of erythrocytes is required.

Preparation of buffer solution

Dissolve 1 buffer tablet in 1 L distilled water. The tablets are very solid pressed, to keep the content and the buffer strength at the end stable. It take some time to dissolve the tablet in the distilled water. The buffer solution is stable for 4 weeks and should be replaced by a fresh buffer solution after that time.

Ordering information

Product	Package size	Cat. No.
Buffer tablets acc. to Weise pH 7.2	1 pack (100 tablets)	**1.09468.0100**
Buffer tablets acc. to Weise pH 6.8	1 pack (100 tablets)	**1.11374.0100**
Buffer tablets acc. to Weise pH 6.4	1 pack (100 tablets)	**1.11373.0100**

Panoptic methods

Standard staining solutions and dry dye mixtures for differential blood and bone marrow smears

May-Grünwald's eosin methylene blue solution

Preparation

1. **Buffer solution**
   Dissolve 1 buffer tablet in 1 L distilled water.

2. **Dilute May-Grünwald's solution for manual staining**
   Dilute 30 mL May-Grünwald's eosin methylene blue solution with 150 mL distilled water and add 20 mL buffer solution.

3. **Dilute May-Grünwald's solution for staining with staining automat**
   Slowly add 30 mL buffer solution and 220 mL distilled water to 50 mL May-Grünwald's eosin methylene blue solution, mix and leave to stand for 10 min.

4. **May-Grünwald's eosin methylene blue solution**
   Dissolve 0.25 g May-Grünwald's eosin methylene blue in 100 mL methanol while warming gently on a water bath at 60°C. Stir for 1 h, leave to stand for 24 h and filter.

5. **Dilute Giemsa's solution for manual staining**
   Dilute 10 mL Giemsa's azure eosin methylene blue solution with 190 mL buffer solution, mix well, leave to stand for 10 min, and filter if necessary.

Procedure

Air-dried smears, fixed in methanol

Staining rack

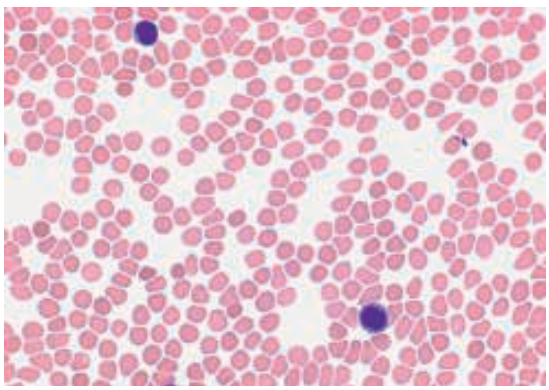
Reagents	Time
May-Grünwald's solution	3 min
Buffer solution (1 mL) add, mix, stain	6 min
Rinse with buffer solution	1 min
Dry	

Staining jar

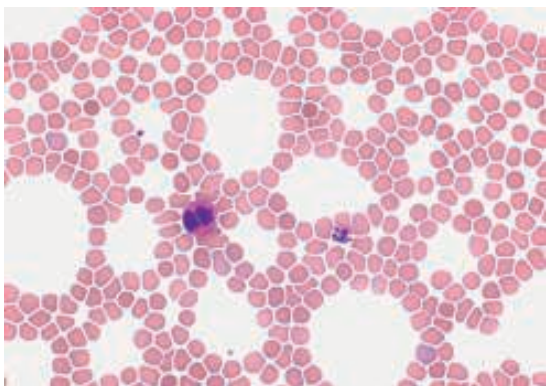
Reagents	Time
May-Grünwald's solution	3 min
Dilute May-Grünwald's solution	6 min
Rinse with buffer solution	2 x 1 min
Dry	

Staining with staining automat

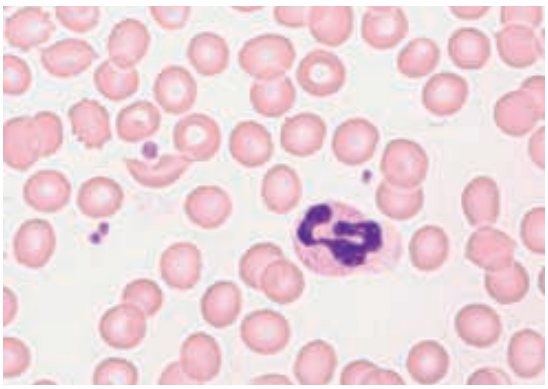
Reagents	Time
May-Grünwald's solution	3 min
Dilute May-Grünwald's solution	6 min
Buffer solution	1 min
Running water (rinse)	2 min
Dry	3 min



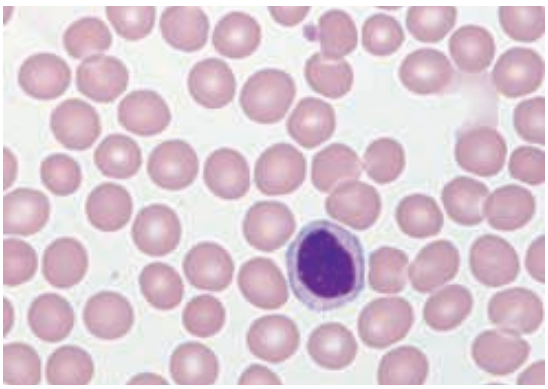
Blood smear, May-Grünwald's stain



Blood smear, May-Grünwald's stain



Blood smear, Pappenheim's stain – pH 6.8



Blood smear, Pappenheim's stain – pH 7.2

Pappenheim's staining: Staining with May-Grünwald's solution and Giemsa's solution

Staining of blood and bone marrow smears and clinical-cytological specimens

Staining rack

Reagents	Time
Cover the smear with 1 mL May-Grünwald's-solution	3 min
Add 1 mL buffer solution, mix and stain	3 – 5 min
Cover with dilute Giemsa's solution, stain	15 – 20 min
Rinse with buffer solution	1 min
Dry	

Staining jar

Reagents	Time
May-Grünwald's solution	3 – 5 min
Dilute Giemsa's solution	15 – 20 min
Rinse with buffer solution	2 x 1 min
Dry	

Results

Cell type	May-Grünwald's	Pappenheim's
Nuclei	red to violet	purple to violet
Lymphocytes	plasma blue	plasma blue
Monocytes	plasma dove-blue	plasma dove-blue
Neutrophilic granulocytes	granules light violet	granules light violet
Eosinophilic granulocytes	granules brick-red to red-brown	granules brick-red to red-brown
Basophilic granulocytes	granules dark violet to black	granules dark violet to black
Thrombocytes	violet	violet
Erythrocytes	reddish	reddish

Ordering information

Product	Package size	Cat. No.
May-Grünwald's eosin methylene blue solution	100 mL	**1.01424.0100**
	500 mL	**1.01424.0500**
	1 L	**1.01424.1000**
	2.5 L	**1.01424.2500**
May-Grünwald's eosin methylene blue	25 g	**1.01352.0025**
	100 g	**1.01352.0100**
Giemsa's azure eosin methylene blue solution	100 mL	**1.09204.0100**
	500 mL	**1.09204.0500**
	1 L	**1.09204.1000**
	2.5 L	**1.09204.2500**

Giemsa's azure eosin methylene blue solution

Staining of blood and bone marrow smears, paraffin sections and clinical-cytological specimens

Preparation

1. **Buffer solution**
   Dissolve 1 buffer tablet in 1 L distilled water.

2. **Dilute Giemsa's solution for manual staining**
   Dilute 10 mL Giemsa's azure eosin methylene blue solution with 190 mL buffer solution, mix well, leave to stand for 10 min, and filter if necessary.

3. **Dilute Giemsa's solution for staining with staining automat**
   Slowly add 25 mL Giemsa's solution to 275 mL buffer solution, mix and leave to stand for 10 min, and filter if necessary.

4. **Giemsa's azure eosin methylene blue solution**
   Dissolve 0.76 g Giemsa's azure eosin methylene blue in 50 mL glycerol and heat for 3 h at 60°C on a water bath, add 50 mL methanol, leave to stand for 5 days and filter.

Procedure

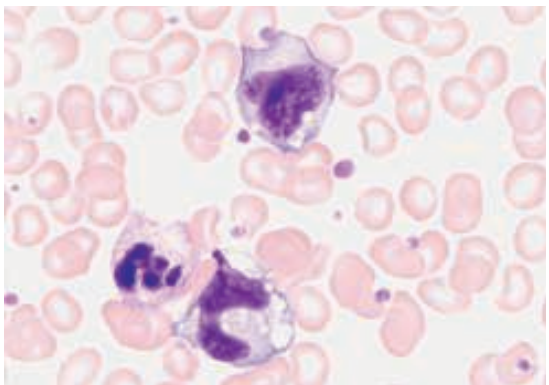
Air-dried smears

Staining rack/Staining jar

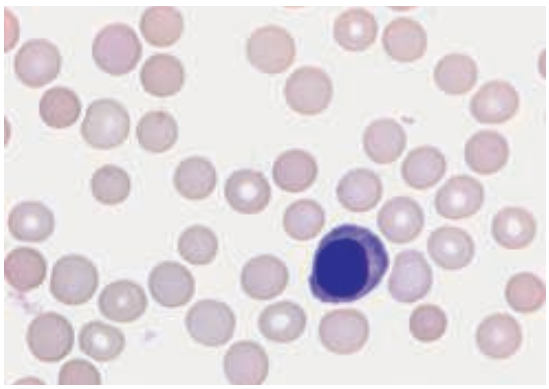
Reagents	Time
Methanol	3 – 5 min
Dilute Giemsa's solution	15 – 20 min
Rinse with buffer solution	2 x 1 min
Dry	

Staining with staining automat

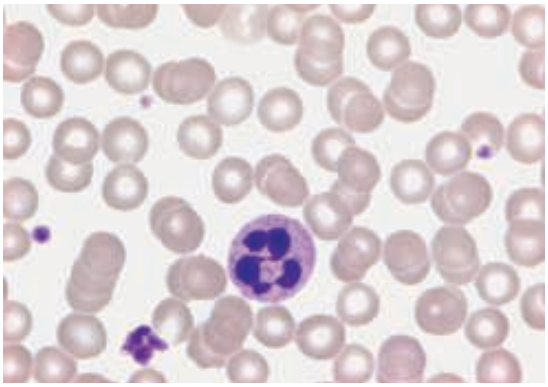
Reagents	Time
Methanol	3 min
Dilute Giemsa's solution	15 – 20 min
Buffer solution	1 min
Running water (rinse)	2 min
Dry	3 min



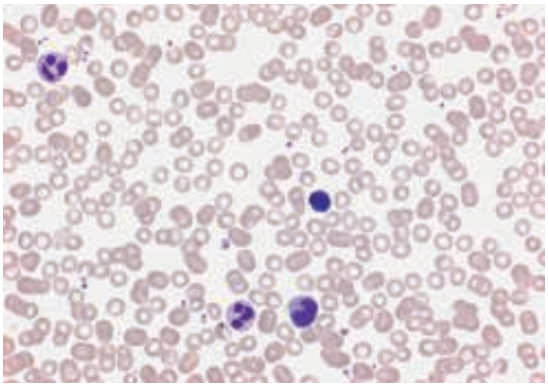
Blood smear, Giemsa's stain



Blood smear, Giemsa's stain



Blood smear, Giemsa's stain



Blood smear, Giemsa's stain

Pappenheim's staining – Staining with May-Grünwald's solution and Giemsa's solution

Staining rack and staining jar: see May-Grünwald's solution

Staining with staining automat

Reagents	Time
May-Grünwald's solution	4 min
Dilute Giemsa's solution	20 min
Rinse with buffer solution	1 min
Running water (rinse)	2 min
Dry	3 min

Results with phosphate buffer acc. to Weise pH 6.8

Cell type	Giemsa's staining	Pappenheim's staining
Nuclei	red to violet	purple to violet
Lymphocytes	plasma blue	plasma blue
Monocytes	plasma dove-blue	plasma dove-blue
Neutrophilic granulocytes	granules light violet	granules light violet
Eosinophilic granulocytes	granules red to gray-blue	granules brick-red to dark violet
Basophilic granulocytes	granules dark-violet	granules dark violet to black
Thrombocytes	violet	violet
Erythrocytes	reddish	reddish
Blood parasites	nuclei bright red	

Panoptic methods
Staining – Giemsa's azure eosin methylene blue

Giemsa's staining of paraffin sections of bone marrow or other suitable sample material

Step	Time
Deparaffinate and rehydrate the sections using standard methods	
Distilled water	10 sec.
Undiluted, filtered Giemsa's azur eosin methylene blue solution	15 min
0.1 % acetic acid	10 sec.
Distilled water	10 sec.
2-propanol	10 sec.
2-propanol	10 sec.
2-propanol	10 sec.
Xylene or Neo-Clear®	5 min
Xylene or Neo-Clear®	5 min
Use Entellan® new to cover the preparations moistened with xylene and use Neo-Mount® to cover those moistened with Neo-Clear®	

Notes on Giemsa's staining of paraffin sections

Always employ separate xylene or Neo-Clear® rinse baths when Giemsa's staining paraffin sections as any ethanol traces in the solutions may result in the preparations being discolored.

