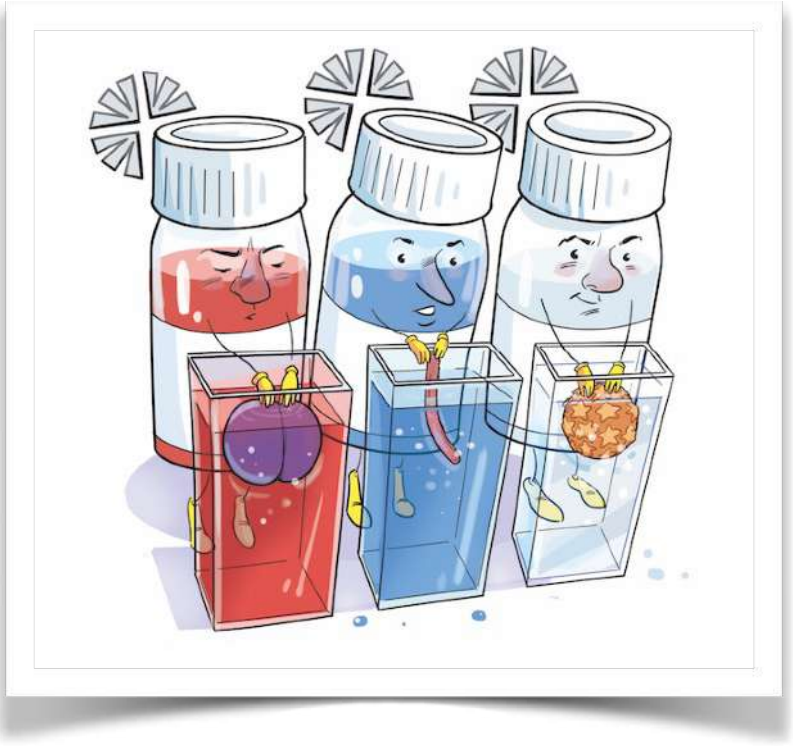
# Staining according to Pappenheim

## Optimized protocol for BIOMED Hemafix®

### A WhitePaper of BIOMED Labordiagnostik GmbH



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# 1. Introduction

The microscopic analysis of blood smears allows conclusions to be drawn about the structure of blood cells that cannot be determined by automatic counting and differentiation alone.

The clinical interpretation of each cytological staining is based on the appearance and staining characteristics and thus the morphology of the cell structures. It should be accompanied by appropriate controls and evaluated within the context of the patient's clinical history and other diagnostic tests by a qualified specialist.

**Pappenheim staining** is referred to as panoptic (all-seeing) or panchromatic (sensitive to all colors) staining, as basophilic, neutrophilic and eosinophilic (also: acidophilic, oxyphilic) cell structures are visualized in a combined staining. Acidic or anionic stains such as eosin stain positively charged cell structures, e.g. proteins, while basic or cationic stains such as methylene blue stain negatively charged cell components such as nucleic acids. Neutrophil components of the cells can be stained with both basic and acidic dyes. Pappenheim staining is a combination of May-Grünwald and Giemsa staining and can therefore also be referred to as MGG (**M**ay-**G**rünwald-**G**iemsa) staining.

The pH value is a significant factor in staining, thus any change in the pH value, the exact composition of the staining solution and the **buffers** used can result in a different staining reaction.dependent
The appropriate pH value is between pH 5.8 and pH 7.2 depending on the type of specimen (bone marrow, blood, other). When selecting the appropriate buffer, the type of specimen and the aim of the staining must always be taken into account.

**May-Grünwald** is a pH-dependent differential stain for air-dried blood smears, bone marrow, histological and cytological preparations. It combines the effects of eosin, methylene blue and methanol.

The **Giemsa** staining solution is a modification of the Romanowsky stain and consists of a combination of the dyes azure A eosinate, azure B eosinate, methylene blue eosinate and methylene blue chloride in methanol. It is also known as azure eosin-methylene blue solution and can be used as an enhanced overview stain for methanol-fixed bone marrow and blood smears or other cytological material (e.g. urine sediment or sputum). The duration of the staining, the pH value of the solution, the buffers used and the fixation can influence the staining result.

