Boule

# Slide preparation for WBC differential by manual microscopy

The reference method for classification of the white blood cells (WBC, also called leucocytes) counted in an automated hematology analyzer is the manual microscopic morphological cell classification according to the Clinical and Laboratory Standard Institute CLSI, 2007 H20-A2 and must be performed by qualified examiners. Boule hematology analyzers do not identify all morphological abnormal cell forms, but they generate alarms/flags that indicate abnormalities in classification of the WBC differential count. In that case, a manual microscopic morphological count is recommended to be performed.

In order to make an accurate manual count, which is meant to be the reference values, a good slide must be made. This entails good blood film preparation and good staining technique. Otherwise the results may not be as accurate.

The purpose with this instruction is to describe the method used by Boule for preparation and staining a blood smear and counting of the WBC subpopulations in a microscope.

## Staining method

A blood smear is treated with dye solutions according to the standardized azure B-eosin Y Romanowsky procedure that includes fixation and staining with May-Grünwalds (containing methanol) and Giemsa's solutions. These stains both contain Azure B and Eosin dyes that, at an appropriate pH (6.4 to 7), color the cell constituents differently. Eosin yields red erythrocytes (or red blood cells; RBC) and eosinophil granules and Azure B gives rise to blue stained chromatin, neutrophil-specific granules, platelets and ribosome-rich cytoplasma as well as to violet basophil granules. The different dye shades in the blood smear help to distinguish between different blood cells and components.

### *Equipment*

1. *Material*
   - Clean, dry, and dust-free object slides of good quality, 25 × 75 mm, 0.8 × 1.2 mm thick (VWR art. 630-2012)
   - Micro cap with 20 µL glass micropipette without any additives /pipette 20 µL
   - Spreader slide with polished edges or mechanical spreader
   - Pencil to write the ID, date and specimen on the slide
   - Fume cupboard
   - Immersion oil
   - Timer
   - Test tube rack, to drain the slides
   - Microscope MD# 690, with 10× to 40× objective for overview and 100× objective for morphological estimation and differential count.
   - COMP-U-DIFF™ cell counter
   - Three slides labelled as A, B and C (or "spare")
2. *Reagents*
   - 2 Blood stain glass cuvettes
   - May-Grünewald stain, undiluted
   - Giemsa stain, diluted 1:20 with distilled water (1-part Giemsa and 19-part distilled water)
   - Glass beaker with distilled water
   - Immersion lens oil
   - Lens paper tissues
   - Absolute alcohol 95%–99% for lens cleaning

## Preparation and staining procedures

### *1. Specimen collocation*

Venous blood is collected in an anticoagulated with $K_2$-EDTA or $K_3$-EDTA. Alternatively, blood collected by skin puncture may be used, but only when it is used on the automated hematology analyzer as well. For skin puncture and capillary blood sampling see "capillary blood sample collection" (1).

## *2. Blood film preparation*

Prepare at least three blood films from each specimen within four hours after blood collection. Two blood films will be used for the procedure and the third will be kept as a spare.

Perform the following:

1. Mix the blood sample tube 20 complete inversions by hand.
2. Aspirate the blood sample with a micropipette placed into a micro-cap or a normal pipette.
3. Apply one blood drop (approximately 10 µL) of well-mixed blood near the end of a glass slide (Figure 1A).
4. Take the spreader slide and drive it against the blood drop. Hold the spreader slide a second to let the blood drop spread out at the edges of the glass slide.
5. Swiftly spread it between spreader slide and smearing slide, holding the latter at an angle of 30°-45°. Avoid deformation of blood cells by squeezing (Fig. 1B).
6. Approx. 3/4 of the slide should be covered with the smear, leaving a small rim along the edges. (Fig. 1B and 1C).
7. Leave the smear to air-dry thoroughly and quickly. Slow drying alters the morphology of the red blood cells, resulting in spike-shaped cells.
8. Use a pencil to write the ID, date, specimen (if dog, cat, etc.) and A, B and C on the frosted end of the respective slide.
9. Stain the smears within the recommended one hour of preparation with Romanowsky stain.