Pretreatment of bone marrow and iliac crest biopsy materials:

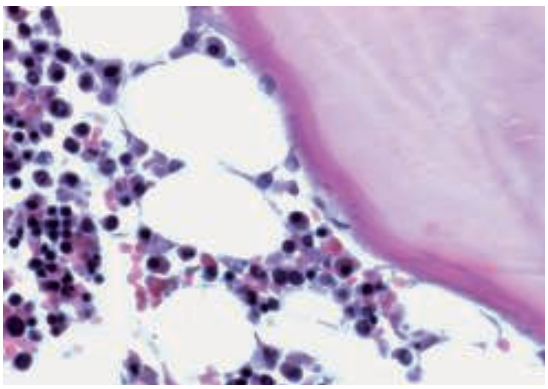
Optimal results can be achieved using a mild OSTEOSOFT® decalcifying solution. To gently remove any calcification, the fixed biopsy materials are first placed in OSTEOSOFT® for 6 hours, after which they are transferred to histoprocessing. Smaller blocks are carefully cut and, if required, are again treated with OSTEOSOFT® for an additional 20 minutes.

Results

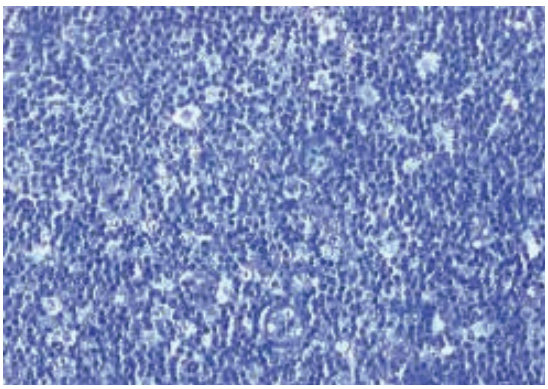
Cell type	Color
Cell nuclei, cells	blue, dark blue
Collagen, osteoid	pale blue
Eosinophilic grains	red
Acidophilic mucopolysaccharides, mastocytes, cartilage matrix	reddish violet
Acidophilic materials	orange red

Ordering information

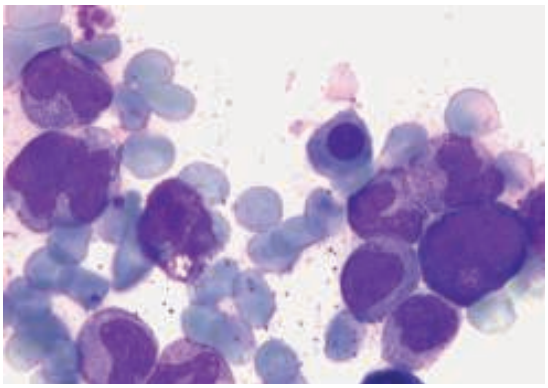
Product	Package size	Cat. No.
Giemsa's azure eosin methylene blue solution	100 mL 500 mL 1 L 2.5 L	**1.09204.0100** **1.09204.0500** **1.09204.1000** **1.09204.2500**
Giemsa's azure eosin methylene blue	25 g 100 g	**1.09203.0025** **1.09203.0100**
May-Grünwald's eosin methylene blue solution	100 mL 500 mL 1 L 2.5 L	**1.01424.0100** **1.01424.0500** **1.01424.1000** **1.01424.2500**
Methanol for analysis EMSURE® ACS, ISO, Reag. Ph Eur	1 L 2.5 L	**1.06009.1000** **1.06009.2500**
Glycerol 85 % suitable for use as excipient EMPROVE® exp Ph Eur, BP	1 L 2.5 L	**1.04091.1000** **1.04091.2500**



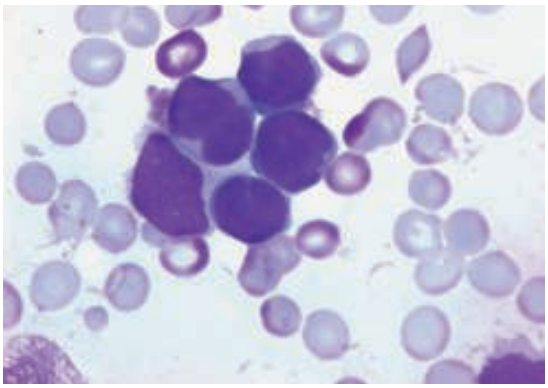
Bone marrow section, Giemsa's stain



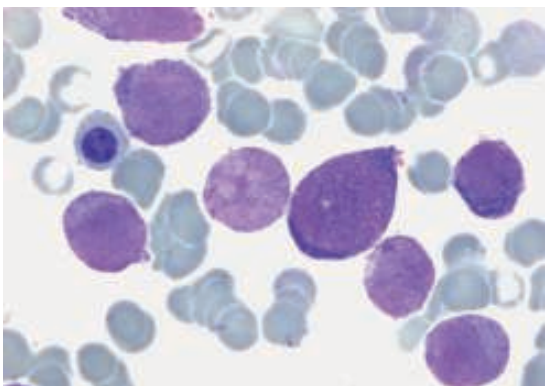
Lymph node section, Giemsa's stain



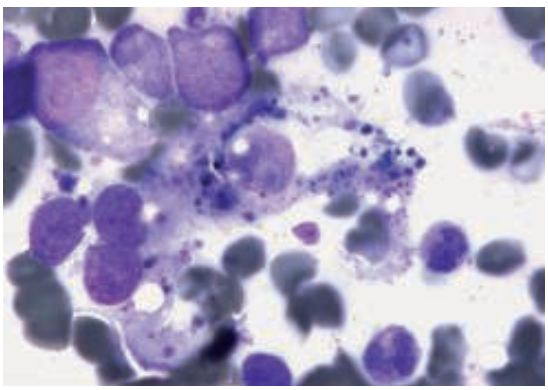
Blood smear, Pappenheim's stain



Blood smear, Pappenheim's stain



Blood smear, Pappenheim's stain



Blood smear, Pappenheim's stain

Wright's eosin methylene blue solution

Staining of blood and bone marrow smears and clinical-cytological specimens

Preparation

1. **Buffer solution**
   Dissolve 1 buffer tablet in 1 L distilled water.

2. **Dilute Wright's solution for manual staining**
   Add 20 mL buffer solution and 150 mL distilled water to 30 mL Wright's eosin methylene blue solution.

3. **Dilute Wright's solution for staining with automated staining**
   Add 30 mL buffer solution and 220 mL distilled water to 50 mL Wright's eosin methylene blue solution.

4. **Wright's eosin methylene blue solution**
   Dissolve 0.25 g Wright's eosin methylene blue in 100 mL methanol, warm gently on a water bath for 20 – 30 min or until the powder is dissolved, filter before use.

Procedure

Air-dried smears, fixed in methanol

Staining rack

Reagents	Time
Wright's solution	1 min
Buffer solution (1 mL) add, mix, stain	4 min
Rinse with buffer solution	1 min
Dry	

Staining jar

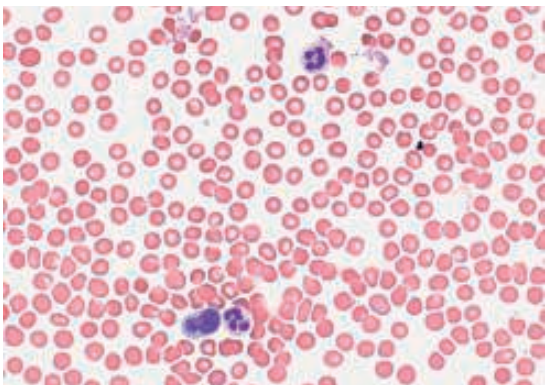
Reagents	Time
Wright's solution	3 min
Dilute Wright's solution	6 min
Rinse with buffer solution	2 x 1 min
Dry	

Staining with staining automat

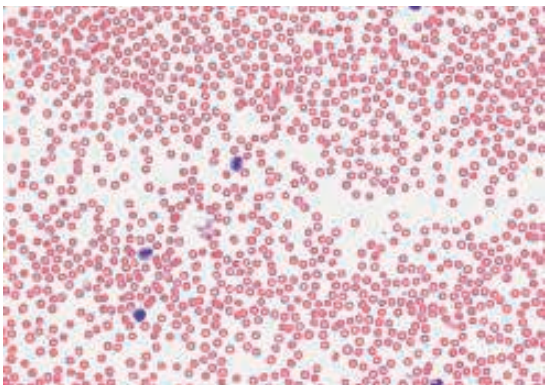
Reagents	Time
Wright's solution	3 min
Dilute Wright's solution	6 min
Buffer solution	1 min
Running water (rinse)	2 min
Dry	3 min

Result

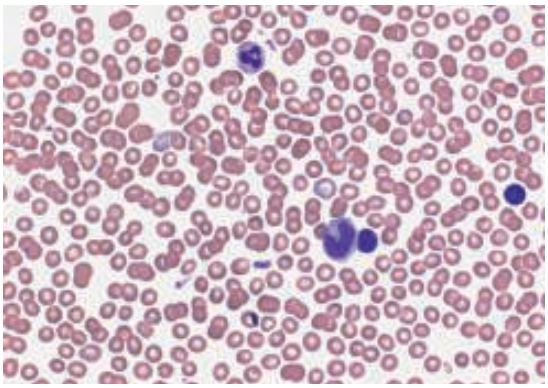
Cell type	Color
Nuclei	red to violet
Lymphocytes	plasma blue
Monocytes	plasma gray-blue
Neutrophilic granulocytes	granules light violet
Eosinophilic granulocytes	granules brick-red to red-brown
Basophilic granulocytes	granules dark violet to black
Thrombocytes	violet
Erythrocytes	reddish



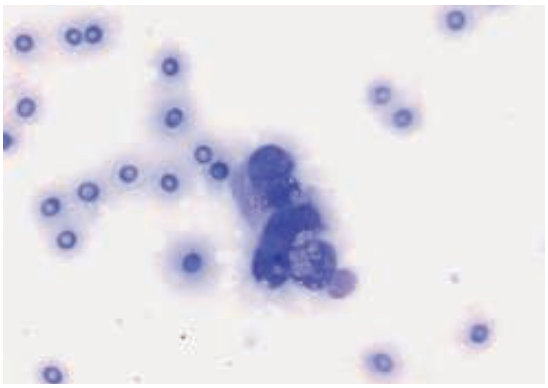
Blood smear, Wright's stain



Blood smear, Wright's stain



Blood smear, Wright's stain



FNAB (Douglas), Wright's stain

Ordering information

Product	Package size	Cat. No.
Wright's eosin methylene blue solution	100 mL	**1.01383.0100**
	500 mL	**1.01383.0500**
	2.5 L	**1.01383.2500**
Wright's eosin methylene blue	25 g	**1.09278.0025**

Leishman's eosin methylene blue solution

Staining of blood and bone marrow smears and clinical-cytological specimen

Preparation

1. **Buffer solution**
   Dissolve 1 buffer tablet in 1 L distilled water.

2. **Dilute Leishman's solution for manual staining**
   Dilute 30 mL Leishman's eosin methylene blue solution with 150 mL distilled water and add 20 mL buffer solution.

3. **Dilute Leishman's solution for staining with an automated stainer**
   Slowly add 30 mL buffer solution and 220 mL distilled water to 50 mL Leishman's eosin methylene blue solution, mix and leave to stand for 10 min.

4. **Leishman's eosin methylene blue solution**
   Dissolve 0.12 g Leishman's eosin methylene blue in 100 mL methanol while warming gently on a water bath at 40°C, leave 5 days to mature, and filter.

Procedure

Air-dried smears, fixed with methanol

Staining rack

Reagents	Time
Leishman's solution	1 min
Buffer solution (2 mL) add, mix, stain	5 min
Rinse with buffer solution	1 min
Dry	

Staining jar

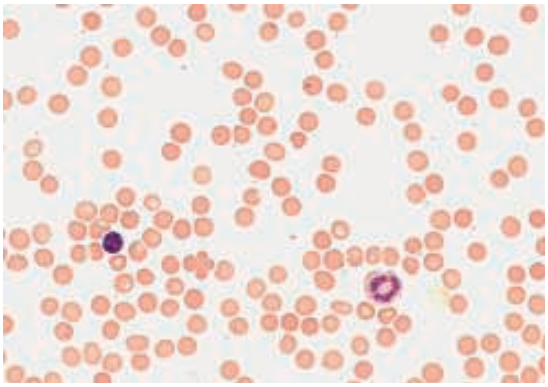
Reagents	Time
Leishman's solution	3 min
Dilute Leishman's solution	6 min
Rinse with buffer solution	2 x 1 min
Dry	

Staining with staining automat

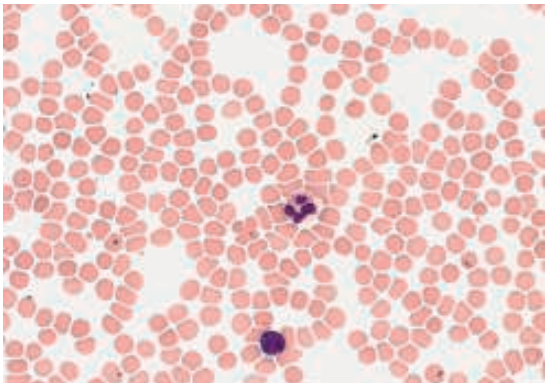
Reagents	Time
Leishman's solution	3 min
Dilute Leishman's solution	6 min
Buffer solution	1 min
Running water (rinse)	2 min
Dry	3 min

Result

Cell type	Color
Nuclei	red to violet
Lymphocytes	plasma blue
Monocytes	plasma gray-blue
Neutrophilic granulocytes	granules light violet
Eosinophilic granulocytes	granules brick-red to red-brown
Basophilic granulocytes	granules dark-violet
Thrombocytes	violet
Erythrocytes	reddish



Blood smear, Leishman's stain



Blood smear, Leishman's stain

Ordering information

Product	Package size	Cat. No.
Leishman's eosin methylene blue solution	500 mL	**1.05387.0500**
Leishman's eosin methylene blue	10 g	**1.01350.0010**

Hemacolor® – Staining set for hematological and clinical specimens

Hemacolor® is a fast staining kit which allows in less then 1 min a staining where the result is brilliant and comparable to a Pappenheim's staining. Hemacolor® contains a fixing solution (methanol), a red (eosin Y) and a blue (methylene blue and azure) staining solution and buffer tablets according to Weise pH 7.2. The fact that the staining solutions are separately applied has the advantage that no dye precipitates occur, which are available in standard hematological staining methods normally. The addition of buffer tablets ensure that the stain is very stable and highly reproducible.