Pappenheim staining combines the effects of both staining solutions and covers the spectrum of Giemsa and May-Grünwald staining. It causes the pH-dependent staining of granulocytes (polymorphonuclear leukocytes: neutrophils, eosinophils and basophils) and the specific staining of cell nuclei and cytoplasm. Thus, it enables the differentiation of erythrocytes, lymphocytes, monocytes, the various granulocytes and thrombocytes, as well as parasites and microorganisms found in the blood.

Die Hemafix® **rapid staining** is based on a modification of the Pappenheim staining method and can be used pH-independently without the requirement for buffer solutions.

Staining can be carried out by dipping or injection, manually or with an automatic stainer.

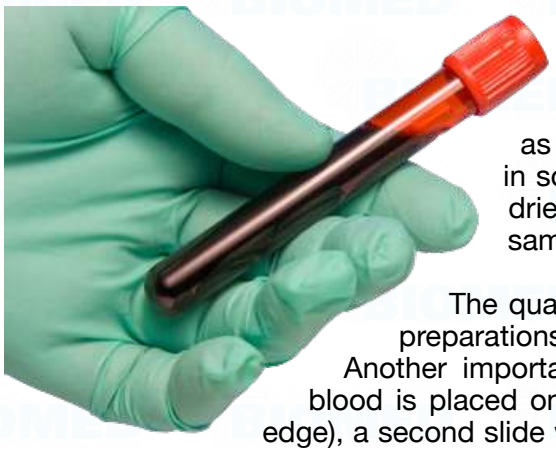
# 2. Material

## 2.1. Preparations

Pappenheim staining enables reliable staining of air-dried smears of body fluids and tissues. It is suitable for thin preparations such as blood smears or cytocentrifuge preparations, for preparations with tissue components such as bone marrow smears and for denser preparations such as tissue samples taken by biopsy or puncture for organ cytology or lymph node cytology.

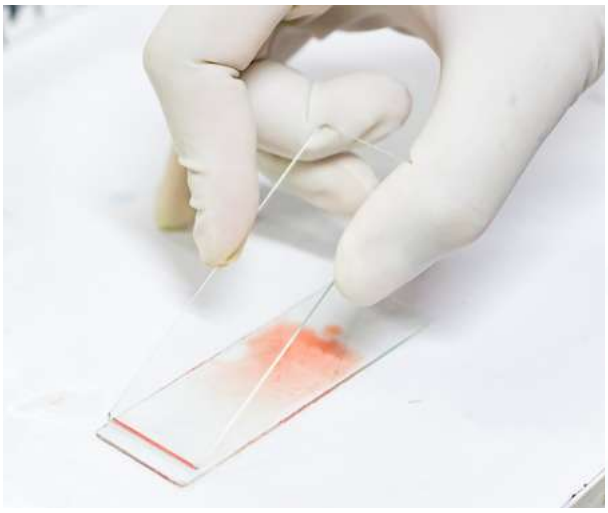
Hemafix® rapid staining (modified Pappenheim method) is well adapted for rapid overview staining of blood smears.

All samples must be taken, processed, prepared and dried in accordance with the state of the art and approved laboratory methods.



Fresh capillary or venous blood coagulated with EDTA is recommended as sample material for blood smears. Samples coagulated with Na-citrate, Na-oxalate or heparin are not suitable for these analyses, as these anticoagulants can seriously distort the staining in some cases. Capillary blood should be smeared and air-dried immediately, venous blood no later than 3 hours after sampling.

The quality of the smear is critical for the evaluation of stained preparations. It is important to use clean, grease-free slides. Another important point is the smearing technique. A small drop of blood is placed on one side of a labeled slide (approx. 1.5 cm from the edge), a second slide with sharp edges is placed at an angle of 45° in front of the drop of blood and then carefully moved into the drop of blood. As soon as the blood has spread over the entire edge, the second slide is continuously moved over the first slide at the same angle. The movement has to be smooth and rapid. There shouldn't be any steps in the smear. A well-spread specimen will show a beard-like fraying of the blood material at the end, where the slide is finally lifted off.
The density of the smear depends on the angle between the first and second slide: the smaller the angle, the thinner the smear will be. If light is allowed to reflect on the dried smear, it should appear greenish. If it appears red, it is too thick. Too rapid movements when smearing can cause the liquid film to break off.
Only when the smear has been carefully air-dried, it can be stained.