The staining solutions are very stable in use. The buffer solution should be replaced and refreshed after 4 weeks. The slides must be moved in the solution because of the short staining/reaction time it is therefore a requirement to get intense and reproducible results.

Sample material

- Air-dried blood and bone marrow smears
- Clinical specimens in cytology, e.g. urine sediment, sputum, FNAB, imprints, lavages

Preparation

Dissolve 1 buffer tablet in 1 L distilled water.

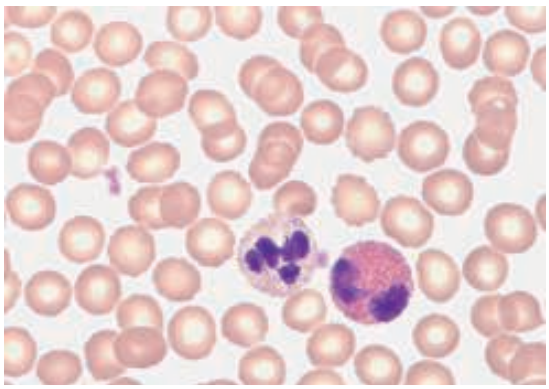
Procedure

Staining in jars

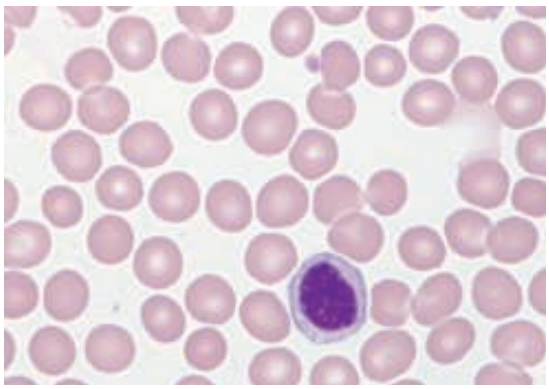
Reagents	Time
Hemacolor® solution 1	5 x 1 sec.
Hemacolor® solution 2	3 x 1 sec.
Hemacolor® solution 3	6 x 1 sec.
Buffer solution pH 7.2	2 x 10 sec.
Dry	

Staining with staining automat

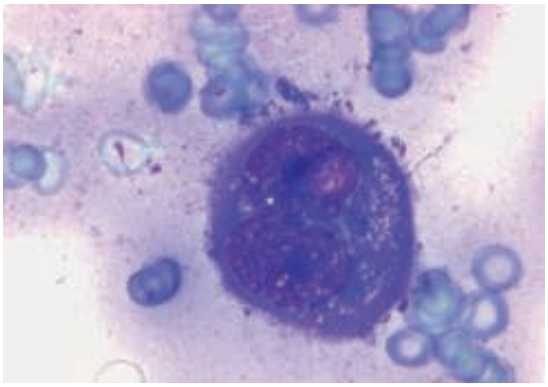
Reagents	Time
Hemacolor® solution 1	30 sec.
Hemacolor® solution 2	6 sec.
Hemacolor® solution 3	4 sec.
Buffer solution pH 7.2	10 sec.
Running water (rinse)	10 sec.
Dry	3 min



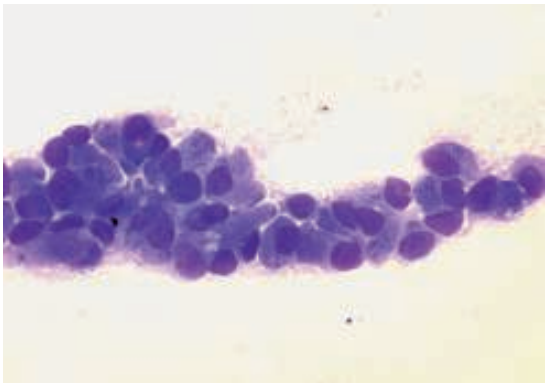
Blood smear, Hemacolor® stain



Blood smear, Hemacolor® stain



Body effusion, Hemacolor® stain



Tumor imprint, Hemacolor® stain

Result

Cell type	Color
Nuclei	red to violet
Lymphocytes	plasma blue
Monocytes	plasma dove-blue
Neutrophilic granulocytes	granules light violet
Eosinophilic granulocytes	granules brick-red to red-brown
Basophilic granulocytes	granules dark violet to black
Thrombocytes	violet
Erythrocytes	reddish

Ordering information

Staining sets	Package size	Cat. No.
Hemacolor® staining set	1 pack	**1.11674.0001**
Kit content:		
- Hemacolor® solutions	(3 x 100 mL)	
- Buffer tablets, pH 7.2	(3 tabs)	
Hemacolor® staining set	1 pack	**1.11661.0001**
Kit content:		
- Hemacolor® solutions	(3 x 500 mL)	
- Buffer tablets, pH 7.2	(6 tabs)	
Single reagents		
Hemacolor® solution 1, fixing solution	2.5 L	**1.11955.2500**
Hemacolor® solution 2, color reagent red	2.5 L	**1.11956.2500**
Hemacolor® solution 3, color reagent blue	2.5 L	**1.11957.2500**
Buffer tablets pH 7.2 acc. to Weise	1 pack (100 tablets)	**1.09468.0100**

Auto-Hemacolor® – Staining set for automatic blood smear staining with the HEMA-TEK* slide stainer

** = HEMA-TEK® 2000 slide stainer (Siemens Diagnostics) must be used. Follow the manufacturer's instructions for installation and service.*

Fixation, staining and rinsing of blood smears can be accomplished with these solutions. The methanolic dye solution also fixes the smears. Staining is achieved by the alkaline dye (azure) binding to the acidic builders of the cells, e.g. chromatin, spongioplasm, and the acidic dye (eosin) binding to the alkaline constituents, e.g. cytoplasm.

Sample material

Air-dried blood and bone marrow smears.

Preparation

The staining procedure is prepared by placing the Auto-Hemacolor® staining set (with folding carton) into the automatic staining system.

Insert the Auto-Hemacolor® staining set into the opening provided in the equipment. The pack must fit squarely. Remove the pre-punched cardboard sections of the carton. Insert the suction cannulas into the closures and push them firmly through as far as the protective ring seals on the suction cannulas. Allow the instrument to run in order to remove air bubbles.

Procedure

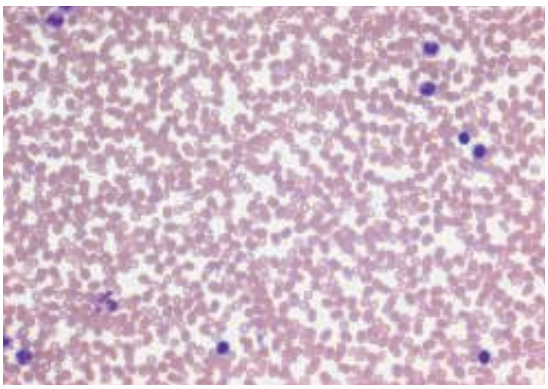
Place the glass slides with the material downwards to the staining strip.

Air-dried blood smears are transported in a fully automatic flow system (no immersion system) over the staining strip. Pass a carefully measured, fresh quantity of dye, buffer and rinse solutions, in this order, into the capillary space between the slides and the staining strip. The solutions are transferred to the staining strip by adjustable peristaltic pumps.

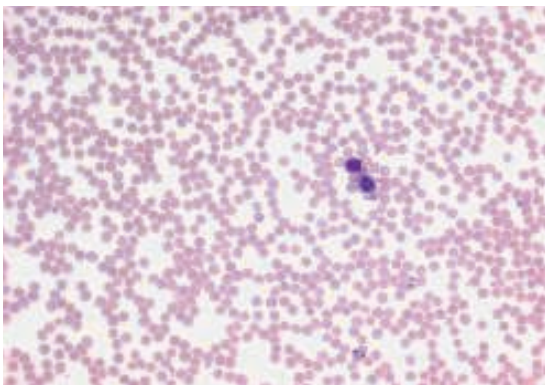
Technical note

If opened packs are not used for longer than 24 h, the puncture openings of the three reagent vessels must be tightly closed to avoid evaporation, which may result in changes in concentration and formation of precipitates. Remove the cannulas, and clean the cannulas and tubes by placing the cannulas in methanol or ethanol and allowing the equipment to run. Insert the clean and dry cannulas as far as the protective ring seals again, and leave them until the next staining; they usually close tightly.

An automatic sign on the equipment is given when the amount of solution left in the equipment is only sufficient for approx. 20 staining. The sign "stain" is extinguished.



Blood smear, Auto-Hemacolor® stain



Blood smear, Auto-Hemacolor® stain

Result

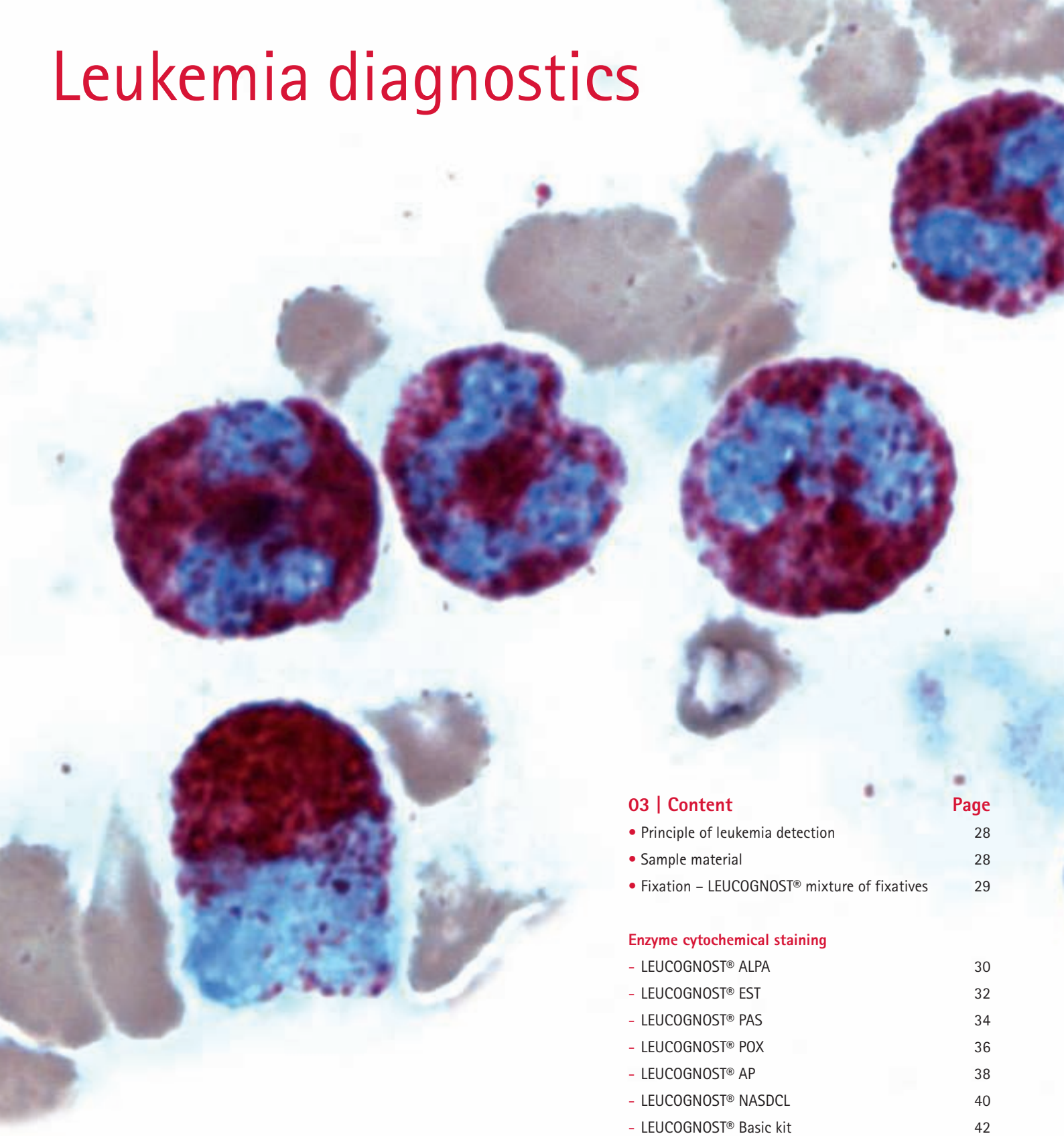
Auto-Hemacolor® gives a comparable staining result to Giemsa's staining.

Cell type	Color
Nuclei	violet
Lymphocytes	plasma blue-gray
Monocytes	plasma gray-blue
Neutrophilic granulocytes	granules red-violet
Eosinophilic granulocytes	granules red-brown
Basophilic granulocytes	granules dark-violet
Thrombocytes	violet
Erythrocytes	beige-brownish

Ordering information

Product	Package size	Cat. No.
Auto-Hemacolor®	1 pack	**1.15213.0001**
Kit content:		
- Staining solution	(200 mL)	
- Buffer solution, pH 7.0	(480 mL)	
- Rinse solution, pH 7.0	(950 mL)	

Leukemia diagnostics



03 | Content

03
Leukemia diagnostics

Enzyme cytochemistry – cytochemical staining serves to detect the location and activity of cellular substances and enzyme systems. In hematology, the PAS, peroxidase, unspecific and specific esterase and acid phosphatase reactions play a key role in the classification of leukemia. A strongly reduced alkaline phosphatase index is characteristic for chronic myeloid leukemia.

These fundamental chromogenic enzyme detection methods were usable in small as well as in big labs with the use of the LEUCOGNOST® staining kits. The LEUCOGNOST® staining kits assist in carrying out the semi-quantitative localization and activity detection of enzyme systems in the cytoplasm of leukemia cells of importance for differential diagnosis. Thus for instance the tedious weighing out of reagents on a microbalance is fundamentally avoided. The quantities of substances in all staining kits are such that all the cytochemical reactions can be carried out without complicated equipment using commercially available 60 mL jars.

In addition to the LEUCOGNOST® Kits with ready-to-use reagents for the determination of alkaline leukocyte phosphatase activity as well PAS, peroxidase, specific and unspecific esterase, and acid phosphatase reactions, all the basic reagents are available as ready-to-use stock solutions. All the reagents are subject to stringent quality criteria and undergo a cytochemical function test. Thought the availability of all the items of the same source and the accompanying charts for the evaluation of the result, it is possible to achieve a high degree of standardization in the methods.

Principle of leukemia differentiation

Leukemias are autonomous tumors of the hematopoietic system, mostly of the white blood cell series.
Leukemias are always diagnosed from blood and bone marrow smears panoptically stained according to a hematological standard method. While the recognition of mature leukemia of the chronic lymphatic leukemia type or a chronic myeloid leukemia type is usually unproblematic, cytological fine diagnosis within the group of hemoblastic immature-cell leukemia often causes considerable difficulty. Thus errors in differential diagnosis between acute lymphatic leukemia and acute myeloid leukemia without certain evidence of constant reliable morphological differential criteria such as Auer rods, primary granulation and maturation tendency are unavoidable.

To permit better checking of suspect differential therapeutic effects in the treatment of acute leukemia and to take full advantage of their use if indicated, standardized classification of the hematopoietic immature-cell neoplasias is essential. For over 4 decades, stem-line specific enzyme and substrate detections have been used with the aid of cytochemical stains in the cytoplasms of leukemic blasts. The immunological methods with specific fluorescence labelling of the blastic membranes contribute to a subclassification particularly within the group of acute lymphatic leukemias.

Sample material

Only fresh, native blood and bone marrow smears should be used as the starting material for all stains. The use of EDTA as an anticoagulant for example significantly reduces the peroxidase reaction. In cases where the addition of an anticoagulant is required should the amount reduced on a minimum.

The smears must be dried in the air for at least 30 min and fixed according to the relevant instruction prior to the actual cytochemical reaction.

Fixative for enzyme cytochemistry

LEUCOGNOST® fixing mixture is especially developed for the fixation of blood and bone marrow smears using the various LEUCOGNOST® kits. LEUCOGNOST® fixing mixture optimally protects enzyme activities, and the reaction times of the different working solutions are specially matched to the fixing mixture.

Only fresh, native blood and/or bone marrow smears should be used as the starting material for all stains. The use of e.g. EDTA as anticoagulant significantly reduces the peroxidase reaction. In any case it is quite unnecessary to add any anticoagulant substances.

The thin, air-dried blood and/or bone marrow smears should be stored for maximally 3 days prior to the procedure.

The smears must be dried in air for at least 30 minutes and fixed in LEUCOGNOST® fixing mixture according to the relevant instructions prior to the actual cytochemical reaction.

Procedure

Fix the air-dried blood and bone marrow smears in LEUCOGNOST® fixing mixture 1 – 3 min.

Air-dry and process immediately acc. to the protocol or store at +4 to +8°C until required.

Note: The staining protocols and especially the reaction time in the protocol are associated with the fixation of LEUCOGNOST® fixing mixture.

Ordering information

Product	Package size	Cat. No.
LEUCOGNOST® fixing mixture	500 mL	**1.12327.0500**

LEUCOGNOST® ALPA

Detection of the alkaline leukocyte phosphatase activity in leukocytes

The determination of the activity (index) of alkaline leukocyte phosphatase is suitable for the cytochemical differentiation of chronic myeloid leukemia from other diseases of the myeloproliferative type, particularly from myelofibrosis and polycythemia or other inflammatory or tumorous processes. Further, the index of alkaline leukocyte phosphatase represents a simple parameter for prognosis in CML, as it reflects the different phases of activity of the hematological disease.

Principle

Alkaline leucocyte phosphatase (AP) catalyzes the hydrolysis of phosphate esters in alkaline solution. 1-naphthol released from 1-naphthyl phosphate is coupled to a diazonium salt to form a brown azo dye, which is precipitated according to the locality and the AP activity in the cell.

Preparation of staining solution*

Solution A

- Dissolve 4 level measuring spoonful (enclosed = 1.1 g) of reagent 1 in 100 mL distilled water.

Solution B

- Wash the contents of one bottle of reagent 2 into the staining cuvette with 15 mL of solution A.

Solution C

- Wash the contents of one bottle of reagent 3 into a conical flask with 45 mL of solution A, shake vigorously for 2 minutes and filter into the staining cell containing solution B through a full- flow filter.

Mix solutions (A + B + C) well

- The reagent solution is stable for a maximum of 1 1/2 hours. The reagent solution is red brown and rapidly becomes turbid. The turbidity, however, does not influence the staining quality.

Procedure

Steps		Time
1.	Fix the air-dried blood and bone marrow smears in LEUCOGNOST® fixing mixture	1 – 3 min
2.	Wash under running tap water	10 sec.
3.	Air dry	
4.	Place in freshly prepared **staining solution***	10 – 15 min
5.	Rinse with distilled water and air dry	
6.	Stain with Mayer's hemalum solution	5 min
7.	Rinse with tap water	1 – 3 min
8.	Air dry and cover with Aquatex® and a cover glass	

Result

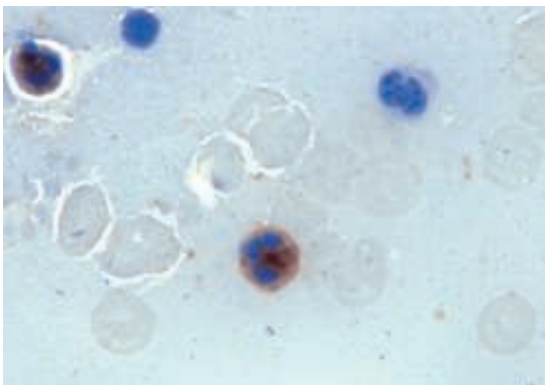
The brown reaction product is only present in the final mature stages of granulopoiesis. Assess 100 neutrophile ones with segmented nuclei; in the event of neutropenia at the most up to 10 % with rod nuclei. Count according to the degree of staining using the following 5 color intensity steps.

Color intensity steps	
0	no reaction
1	single to few granules
2	many granules localized
3	granules diffuse distributed
4	cell completely overcasted with granules
5	maximum number of granules, nucleus frequently no longer visible

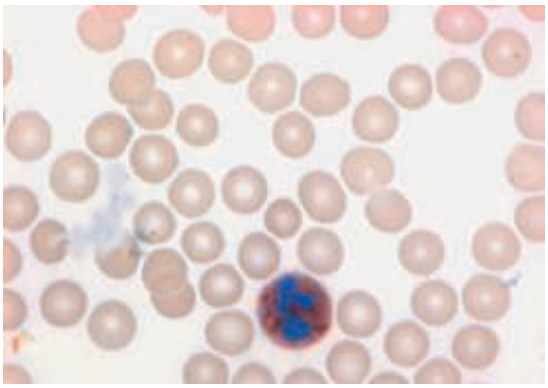
Multiply the percentages determined by the factors for the corresponding reaction classes and add the products to obtain the dimension less ALPA index.

Normal range: 10 to 100

A reduced index is pathognomonic for the active disease phase of chronic myeloid leukemia. Only hemolytic anemias, iron deficiency anemias or individual virus diseases produce comparable low index values. Normal and increased values always allow a number of interpretations, so that they are of no significance for differential diagnosis. Chronic myeloid leukemia in remission can also produce normal or even increased ALPA values. In general the index is higher, the more extensively necrobiotic catabolic processes (e.g. inflammatory tissue liquefaction) proceed in inflammatory or tumorous processes.



Blood smear, LEUCOGNOST® ALPA



Blood smear, LEUCOGNOST® ALPA

Ordering information

Product	Package size	Cat. No.
LEUCOGNOST® ALPA	1 pack (for 12 staining batches)	**1.16300.0002**

Kit content:

- Reagent 1: Tris(hydroxymethyl)-aminomethane
- Reagent 2: 1-naphthyl phosphate sodium salt
- Reagent 3: Variamin® blue salt B

LEUCOGNOST® EST

Detection of the alpha-naphthyl acetate esterase reaction in leukocytes

Esterase reactions with different substrates facilitate differentiation between myeloblastic and monoblastic leukemia. Apart from the naphthol AS-D chloroacetate esterase reaction, whose reliability is comparable with that of the peroxidase reaction, the 1-naphthyl acetate esterase reaction is the most suitable for identifying monoblastic types of leukemia.

1-Naphthyl acetate esterases accelerate the hydrolytic cleavage of 1-naphthyl acetate to form acetic acid and 1-naphthol, which couples with a diazonium salt to form a red brown azo dye which is insoluble in water.

Preparation of staining solution*

Solution A

- Dissolve 2 level measuring spoonful (enclosed, 0.8 g) of reagent 1 in 60 mL of distilled water.

Solution B

- Dissolve the contents of 1 bottle of reagent 2 in 2 mL acetone, add to 60 mL of **solution A** and shake vigorously for 1 minute.

Solution C

- Mix 4 – 5 drops (0.2 mL) of reagent 3 and reagent 4, respectively, in an empty bottle of reagent 2 and wait 1 minute (diazotization time).

Mix solutions B and C and filter through a full flow filter into the staining cell.

Note: The staining solution is stable for a maximum of 2 1/2 hours. The staining must be conducted within 15 minutes after preparing the reagent solution. The staining solutions must be freshly prepared immediately before each staining process.

Procedure

Steps		Time
1.	Fix the air dried blood and/or bone marrow smears in LEUCOGNOST® fixing mixture	1 – 3 min
2.	Wash with distilled water	1 min
3.	Place in freshly prepared **staining solution*** and incubate in the dark	1 – 2 h
4.	Wash with distilled water	10 sec.
5.	Stain with Mayer's hemalum solution	30 min
6.	Wash under tap water	2 min
7.	Air dry and cover with Aquatex® and a cover glass	

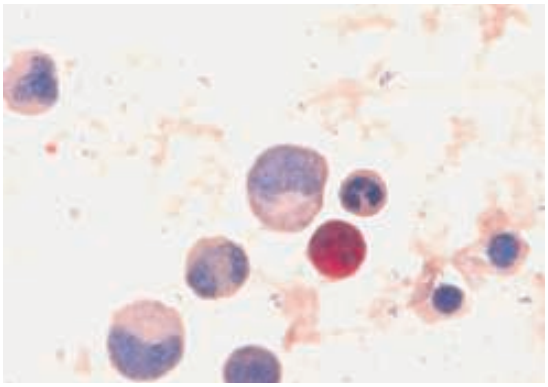
The stain is stable for about 5 days without embedding and for only a few hours when covered with immersion oil. The stability can be extended to several months with the use of embedding agent and a cover glass.

Result

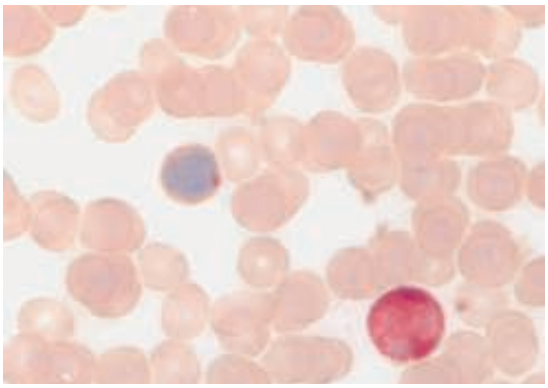
1-naphthyl acetate esterase reacts weakly in all hematopoietic cells. In particular monocytes, plasma cells, erythroblasts and megakaryocytes react more strongly. The red-brown granular color reaction in this kit is adjusted such that practically only leukemia monoblasts/monocytes with the highest reactivity become stained.

To classify acute leukemia, determine the percentage of esterase-positive blasts and taking into consideration simultaneous differently graded peroxidase reactions, place in one of the categories below:

Categories	
Peroxidase type	below 25 % EST-pos. blasts AML, AProL
POX-EST mixed type	25 % – 50 % EST-pos. blasts AMMoL
Esterase type	over 50 % EST-pos. blasts AmoL



Blood smear, LEUCOGNOST® EST



Blood smear, LEUCOGNOST® EST

Ordering information

Product	Package size	Cat. No.
LEUCOGNOST® EST	1 pack (for 12 staining batches)	**1.16301.0002**

Kit content:

- Reagent 1: Phosphate buffer
- Reagent 2: 1-naphthyl acetate
- Reagent 3: Pararosaniline-HCl solution
- Reagent 4: Nitrite solution

LEUCOGNOST® PAS

Detection of the periodic acid Schiff reaction in leukocytes

The PAS reaction is an important method for the identification of lymphatic cell elements. Next to peroxidase and esterase reactions, it is one of the three basic cytochemical staining methods important for differential diagnosis that are regularly carried out in acute cases of leukemia. Smears already stained by the Pappenheim's method can additionally be stained with PAS and the stains subsequently removed with 1 % periodic acid.