Staining should be started no later than 24 - 48 hours of smearing to avoid a significant change in staining behavior. For later staining, the preparations should be fixed immediately after air drying for a suitable length of time depending on the thickness of the preparation.
The following protocols are for thin to medium density smear preparations.

Cytological samples can be fresh bone marrow smears or clinical cytological material such as urine sediment, sputum, etc. Smears from fine needle aspiration, biopsies and lavages are also suitable.

For the analysis of CSF sediment, CSF (also: Liquor cerebrospinalis, cerebrospinal fluid) should be processed immediately (up to 1 h after collection). CSF is a clear fluid with little cellular substance. The most common cells in the CSF of healthy persons are lymphocytes, less frequently monocytes. Increased numbers of leukocytes or the presence of erythrocytes in the CSF indicate a medical condition.

## 2.2. Staining Solutions


Staining according to **Pappenheim**:
Fixing solution: e.g. Hemafix® Fixierlösung
May-Grünwald Solution: e.g. Hemafix® May-Grünwald
Giemsa Solution: e.g. Hemafix® Giemsa
Buffer solution according to Weise: e. g. Hemafix® Puffer pH 7.2

**Rapid staining** modified according to Pappenheim:
Fixing solution: e.g. Hemafix® Fixierlösung
First staining: e.g. Hemafix® Rot
Second staining: e.g. Hemafix® Blau

## 2.3. Additional Materials required

The additional materials required must be suitable to meet the requirements of a medical diagnostic laboratory.
Capillaries for blood collection
Grease-free, clean microscope slides
If necessary, cover glass in a compatible size and mounting medium, e.g. Euparal or Entellan.
Transmitted light microscope for medical and biological use in laboratories.
Immersion oil if necessary.
Staining cuvettes or a suitable staining device.

The protocol was tested manually in staining cuvettes and on the following staining machines:

- Dagatron AT-2000H Hematology Auto Stainer
- Dagatron AT-3004 GRAM/MGG Dual Stainer
- Dagatron AT-2000I INDIVIDUAL

# 3. Method

Both Pappenheim staining and rapid staining can be carried out by injection or dipping. The solutions are applied to the preparation accordingly or the entire preparation is dipped into the solution.

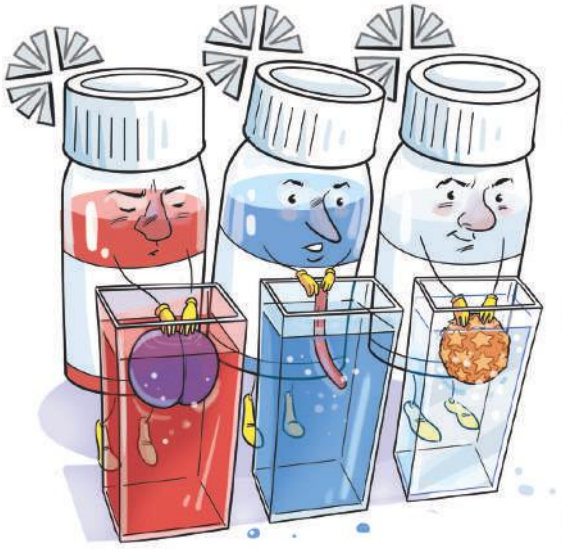
## 3.1. Fixing

The air-dried preparations are fixed as soon as possible after drying (after 24 - 48 h at the latest) using a fixing solution containing methanol. The preparations for this purpose are quickly covered with the fixing solution or dipped into it, followed by an incubation of 10 min. For thicker preparations, the duration of fixation should be increased.
If staining is planned later, the preparation should be dried, otherwise staining can follow immediately.

## 3.2. Manual Staining

Manual staining can be carried out in staining cuvettes or by applying the solutions using a pipette. For a larger number of preparations, the use of staining cuvettes is recommended; for few preparations, the method of application using a pipette is economical and should therefore be considered. The following protocol has been optimized with Hemafix® solutions, but can also be used with other staining solutions. The staining solutions used must be prepared and used according to the manufacturer's instructions.

**Pappenheim Staining**
The solutions Hemafix® Fixierlösung and Hemafix® Puffer pH 7.2 are ready to use.
The Hemafix® May-Grünwald staining solution is diluted with Hemafix® Puffer pH 7.2 in a ratio of 1:2 (1 part Hemafix® May-Grünwald + 1 part Hemafix® Puffer).
Hemafix® Giemsa solution is diluted in a ratio of 1:4 (corresponding to 1 part Hemafix® Giemsa + 3 parts Hemafix® Puffer).



The required amount of Hemafix® May-Grünwald and Hemafix® Giemsa solutions should be filtered with a pleated filter for manual application in order to avoid artifact formation.

Staining steps:
- Fixation in fixing solution for 10 min.
- Incubation in May-Grünwald solution for 7 min.
- Rinsing in buffer solution for 3 min.
- Incubation in Giemsa solution for 20 min.
- Briefly (time can be adjusted individually) rinse in buffer solution according to Weise (pH 7.2).
- Rinse 2 x for 3 min in distilled water.
- Air-drying the preparations.

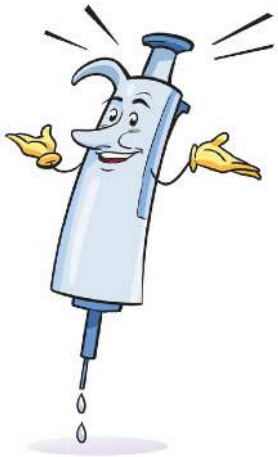
The incubation times of the staining solutions can be individually adjusted.
If longer storage of the preparations is desired, they can be covered with the aid of a mounting medium and a suitable cover glass to preserve them.

**Rapid Staining modified according to Pappenheim**
The solutions Hemafix® Fixierlösung, Hemafix® Rot and Hemafix® Blau are ready to use, they are buffered and do not result in color changes due to pH shifts (water, etc.).
The amount of solutions Rot and Blau required for Hemafix® rapid staining should be filtered with a pleated filter for manual use to avoid artifact formation.

Staining steps:
- Fixation in fixing solution for 5 - 10 sec.
- Incubation in Hemafix® Rot for 5 - 10 sec.
- Incubation in Hemafix® Blau for 5 - 10 sec.
- Briefly rinse in in distilled water.
- Air-drying the preparations.

The incubation times of the staining solutions can be individually adjusted.
If longer storage of the preparations is desired, they can be covered with the aid of a mounting medium and a suitable cover glass to preserve them.

## 3.3. Staining in Staining Devices from Dagatron



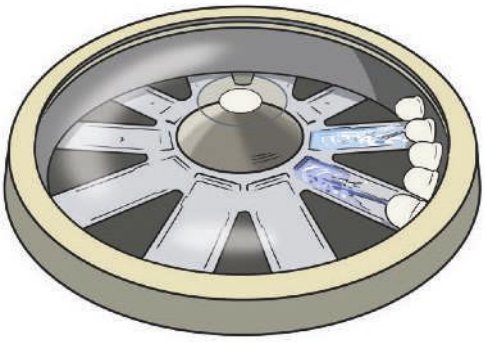
In the Dagatron stainers, the staining process, including drying, runs automatically thanks to the internal programming of the device. The staining solutions are injected in the device in the same way as they are applied with a pipette during manual staining. Simultaneous staining of up to 10 or, with an additional slide wheel, up to 20 preparations is possible. The staining program is already pre-installed in the device, but the injection and incubation times can be set individually. The last staining times applied are used again for the next staining.

The following protocol has been optimized with Hemafix® solutions, but can also be used with other staining solutions in the original Dagatron bottles. The staining solutions used must be prepared and used according to the manufacturer's instructions.