Periodic acid cleaves neighboring carbon-carbon bonds in polysaccharides (glycogen) when hydroxyl groups are attached to both carbon atoms. The alcoholic groups are then oxidized to aldehydes, which can subsequently be clearly revealed with Schiff's reagent (fuchsin sulfurous acid), producing a red stain.

Preparation of staining solutions*

Solution A

- Dissolve the contents of 1 bottle of reagent 1 in 60 mL of distilled water and transfer to a staining cuvette.

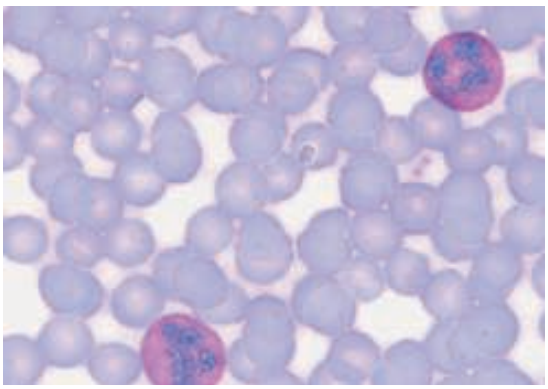
Solution B

- Dissolve the contents of 1 bottle of reagent 2 in 60 mL of distilled water, transfer to a staining cuvette, add 2 mL of reagent 3 and mix. All the reagent solutions are colorless and stable for 3 hours.

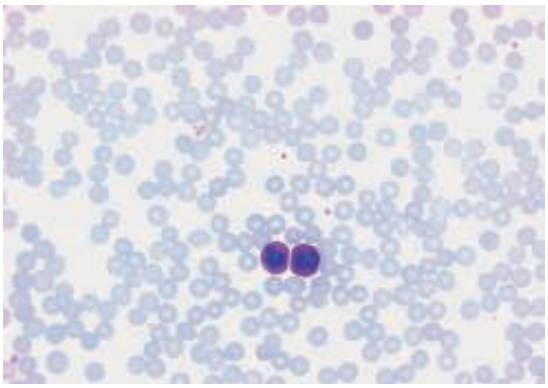
Procedure

Steps		Time
1.	Fix the air dried blood and bone marrow smears in LEUCOGNOST® fixing mixture	1 – 3 min
2.	Wash under running tap water	10 sec.
3.	Place in **solution A***	30 min
4.	Wash with distilled water	10 sec.
5.	Place in **solution B***	1 min
6.	Wash with distilled water	10 sec.
7.	Stain in Schiff's reagent (20 to 25°C, incubate in the dark	30 min
8.	Wash in distilled water	10 sec.
9.	Place in **solution B***	2 min
10.	Place in distilled water	3 min
11.	Stain with Mayer's hemalum solution	3 min
12.	Wash under running tap water	3 – 5 min
13.	Air dry and cover with Aquatex® and a cover glass	

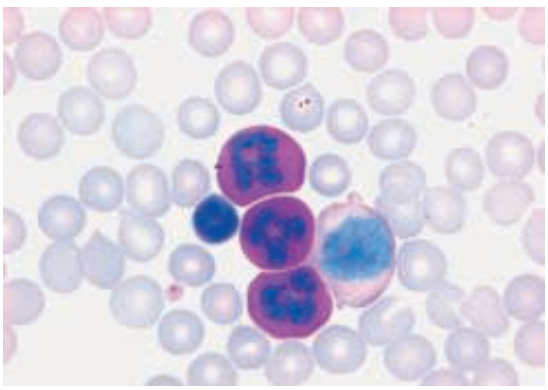
The stain is stable for about 30 days without embedding and for only 3 days when covered with immersion oil. The stability can be extended to several months with the use of embedding agent and a cover glass.



Blood smear, LEUCOGNOST® PAS



Blood smear, LEUCOGNOST® PAS



Blood smear, LEUCOGNOST® PAS

Result

All polysaccharide and in particular glycogen containing structures are stained bright red. Blast populations that at least partly show a characteristic coarse grained PAS positive granulation generally belong to the lymphatic series. Leukemia blasts of the myeloid series are diffuse to fine grained, sometimes also coarse plaqued and PAS positive. Normal myeloblasts, eosinophilic ones and cells of the unaffected red blood cell series are PAS negative by contrast. Promyelocytes, monocytes, basophilic ones and the entire neutrophilic development series demonstrate a diffuse red coloration that shows up bright red with increasing maturity. Erythroblasts in erythroleukemia and some extremely hyper regenerative anaemias can demonstrate a conspicuous PAS reaction.

Ordering information

Product	Package size	Cat. No.
LEUCOGNOST® PAS	1 pack (for 12 staining batches)	**1.16302.0002**
Kit content:		
- Reagent 1: Periodic acid		
- Reagent 2: Potassium disulfite		
- Reagent 3: Hydrochloric acid		
Schiff's reagent	500 mL	**1.09033.0500**
	2.5 L	**1.09033.2500**

LEUCOGNOST® POX

Detection of the peroxidase reaction in leukocytes

The peroxidase reaction, specially the cytochemically significant myeloperoxidase reaction, is used to detect myeloid cell elements, where it is possible to obtain a good estimate of the degree of maturity of the maturing granulocytes from the intensity of the black brown color reaction.

Peroxidase are lysosomal catalases which transfer hydrogen from a suitable donor (previously the carcinogen benzidine, here: 4-chloro-1-naphthol) to a peroxide (here: hydrogen peroxide). The donor 4-chloro-1-naphthol is oxidized and converted to a black brown insoluble dye which can be regarded as an indicator of the peroxidase activity.

Material

Only fresh, native blood and/or bone marrow smears should be used as the starting material for all stains. The use of EDTA as an anticoagulant for example significantly reduces the peroxidase reaction. It is in any case quite unnecessary to add anticoagulant substances. Fine air dried blood and/or bone marrow smears not more than 3 days old are required. The smears must be dried in the air for at least 30 minutes and fixed according to the relevant instructions prior to the actual cytochemical reaction.

Preparation of staining solution*

Dissolve the contents of 1 bottle of reagent 1 in 15 mL of ethanol and transfer to the staining cuvette.

Add with stirring, 45 mL distilled water, 10 drops of reagent 2 and 2 drops of reagent 3.

Note: The reagent solution is colorless and stable for 3 hours.

Procedure

Steps		Time
1.	Fix the air dried blood or bone marrow smears in LEUCOGNOST® fixing mixture	1 min
2.	Wash under running tap water	10 sec.
3.	Place in freshly prepared **staining solution***	10 min
4.	Rinse with distilled water Dry in the air	10 sec.
5.	Stain with Mayer's hemalum solution	2 min
6.	Wash with tap water	3 – 5 min
7.	Air dry and cover with Aquatex® and cover glass	

The stain is stable for about 3 days without embedding and for only a few hours when covered with immersion oil. The stability can be extended to several months with the use of embedding agent like Aquatex® and a cover glass.

Result

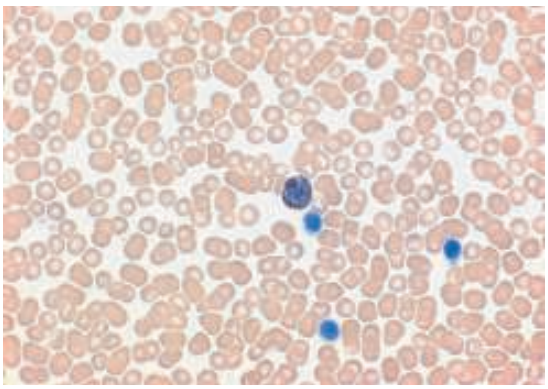
All cells in the neutrophilic and particularly the eosinophilic series of maturity starting with promyelocytes have black brown colored granules and are thus clearly peroxidase positive. The more mature myeloblasts also can contain peroxidase positive fermentation islands in their cytoplasms, even in cases in which the Pappenheim's staining method shows primary granulation at the early stage of development. The great majority of normal monocytes also reacts peroxidase positively. Their coloration is, however, significantly weaker than that of the neutrophilic and eosinophilic granulocytes. Basophilic granulocytes and all cells of the lymphatic and erythropoietic series are peroxidase negative.

Leukemia blast populations which react partly or completely peroxidase-positively are evidence of acute myeloid leukemia, as the lymphoblasts and lymphoid cells of significance in differential diagnosis are always peroxidase-negative. Auer bodies appear conspicuously strongly. In the absence of a peroxidase reaction, however, acute myeloid leukemia cannot be ruled out from the beginning.

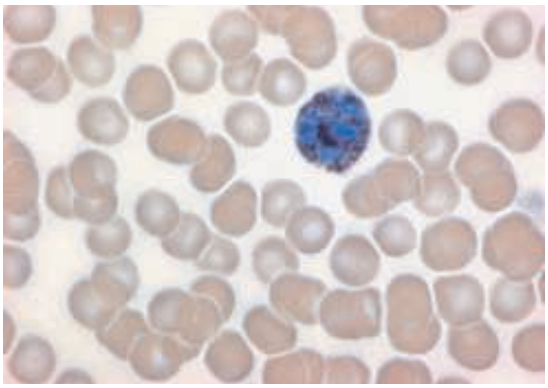
In order to positively identify acute myeloid leukemia with an esterase reaction of less than 50 % positivity as myeloblastic, promyelocytic or myelomonocytic leukemia, the exact percentage of peroxidase positive cells in each blast population must be counted. Additional subdivision of the positive cells according to degree of intensity is not required.

In peroxidase positive leukemia, there are 3 reaction types:

POX type 1
- up to 5 % POX-positive blasts - AML without maturation tendency; AUL or ALL not excluded
POX type 2
- 5 % to 65 % POX-positive blasts - AML without maturation tendency or AMMoL
POX type 3
- over 65 % POX-positive blasts - AML with maturation tendency up to AProL



Blood smear, LEUCOGNOST® POX



Blood smear, LEUCOGNOST® POX

Ordering information

Product	Package size	Cat. No.
LEUCOGNOST® POX	1 pack (for 12 staining batches)	**1.16303.0002**
Kit content: - Reagent 1: 4-chloro-1-naphthol - Reagent 2: Tris(hydroxymethyl-aminomethane)-HCl buffer - Reagent 3: Hydrogen peroxide solution		

LEUCOGNOST® AP

Detection of the acid phosphatase reaction in leukocytes

Acid phosphatase demonstrates specific activity in almost all hematopoietic cell elements (with the exception of neutrophilic and eosinophilic elements) and this is a particularly pronounced characteristic in T-lymphoblastic cells and plasmocytoma cells.

Acid phosphatase catalyses the hydrolysis of phosphate esters in an acidic medium. Under suitable conditions, naphthol AS-BI is released from naphtho-AS-OL phosphate and coupled with a diazonium salt to give a red brown azo dye which is precipitated in the cell.

Preparation of staining solution*

Dissolve the following in sequence in 60 mL of distilled water: 2 mL of reagent 1 and 3 level measuring spoonful (enclosed, 0.8 g) of reagent 2.

Mix 4 – 5 drops of reagent 3 and reagent 4, respectively, in a small test tube, wait 1 minute and then add to the solution.

Filter the reagent solution into the staining cuvette through a full flow filter.

Note: The reagent solution is stable for a maximum of 3 1/2 hours. The staining must be conducted within 15 minutes of preparing the reagent solution.

Procedure

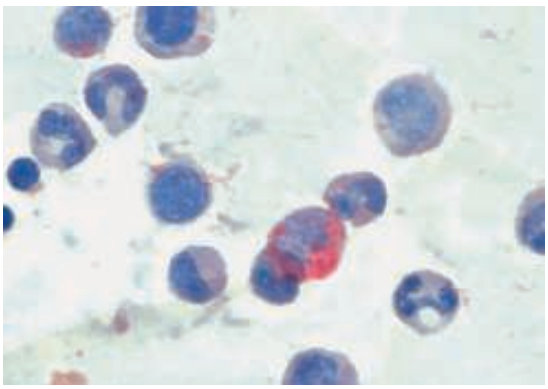
Procedure without inhibition by tartrate

Steps		Time
1.	Fix the air dried blood and bone marrow smears in LEUCOGNOST® fixing mixture	1 – 3 min
2.	Wash with distilled water	1 min
3.	Place in freshly prepared **staining solution*** and incubate in the dark	2 – 3 h
4.	Wash with distilled water	10 sec.
5.	Stain with Mayer's hemalum solution	15 min
6.	Wash under tap water	2 min
7.	Air dry and cover with Aquatex® and a cover glass	

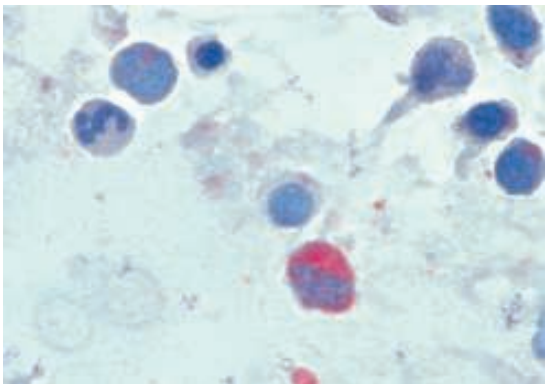
The stain is stable for about 10 days without embedding and for only a few hours when covered with immersion oil. The stability can be extended to several months with the use of embedding agent and a cover glass.

Procedure with inhibition by tartrate

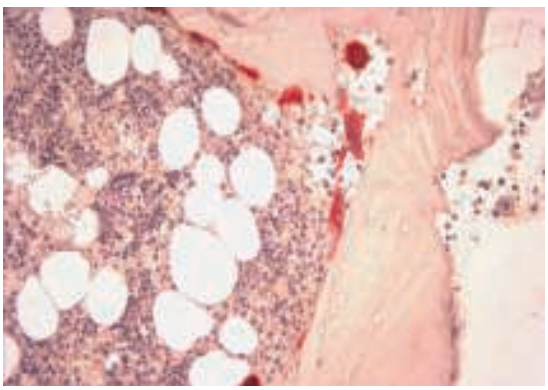
The individual reaction steps and solutions are identical with those of the procedure without tartrate inhibition. Only the staining solution is slightly modified by dissolving a further 4 level measuring spoonful (enclosed = 0.35 g) of reagent 5.



Blood smear, LEUCOGNOST® AP



Blood smear, LEUCOGNOST® AP



Bone marrow biopsy, LEUCOGNOST® AP

Result

In contrast to other lymphatic cell elements, T-lymphoblastic cells demonstrate characteristic red brown fermentation islands. Thus with the aid of acid phosphatase, it is in many cases possible to obtain a clear identification of otherwise cytochemically undifferentiable leukemia.

Addition of tartrate to the reaction mixture inhibits the normal phosphatase activity so that little or no coloration takes place in the blood and bone marrow cells. The acid phosphatase (isoenzyme 5) alone in the characteristic cells of hair cell leukemia is "tartrate resistant" in this procedure and can therefore be used as a diagnostic characteristic.

Ordering information

Product	Package size	Cat. No.
LEUCOGNOST® AP	1 pack (for 12 staining batches)	**1.16304.0002**

Kit content:

- Reagent 1: Naphthol AS-OL phosphoric acid
- Reagent 2: Sodium acetate
- Reagent 3: Pararosaniline-HCl solution (2 N)
- Reagent 4: Nitrite solution 4 %
- Reagent 5: Di-sodium tartrate

LEUCOGNOST® NASDCL

For the detection of naphthol AS-D chloroacetate esterase in leukocytes

Naphthol AS-D chloroacetate esterase brings about the enzymatic hydrolysis of naphthol AS-D chloroacetate to a naphthol compound. This in turn reacts with a diazonium salt to form an insoluble red-violet dye.

Preparation of staining solution*

Solution A

- Dilute 10 mL of reagent 1 with 60 mL distilled water. Add the contents of bottle 2 and rinse out the bottle 2 – 3 times with a few milliliter of buffer.

Solution B

- Add 15 drops of reagent 3 to bottle 4, mix and allow to incubate for 2 minutes.

Solution C

- Add solution B to solution A and rinse out the bottle 2 – 3 times with a few milliliter of substrate buffer mixture.

Prepare the staining solution immediately prior to use.

Procedure

Fixation

Steps		Time
1.	Immerse the air-dried smear in LEUCOGNOST® fixing solution	5 min
2.	Immerse in distilled water	5 min
3.	Air-dry and process immediately or store at +4 to +8°C until required.	5 min

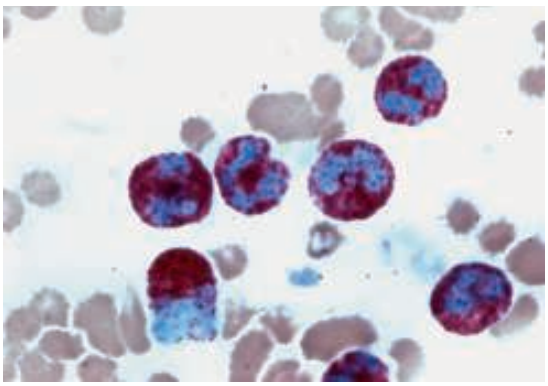
Staining procedure

Steps		Time
1.	Incubate in the freshly prepared **staining solution*** at room temperature	30 min
2.	Place in distilled water	5 min
3.	Counter-stain in Mayer's hemalum	5 min
4.	Rinse with tap water	5 min
5.	Air-dry and cover with Aquatex® and cover glass	

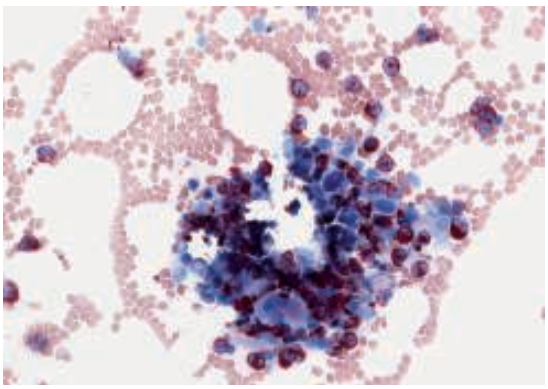
If the smear has not been mounted, the stain is stable for a few days only; if immersed in oil, the stain is stable for a few hours only. Use of a mounting agent and cover glass prolongs stability to several months.

Result

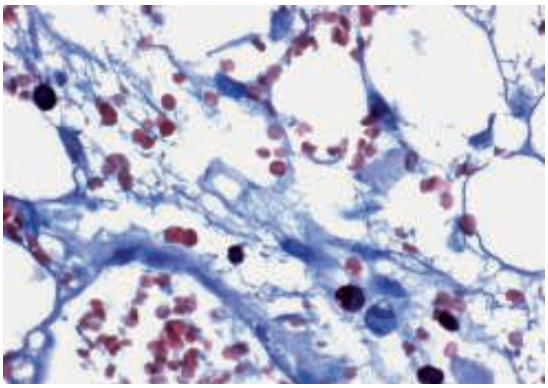
Naphthol AS-D chloroacetate esterase reacts clearly with both mature and immature granulocytes. Intensive enzyme activity can also be shown in the case of myelocytes, metamyelocytes, stab cells and mast cells. Activity may be detected in myeloblastic leukemia cells, promyelocytes and Auer bodies. Monocytes show such activity only rarely. Eosinophiles, basophiles, megakaryocytes, lymphocytes, plasma cells and red cell precursors show no or at most very weak reaction.



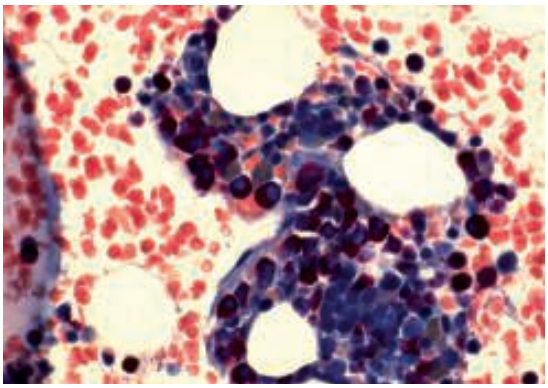
Blood smear, LEUCOGNOST® NASDCL



Blood smear, LEUCOGNOST® NASDCL



Bone marrow biopsy, LEUCOGNOST® NASDCL



Bone marrow biopsy, LEUCOGNOST® NASDCL

Ordering information

Product	Package size	Cat. No.
LEUCOGNOST® NASDCL	1 pack (for 12 staining batches)	**1.16198.0001**

Kit content:

- Reagent 1: Tris buffer concentrate
- Reagent 2: Naphthol AS-D chloroacetate
- Reagent 3: Sodium nitrite solution
- Reagent 4: Fast Red violet LB salt solution

LEUCOGNOST® Basic kit

The LEUCOGNOST® Basic kit contains reagents that are used with the various LEUCOGNOST® kits and are specially matched in quantity to the kits. These reagents fit perfectly to the single LEUCOGNOST® kits and allow intense and reproducible results. The LEUCOGNOST® Basic kit complete the available LEUCOGNOST® kits.

Ordering information

Product	Package size	Cat. No.
LEUCOGNOST® Basic kit	1 pack	**1.16305.0001**
Kit content:		
- Reagent 1: Mayer's hemalum (500 mL)		
- Reagent 2: LEUCOGNOST® fixing mixture (2 x 500 mL)		
- Reagent 3: Schiff's reagent (500 mL)		
- Reagent 4: Ethanol absolute (500 mL)		
- Reagent 5: Acetone (20 mL)		
- Reagent 6: Aquatex® (20 mL)		

Positive and negative controls

To confirm the result in the face of possible unspecific reactions, it is necessary to conduct a positive and negative control with each cytochemical staining batch.

Positive control

The simplest method here is to simultaneously stain normal blood and bone marrow smears. The cells of these smears with their typical color reactions and color intensities serve as references. In addition, normal cell types in the pathological preparation provide a good internal standard.

Example: A normal blood smear is included in the peroxidase reaction. Segmented leukocytes must give a strong positive reaction, lymphocytes a negative reaction. If the control gives both these results, it can be assumed that the peroxidase stain was conducted properly.

Negative control

To do this, a second smear from the patient is used. The smear is treated in the same way as the first, except that the actual substrate is left out.

Example: A second smear from the patient is stained alongside the first in the esterase reaction. In this parallel stain, however, the substrate, alpha-naphthyl acetate, is left out so that no color reaction can take place. If it still takes place, an unspecific reaction is involved which must not be assessed as positive in the first smear either.

Classification of immature-cell leukemia

Acute leukemia is always diagnosed independently of the total number of cells on the basis of their blast population in panoptically stained blood and bone marrow smears. Further attempts at the differentiation only on basis of cytomorphological criteria are, however, not very reliable. The classification of acute leukemia is therefore heavily based on the stem-line specific enzyme detections within the cytoplasma of leukemic blasts. However, a widely held misunderstanding must be pointed out: cytochemical stains cannot be used as an unselected screening method for better primary detection of acute leukemia.

Cytochemical stain of normal cells

The majority of cases of acute leukemia in adults detected from blasts can be accurately classified from a typical pattern of findings based on PAS, Peroxidase (POX), and alpha-naphthyl acetate esterase (EST) reactions – see table "Cytochemical stain of normal cells" below.

Cytochemical stain of normal cells

Cell type	POX Peroxidase Sudan Black B	PAS Periodic acid Schiff reaction	ALPA Alkaline phosphatase	AP Acid phosphatase	EST Alpha-naphthyl acetate esterase	NASDCL Naphthol AS-D chloroacetate esterase
leukocyte	granular ++++	fine granular +++	+ to ++++	diffuse (+)	(+)	+
metamyelocyte	granular ++++	fine granular +++	negative to +	diffuse (+)	negative	+
myelocyte	granular ++++	fine granular ++	negative to +	diffuse +	negative	++
promyelocyte	granular ++	fine granular +	negative to +	diffuse +	negative	+
myeloblast	negative to granular +	negative	negative	negative	(+)	(+)
eosinophilic	eosinophil granules positive	negative to +	negative	diffuse ++	negative	negative
basophilic	granular ++++	negative to +	negative	negative	negative	negative
monocyte	weak granular +	negative to +	negative	+ to ++	(+)	negative
megakaryocyte	negative	fine granular +	negative	diffuse +++	++++	negative
normoblast	negative	negative	negative	++	++	negative
lymphocyte	negative	a few fine to middle +	negative	in T cells	(+) focal	negative
plasma cells	negative	negative	negative	diffuse ++		negative

Cytochemical stain of acute leukemia

In general there is no problem in determining to the particular cytochemical subtype and therefore making a differential diagnosis in acute leukemia on basis of the cytochemical constellation in approx. 95 % of cases – see table "Cytochemical stain of acute leukemia" below.

Cytochemical stain of acute leukemia

FAB-Classification	POX Peroxidase Sudan Black	PAS Periodic acid Schiff reaction	EST Alpha-naphthyl acetate esterase	NASDCL Naphthol AS-D chloroacetate esterase	AP Acid phosphatase
M1	≥ 3 % +	negative to fine granular +	negative	negative	negative
M2	++	negative to fine granular +	negative	+ to ++	negative
M3, M3 Var	+++	negative to fine granular +	negative	+ to ++	negative
M4, M4 Eo	> 5 % +	negative to fine granular +	++	(+)	negative
M5a, b	±	negative to fine granular +	+++	++++	negative
M6	+ in myeloic blasts	coarse positive (erythropoiesis)	negative	negative to (+)	negative
M7	negative	±	±	negative	±
ALL	< 1 % +	coarse positive < 5 %	negative	negative	polar positive (T)
AUL	negative	negative	negative	negative	negative

(positive = focal)

FAB classification of acute leukemia

In approximately 5 % of leukocytes neoplasias with infiltration of bone marrow and release into the blood stream, it is not possible to differentiate between immature lymphatic leukemia with negative PAS reaction and immature myeloid leukemia of the peroxidase -1- type (>/= 5 % POX positive blasts). These cases must be placed into a special group, "cytochemical undifferentiated leukemia". Sometimes it is possible with the aid of an additional characteristic acid phosphatase reaction and particularly with the aid of immunological determination methods, to reach a fine diagnosis, usually indicating acute lymphatic leukemia.

In this connection attention is drawn to the increasing use of a leukemia classification developed by a French-American-British group of hematologists under the leadership of Bennet (1976), the so-called FAB classification of acute leukemia. It is mainly based on the assessment of comprehensive cytomorphological criteria, the cytochemical findings being allocated only a secondary function.