**Pappenheim Staining**
The solutions Hemafix® Fixierlösung and Hemafix® Puffer pH 7.2 are ready to use.
The Hemafix® May-Grünwald staining solution is diluted with Hemafix® Puffer pH 7.2 in a ratio of 1:2 (1 part Hemafix® May-Grünwald + 1 part Hemafix® Puffer).
Hemafix® Giemsa solution is diluted in a ratio of 1:4 (corresponding to 1 part Hemafix® Giemsa + 3 parts Hemafix® Puffer).

**Rapid Staining modified according to Pappenheim**
The solutions Hemafix® Fixierlösung, Hemafix® Rot and Hemafix® Blau are ready to use, they are buffered and do not result in color changes due to pH shifts (water, etc.).

Filtering the solutions before use is not necessary for the injection method using the Dagatron staining device, as the Dagatron staining system has built-in reagent filters.



After smearing and fixing the samples on the slides, they are placed individually on the slide holders in the slide wheel of the stainer. For multi-stainers, it is necessary to select the desired staining method and then press the "STAIN" button. The stainer automatically carries out the programmed staining process (see also stainer operating instructions). After successful staining, the preparation only needs to be analyzed under a microscope in order to differentiate the various blood cells according to color and structure.

If longer storage of the preparations is desired, they can be covered with the aid of a mounting medium and a suitable cover glass to preserve them.

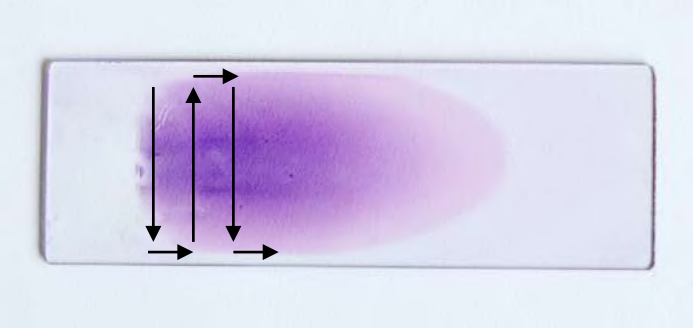
### 3.4. Mounting for Stabilization

When the preparations are ready for histological, cytological or hematological assessment, the last step of covering the preparations with a suitable cover glass can be completed. To prevent the cover glass from moving against the slide, the cover glass is fixed to the slide using a mounting medium. This improves the handling of the preparations, but also enables longer-term storage of the fixed preparations, as the mounting medium protects the sample from damage caused by moisture, oxygen and light. Nevertheless, classically stained microscopic preparations should be stored dry, protected from light and at room temperature in order to achieve a long shelf life. For example, slide folders made of cardboard or plastic or slide boxes made of plastic or wood are suitable for this purpose.

# 4. Analysis and evaluation

## 4.1. Analysis

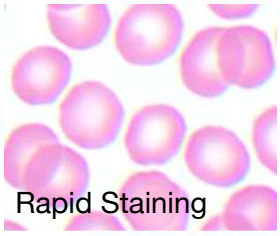
First of all, the quality of the specimen can be examined macroscopically; the area to be analyzed is determined based on the quality and thickness of the smear. An initial, superficial assessment of the staining with regard to the strength of the staining and uniformity is also already possible.
A suitable transmitted light microscope should be used for a more precise evaluation of the specimens. At low magnification (objective 10x or 20x) the smear quality and the staining quality can be determined. It is crucial that the cells are as isolated as possible, i.e. not overlapping, and that they have not been mechanically destroyed during smearing. The appropriate area for the differentiation of the cells can be defined.
At higher magnification (objective 50x or 100x, oil immersion if possible), the cells can be differentiated on the basis of their size, structure and staining. To prevent repeated analysis of the same area, the preparation is screened in a meandering pattern.

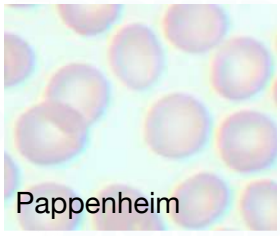


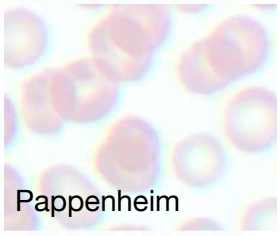
## 4.2. Staining results

Different cell types and cell structures can be differentiated using Pappenheim staining and hematological rapid staining modified according to Pappenheim:

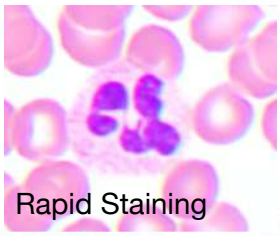
- **Erythrocytes**: 99 % of blood cells
  Shape: Biconcave, round disks
  Size: ⌀ 6 - 8 µm, thickness 1 - 2 µm



- **Leukocytes**: 1 % of blood cells
  - **Granulocytes**: 60 - 70 % of Leukocytes
    Shape: Spherical
    - **Neutrophiles**: 50 - 65 % of Leukocytes
      Size: 9 - 15 µm
      Granules: - Primary (azurophilic, acidic granules)
      - Secondary ("specific granules", do not react with basic or acidic stains)
      - 3 more granules
      Nucleus: - Rod nuclei (rod-shaped, unsegmented nucleus, "young" cells)
      - Segmental nuclei (2-5 segments, mature cells)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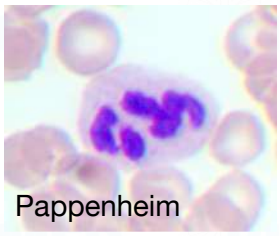
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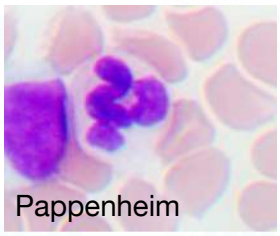
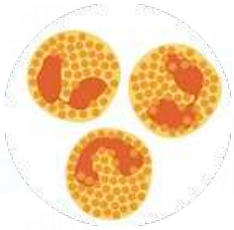
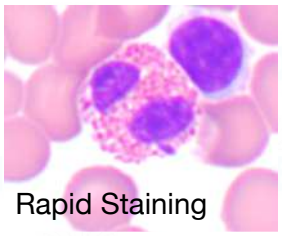

Pappenheim

    - **Eosinophiles**: 2 - 5 % of Leukocytes
      Size: 8 - 10 µm
      Granules: Numerous, large, eosinophilic (red-orange in color)
      Nucleus: Rod-shaped or (mostly) double-segmented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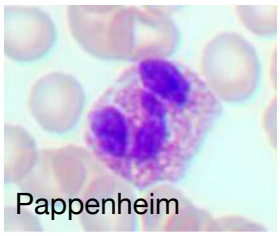
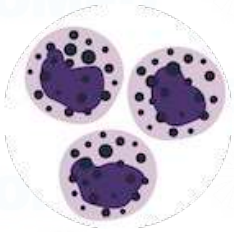
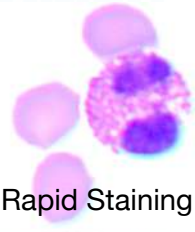

Pappenheim

    - **Basophiles**: 0 - 2 % of Leukocytes
      Shape: Round to oval
      Size: ~10 µm
      Granules: Intracellular, basophilic (blue-violet), numerous and rough, often covering the nucleus
      Nucleus: Mostly cloverleaf or spectacle-shaped, roughly segmented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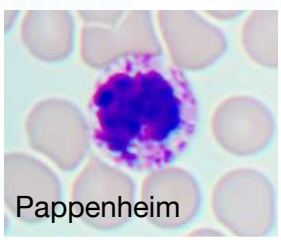
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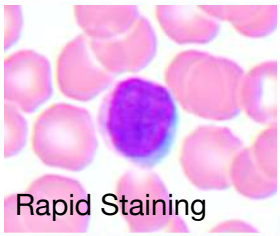
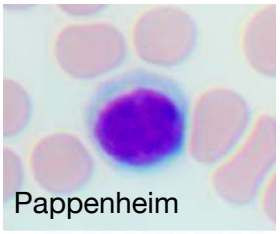
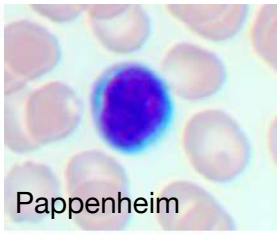
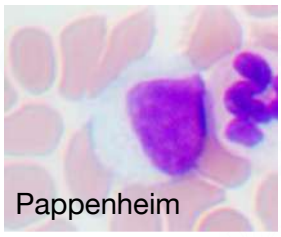
- **Lymphocytes**: 20 - 30 % of Leukocytes
  Shape: Round-oval
  Size: 8 - 10 µm
  Nucleus: Round, relatively large (rarely incurved)