In accordance with this FAB classification, acute leukemia is divided into 2 main groups with 3 or 6 subgroups. The one main group includes acute lymphoblastic leukemia (ALL) with the subtypes L1 to L3, and the other main group contains acute myeloid leukemia (AML) or acute non-lymphoblastic leukemia (ANLL) with the high inhomogeneous subgroup M1 to M6 including erythroleukemia and megakaryocytic leukemia – see table "FAB classification" on the next page. More recently, the megakaryocytic FAB M7 has been differentiated from FAB M6 erythroleukemia.

Nevertheless, even with the FAB classification there is still a not inconsiderable percentage (approx. 3 %) of little differentiated leukemias which cannot easily be placed in the L2 or M1 subgroup. These morphological and cytochemical non-classifiable cases of acute leukemia are combined according to a new definition in the subgroup M0.

FAB classification of acute leukemia is in correlation to the cytomorphological and cytochemical differentiation characteristics on which they are based.

FAB-Classification	Characteristic	Cytochemical main reaction	Incidence (%)	Overall abbreviation
Acute lymphoblastic leukemia (ALL)				
L1	small cells (child. L.)	PAS, AP	65	ALL
L2	mixed cells, often undiff.	PAS + undiff.	30	ALL, AUL
L3	coarse cells, Burkitt type	PAS	5	ALL
Acute myeloid leukemia (AML) or acute non-lymphoblastic leukemia (ANLL)				
M1	myeloblastic, immature	POX-1	14	AML (immature)
M2	myeloblastic, mature	POX-2, POX-3, Sudan Black	30	AML (maturing)
M3	promyelocytic, hypergranular	POX-3, Sudan Black	6	AProl
M4	myelomonocytic	POX-EST, NASDCI	33	AMMol
M5	monocytic (A = immature, B = mature)	EST	14	AMoL
M6	erythroblastic, megakaryocytic	PAS (erythrobl.)	3	AEL

The two most commonly used classification schemata for AML are the older French-American-British (FAB) system and the newer World Health Organization (WHO) system.

Myelodysplastic syndrome

Myelodysplastic syndromes (MDS) are diseases that increase with the increase of age population. Thus demographic development indicate significant rise in the number of these disease. Enzyme cytochemical staining techniques can be used in hematological tests for the differentiation of MDS. Beside iron staining, naphthol AS-D chloroacetate esterase (NASDCL) are important methods for MDS diagnosis, as enzyme pattern abnormalities suggestive of MDS can be visualized by both positive and negative reaction.

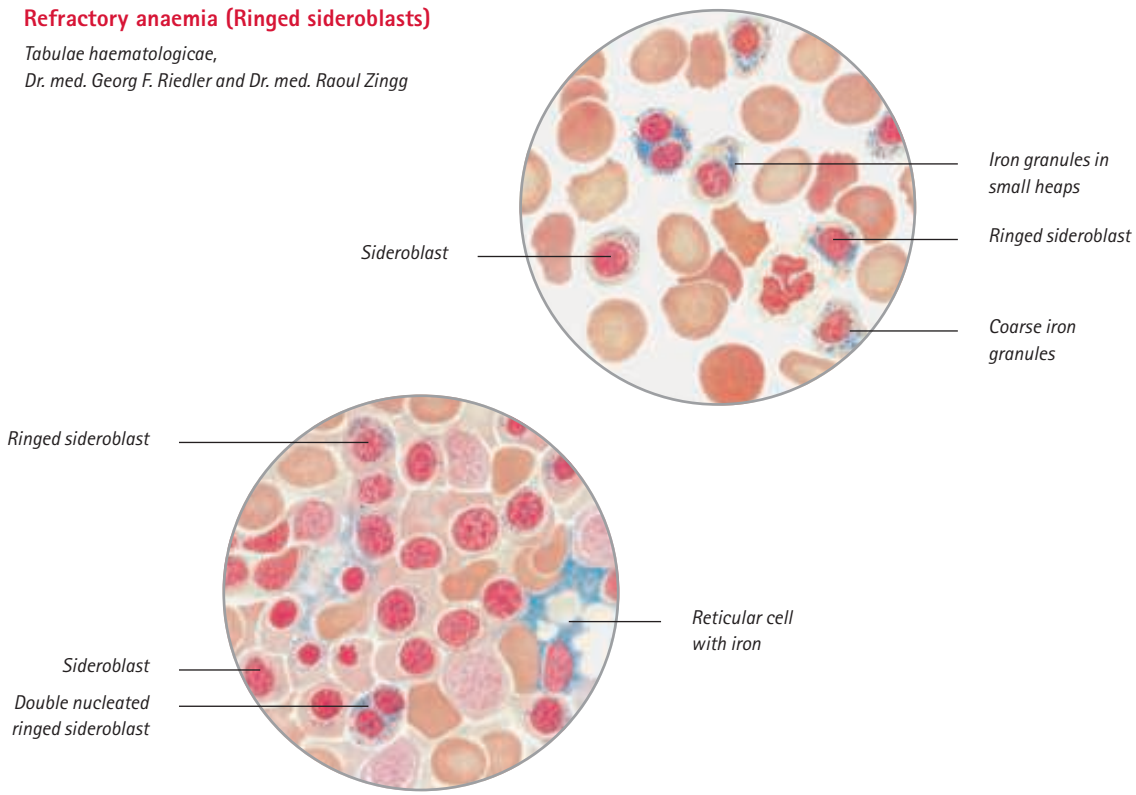
In refractory anemia, more then 15 % of all nucleated red blood cells are in the bone marrow ringed sideroblasts. Ringed sideroblasts are nucleated red cell precursors which on light microscopy have at least five granules of hemosiderin. The granules are stained blue with the Berlin blue stain/Prussian blue stain for iron. In refractory anemia with excess of blasts (RAEB) may be seen ringed sideroblasts.

MDS diagnosis types:

1. Refractory anemia (RA)
2. Refractory anemia with ringed sideroblasts (RARS, D6)
3. Refractory anemia with excess blasts (RAEB)
4. Chronic myelomonocytic leukemia (CMML)
5. Refractory anemia with excess blasts in transformation (RAEBT)

Refractory anaemia (Ringed sideroblasts)

Tabulae haematologicae,
Dr. med. Georg F. Riedler and Dr. med. Raoul Zingg



HEMATOGNOST Fe®

Staining kit for the detection of free ionic iron (Fe^{3+}) in cells

In the Prussian blue reaction, ionic iron (Fe^{3+}) not bound to the heme structure reacts with potassium hexacyanoferrate(II) in hydrochloric solution. It precipitates as an insoluble complex salt in the blood, bone marrow, or tissue cells and thus localizes free cellular iron.

$$4\ Fe^{3+} + 3\ K_4Fe(CN)_6 = Fe_4[Fe(CN)_6]_3 + 12\ K^+$$

As a measure to achieve the best possible visual differentiation of the iron deposits in the cytoplasm, this is counterstained with nuclear fast red solution, resulting in a tender pink color.

Material

Only fresh, native blood or bone marrow smears should be used as the starting material for all stains. The use of e.g. EDTA as anticoagulant significantly reduces the peroxidase reaction. In any case, it is not recommended to add any anticoagulant substances.
Paraffin sections should be approximately 5 – 6 µm from the tissue specimen.

Sample preparation

Smear specimens	
Please use thin, air-dried blood or bone marrow smears that have been stored **not longer than three days**	
The smears must be dried in air for **at least 30 minutes and at most 4 hours** and be fixed with methanol	
Fix the air-dried blood and bone marrow smears in methanol	**3 min**
Air-dry	

Histological specimens
Deparaffinize the sections in the typical manner and rehydrate in descending alcohol series

Reagent preparation

Staining solution* for smears and tissue sections

When using the 60-mL Hellendahl cell (with extension), equal volumes are mixed:

Reagent 1
Potassium hexacyanoferrate(II) solution: 30 – 40 mL

Reagent 2
Hydrochloric acid: 30 – 40 mL

Use only freshly prepared solutions. The staining solution must be discarded after each staining procedure.

The staining solution* used for counterstaining

(Reagent 3) is ready-to-use, dilution of the solution is not necessary.

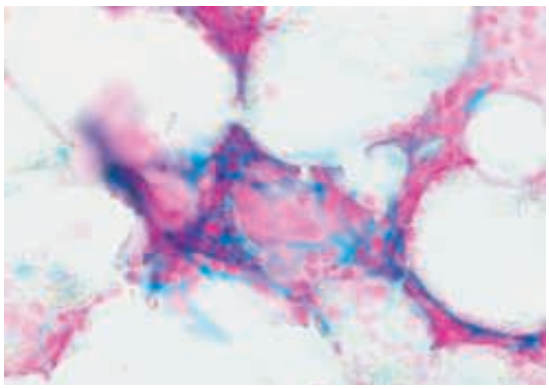
Leukemia diagnostics
HEMATOGNOST Fe®

Procedure
Carrying out the Prussian blue reaction at 37°C yields a sharper definition of the stain precipitates.

Staining smear specimens in the 60-mL Hellendahl cell
The slides should be allowed to drip off well after the individual staining steps, as a measure to avoid any unnecessary cross-contamination of solutions.

Slide with fixed, air-dried smear

Steps		Time
1.	**Staining solution* (mixed 1+1 from reagents 1 and 2)**	20 min
2.	Distilled water rinse	
3.	**Reagent 3 (nuclear fast red solution)***	5 min
4.	Distilled water rinse	
5.	Air-dry (e.g. over night or at 50°C in the drying cabinet)	
	If necessary mount with aqueous mounting agent (e.g. Aquatex®) and cover glass	



Bone marrow smear, HEMATOGNOST Fe®

Staining histological specimens in the 60-mL Hellendahl cell
Slide with histological specimen

Steps		Time
1.	**Staining solution* (mixed 1+1 from reagents 1 and 2)**	20 min
2.	Distilled water rinse out carefully	
3.	**Reagent 3 (nuclear fast red solution)***	5 min
4.	Distilled water rinse	
5.	Ethanol 70 %	1 min
6.	Ethanol 70 %	1 min
7.	Ethanol 96 %	1 min
8.	Ethanol 96 %	1 min
9.	Ethanol 100 %	1 min
10.	Ethanol 100 %	1 min
11.	Xylene or NEO-CLEAR®	5 min
12.	Xylene or NEO-CLEAR®	5 min
13.	Mount the NEO-CLEAR®-wet slides with Neo-Mount® or the xylene-wet slides with Entellan® new and cover glass	

Result

Cell type	Color
Free iron (Fe^{3+})	intensive blue granules
Cell nuclei	pale red
Cytoplasm	tender pink

Evaluation

With the binocular microscope (100x, oil immersion) 1,000 – 2,000 cells of a blood smear are differentiated in a darkened room. The Berliner blue reaction is characterized by an intensive turquoise blue color. The siderocytes are determined in ‰ of the counted erythrocytes. When differentiating bone marrow smears, 100 nucleated red cell precursors should be counted. Besides the quantitative determination of the sideroblasts, the type and abundance of the iron deposits must also be considered (see below).

Normal range

I. In the peripheral blood the normal range for siderocytes in adults is given as 0 – 3 ‰. For newly born babies it is 3 – 17 ‰.

II. Normal range for sideroblasts in bone marrow is 20 – 40 %.

Information to pathological changes in cellular iron content

Increase

Sideroachrestic and hemolytic anemia, pernicious anemia, lead poisoning, spleen ectomy.
Severe overload of an organism with iron causes detection of so called ring sideroblasts. In this case, the iron deposits are coarse-grained and arranged wreath-shaped in the cytoplasm of red cell precursors. Reticular cells are also Fe^{3+} positive.

Decrease

Lack of iron causes a decrease of sideroblasts. Reticular cells are nearly free from detectable iron deposits.

Histology

Increased values of serum ferreting indicate hemochromatosis. Such a suspect has to be confirmed by liver biopsy and determination of the liver iron content. Regularly, a semi-quantitative determination with the Berliner blue reaction is sufficient. Predominantly affected are parenchyma and epithelial cells of the cystic duct. In tissue sections, concentrations of ionic iron as low as 0.002 µg can be detected with the Prussian blue reaction.

Ordering information

Product	Package size	Cat. No.
HEMATOGNOST Fe®	1 pack (for 8 staining batches)	**1.12084.0001**

Kit content:
- Reagent 1: Potassium hexacyanoferrate(II) solution (250 mL)
- Reagent 2: Hydrochloric acid (250 mL)
- Reagent 3: Nuclear fast red solution (2 x 500 mL)

Immersion and mounting

Oil immersion | Mounting with Aquatex®

Oil immersion

Immersion media for microscopy have nearly identical indices as glass. Immersion oils practically eliminate light beam deflection so that the effectiveness of the lens is considerably increased. The refractive index is around 1.5 and the differences for the convenience of the application is based on the different viscosities.

Application

When microscoping, first locate the part of the dry specimen to be investigated. Swing the lens holder away, place a drop of immersion oil on the specimen at the point to be observed and return the lens to their original position. When finished clean the lens and the specimen with ethanol.

Ordering information

Product	Package size	Cat. No.
Immersion oil for microscopy	100 mL	**1.04699.0100**
	500 mL	**1.04699.0500**
Immersion oil acc. to ISO 8036, mod. for microscopy	100 mL	**1.15577.0100**
Oil of cedar wood for microscopy	100 mL	**1.06169.0100**

Mounting with Aquatex®

Specimen which were processed according to an enzymcytochemical method should be preserved with an aqueous mounting agent. Aquatex® is recommended. As soon as the preparation has dried, apply an adequate quantity of Aquatex® (2 – 3 drops), to permit homogeneous distribution over the entire smear. Apply a clean cover glass avoiding the inclusion of air bubbles. After embedding leave the preparation to lie horizontally for about 20 – 30 min until it has set. The slide can then already be immersed in immersion oil and assessed or placed vertically, in a collection.