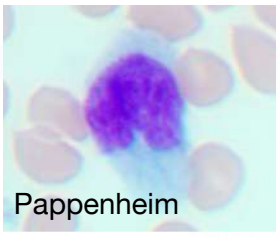





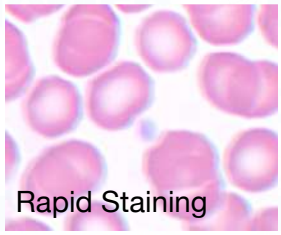


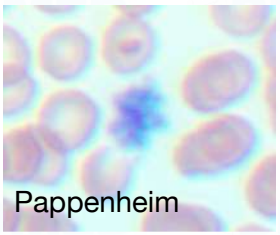

- **Monocytes**: 2 - 6 % of Leukocytes
  Shape: Mostly round
  Size: 12 - 20 µm (largest cells in blood)
  Nucleus: Multiform, spherical or bean-shaped

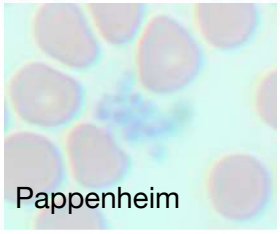






- **Thrombocytes**: 150.000 - 350.000 Platelets per µL of blood
  Shape: Flat, uneven round to oval platelets
  Size: 1 - 4 µm (Smallest cells in the blood)
  Nucleus: Nucleus-free cytoplasmic fragments






## 4.3. Evaluation

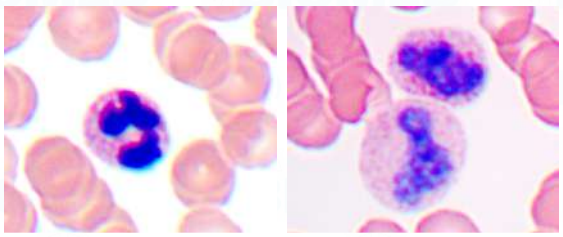
The preparations are evaluated according to the usual internal laboratory routine.
With regard to the erythrocytes, for example, changes in shape, possible erythrocyte inclusions and color changes or changes in the nucleated red precursors (erythroblasts) can be detected. In addition to determining the relative proportions of individual leukocyte subtypes in the total leukocytes, morphological characteristics of the cells can be recognized in the microscopic examination, which can provide important indications of reactive events (e.g. infections) but also of neoplastic processes (e.g. leukemias).

# 5. Tips and Troubleshooting

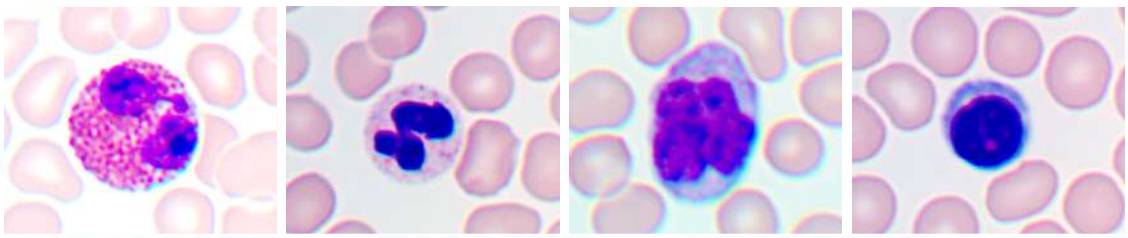
## 5.1. Tips for using Pappenheim Staining