Stained slides prepared in this way maintain their colors for months. A slight yellowing of the mounting agent or small hair-line cracks in the preparation do not affect the use or the stain.

Ordering information

Product	Package size	Cat. No.
Aquatex®	50 mL	**1.08562.0050**

Storage and documentation

Entellan® and Neo-Mount®

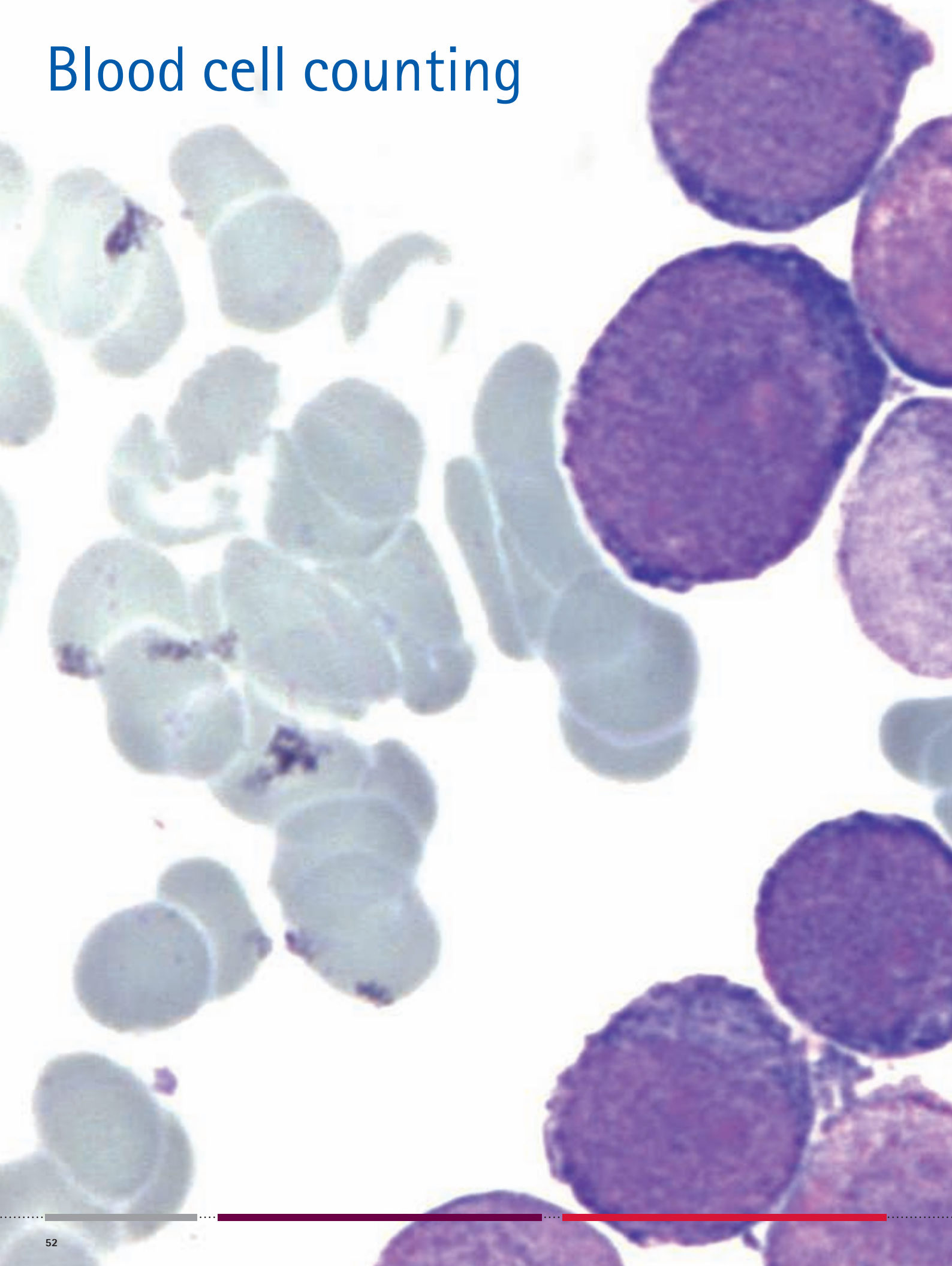
Entellan® and Neo-Mount®

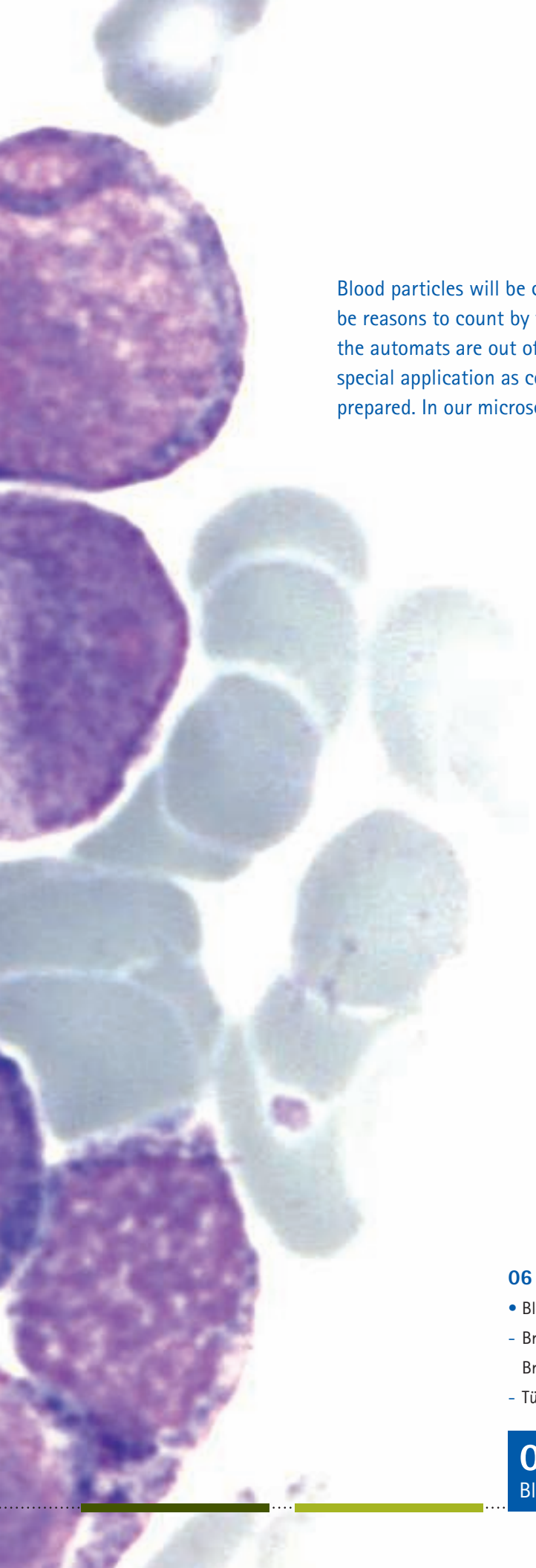
A non-aqueous/permanent mounting medium is recommended. As soon as the slide is completely dried, a few drops of a non-aqueous/ permanent mounting medium are brought up on the preparation. Avoid air bubbles under the cover slip when the slide is covered. After drying time of 20 – 30 min, the smear can be examined under the microscope and stored in an archive. The so preserved preparation remains color stable for a minimum of 10 years.

Ordering information

Product	Package size	Cat. No.
Entellan®	500 mL	**1.07960.0500**
Entellan® new	100 mL	**1.07961.0100**
	500 mL	**1.07961.0500**
	1 L	**1.07961.1000**
Entellan® new for cover slipper	500 mL	**1.00869.0500**
DPX new	500 mL	**1.00579.0500**
Canada balsam	25 mL	**1.01691.0025**
	100 mL	**1.01691.0100**
Neo-Mount®	100 mL	**1.09016.0100**
	500 mL	**1.09016.0500**

Blood cell counting





Blood particles will be counted with automated methods mostly nowadays. There could be reasons to count by the manual methods in counting chambers especially when the automats are out of order, when the number of thrombocytes are very low or for special application as counting in liquor or body effusion or when cell culture are prepared. In our microscopy portfolio are available 2 products for manual used methods.

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Blood cell counting

Brilliant Cresyl Blue solution

Staining of reticulocytes in blood with Brilliant Cresyl Blue Zinc Chloride Double Salt Certistain® – Method for the visualization of erythrocyte regeneration by counting of reticulocytes

Use for the measurement of substantia granulofilamentosa (ribonucleoproteins) with fresh, non-fixed, young erythrocytes (supravital staining). Four stages of substantia granulofilamentosa maturation can be distinguished depending on the stage of reticulocyte development: coiled skein (I), incomplete network (II), complete network (III) and granular form (IV). In peripheral blood the development stages III and IV are found most commonly. When stained with brilliant cresyl blue they display a bluish black network or bluish black dots.

Sample material

Venous blood, in exceptional cases capillary blood

Preparation

- **Brilliant Cresyl Blue solution** Dissolve 1.5 g Brilliant Cresyl Blue $ZnCl_2$ Certistain® in 100 mL physiologic saline solution (0.85 % NaCl), and filter. Keep as a saturated stock solution.
- **Brilliant Cresyl Blue working solution** Dilute the Brilliant Cresyl Blue stock solution 1:80 to 1:200 with physiologic saline solution. For the best dilution make a test before.
- **Single tests** Draw 20 µl blood and 20 µl Brilliant Cresyl Blue solution into a hemoglobin pipette and fill into a small sealable container. Mix thoroughly and after about 30 min prepare a thin smear.
- **Tests in series** Prepare thin smears of Brilliant Cresyl Blue solution on microscope slides using a glass rod. Air-dried slides prepared in this way can be stored for 2 – 3 weeks. For reticulocyte counts smear a small drop of blood quickly over the stain layer, and immediately place the still wet preparation in a moist chamber (Petri dish with damp filter paper). Leave for 5 – 10 min and then allow to dry in air.

Procedure

Counting under the microscope

- Count the reticulocytes per 1,000 erythrocytes with oil immersion under the microscope following a meandering pattern. In order to avoid confusion when counting it is advisable to place a reticulocyte counting grid subdivided into small squares (or a square paper diaphragm) in one of the two eyepieces.
- In peripheral blood the development stages III and IV are found most commonly. When stained with brilliant cresyl blue they display a dark blue network and dark blue dots.

Result

The reticulocyte count is expressed in relation to 1,000 counted erythrocytes (i.e. as $^0/_{00}$). If the erythrocyte count is low, then the absolute reticulocyte count /µl is used.

Calculation

$$\text{Reticulocyte count} = \frac{E/\mu l \times R\ (^0/_{00})}{1{,}000}\ [\text{cells}/\mu l]$$

E = erythrocyte count | R = reticulocyte count

Normal range

Patient	$^0/_{00}$	reticulocyte count/µl
Adults	5 – 15	25,000 – 75,000
Newborn babies	20 – 60	100,000 – 300,000

Ordering information

Product	Package size	Cat. No.
Brilliant Cresyl Blue solution	100 mL	**1.01384.0100**
Brilliant Cresyl Blue $ZnCl_2$ Certistain®	25 g	**1.01368.0025**

Türk's solution

Reagent for manual leukocyte counting

Leukocyte counting is a routine method. The basis of all counting methods is the dilution and preparation of a blood sample of known volume. The erythrocytes are hemolyzed by the acetic acid of Türk's solution and the leukocytes are stained by the dye contained. The required cell type in a defined volume is counted and the number of cells per microliter of blood is then calculated.

Sample material

Anticoagulant venous blood, in exceptional cases capillary blood

Preparation

Filling the pipette

- Draw blood into the leukocyte pipette up to the 1.0 mark, and then draw Türk's solution up to the 11 mark. The dilution is 1:10.
- A dilution of 1:20 can also be used (draw blood up to the 0.5 mark and Türk's solution up to the 11 mark).
- Mix blood and Türk's solution carefully, leave for maximally 1 h.

Filling the counting chamber

- Discard the first 3 drops and fill the counting chamber.

Procedure

Counting under the microscope

- Counting is performed with a x 10 objective, in older microscope models the condenser should be lowered and its front lens swung out.
- Count the leukocytes in the 4 large corner squares, the sides of which each measure 1 mm.
- It is advisable to determine the counts in duplicate; the results should not differ by more than 15 %.

Result

The results are expressed as the means of the duplicate determinations.

Calculation

$$\text{Leukocyte count} = \frac{x \cdot 10 \cdot 10}{4} \quad \text{(dilution 1:10)}$$

$$\text{Leukocyte count} = x \cdot 25 \ [\text{cells}/\mu\text{l}]$$

x = total number of cells counted in the 4 corner squares

Normal range

Patient	Leukocyte count/µl
Adults	4,000 – 9,000
School children	5,000 – 12,000
Small children	6,000 – 15,000
Infants	7,000 – 17,000
Newborn babies	10,000 – 30,000

Ordering information

Product	Package size	Cat. No.
Türk's solution	100 mL, 500 mL	**109277**

Required auxiliaries

- Neubauer counting chamber
- Leukocyte pipette (with white mixing bead)

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