- Both the dyes and the preparation thickness have an influence on the intensity of the staining, so the staining time should be adapted to the respective conditions.
- Insufficient rinsing during the staining process or dirty or greasy slides can lead to inaccurate staining of the preparation.
- The preparations must always be completely and evenly wetted with staining solution in order to obtain uniform, reproducible staining without gaps.
- Old or unfiltered staining solutions can lead to the formation of artifacts. It is therefore always useful to check the quality of the staining solutions before carrying out staining. This includes a visual check for turbidity and contamination of the solutions, for example due to the growth of microorganisms in the case of improper storage.
- If the staining solutions are left in cuvettes for a longer period of time, it is advisable to mix or filter the staining solutions before the next use in order to prevent the formation of artifacts.
- Staining solutions in use should be changed after 3 days at the latest, the buffer solution is best changed after each staining run.
- When using an automated stainer, it is essential to follow the manufacturer's instructions.
- The pH value of the buffer solution has a strong influence on the staining, so it should be checked weekly in accordance with Rili-BÄK, Table B 3-1 - Internal quality assurance. A slight shift in the pH value can influence the reaction of the cell components to the staining solutions to the extent that, for example, a cell component with an alkaline reaction can react acidically and vice versa.

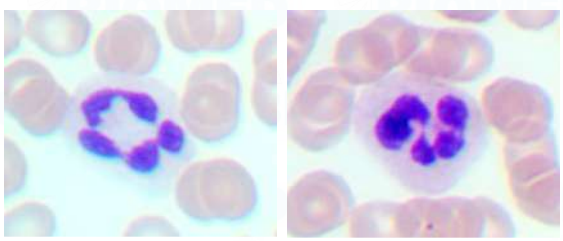
The lower the pH value of the buffer during staining, the stronger the (acidic) red staining and methylene blue is washed out.



Buffer pH 6.4



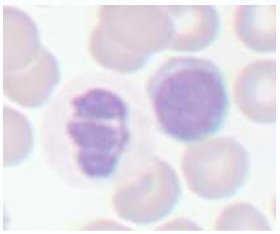
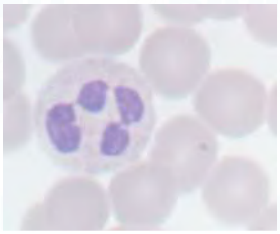
Buffer pH 6.8



Buffer pH 7.2

## 5.2. Troubleshooting

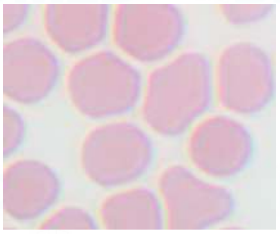
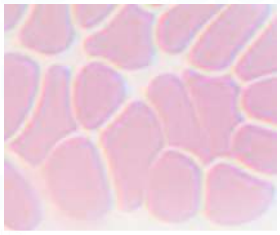
- The staining is too weak (nuclei, erythrocytes, granules) - possible causes:
  - Staining too short
  - Preparation too thick
  - Rinsing too strong
  - Staining solution too old/used
  - Unsuitable staining times

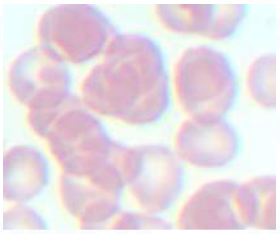
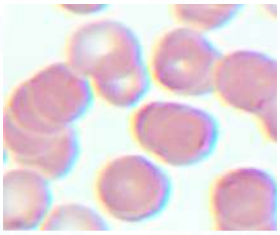
- Staining is too red (erythrocytes bright red):
  - Acidification of the staining, e.g. by pH change in buffer or staining solutions

- Staining is too blue (chromatin, granules):
  - Preparation too thick or old
  - Staining too long
  - Rinsing insufficient
  - pH value of individual solutions too high (distilled water also possible!)

- The cells appear too large, erythrocytes without a dent:
  - Preparation too thin

- The cells appear too small, overlap:
  - Preparation too thick

- Artifacts are visible
  - Staining solutions have not been filtered
  - Mechanical destruction of the cells during spreading
  - Fixation insufficient/diluted
  - Insufficient drying of the preparation
  - Incorrect storage of the sample (too long/chilled)

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