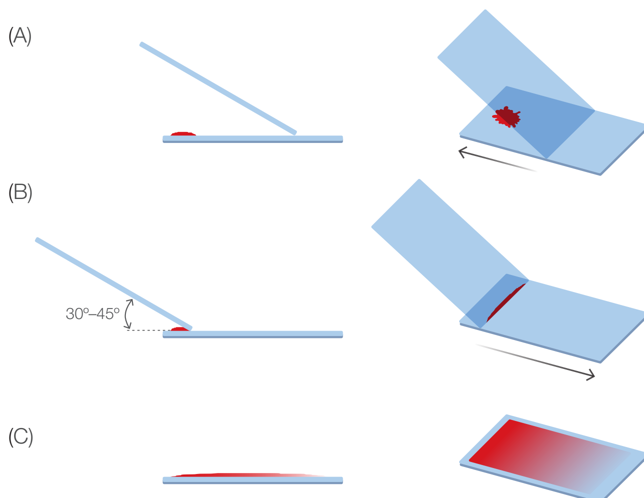


**Fig. 1.** Preparation of a blood smear.

A good blood film preparation will be thick at the drop end and thin at the opposite end. Examples of acceptable blood smear are shown in Fig. 2.

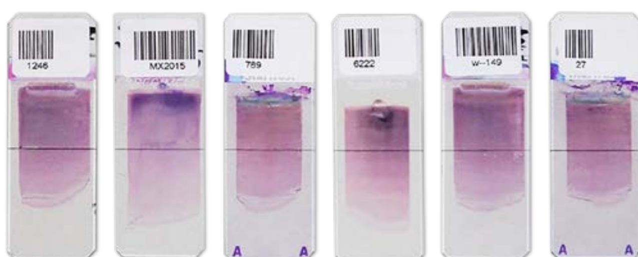

**Fig. 2.** Examples of acceptable blood smears (used with kind permission from CellaVision).

Prepare a new smear slide if the smear looks like one of the examples shown in Fig. 3.

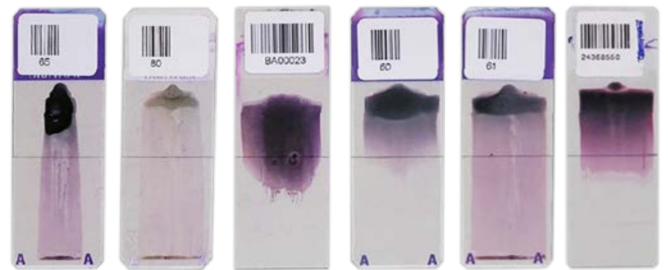

**Fig. 3.** Examples of unacceptable blood smears (used with kind permission from CellaVision).

## *3. Romanowsky Staining*

1. Stain the slides, preferably within one hour after preparation of the smear, as recommended by Equalis, the Swedish external quality assurance (EQA) provider.
2. Pour up May-Grünewald staining into one glass cuvette and Giemsa staining diluted with distilled water 1:20 in another glass cuvette.
3. Pour up a glass beaker filled with distilled water to rinse the slides.
4. Place the dried blood smear slides into the May-Grünewald cuvette in eight minutes.
5. Rinse off the stain carefully from the smears with distilled water.
6. Place the blood smears into the Giemsa cuvette in 15 minutes.
7. Rinse off the stain carefully from the smears with distilled water. It is important to rinse off all the expendable staining color to get a clear blood smear slide.
8. Let the smears completely dry in an upright position.

# Protocol for examining the blood film

After performing the Romanowsky stain, the WBC can be divided into subpopulations based on the morphology/maturity and stainability of the cells. The main subpopulations are the neutrophil (NEU), eosinophil (EOS), and basophil granulocytes (BASO), as well as the lymphocytes (LYM), monocytes (MONO) and all the white blood cell (WBC) precursors.

The morphological examination is performed in a light microscope for classification and qualitative assessment of the WBC, erythrocytes (RBC), and platelets (PLT).

# Microscopic examination of the blood film

1. Adjust the microscopy before counting according to Köhler's principal.
2. The blood film should always be pre-scanned under lower resolution (10× to 40× objective) for unusual or abnormal cells and an acceptable cell distribution.
3. Most 100× oil immersion objectives are capable for resolving cytoplasmic granules and neutrophilic filaments.

4. A droplet of blood should be spread on a slide in such a manner that the cells are located separately, side by side in one layer.
5. The leucocytes should be well preserved. Except for certain forms associated with pathological states (e.g., chronic lymphocytic leukemia), less than 2% by manual smear method of the leucocytes should be disrupted or unidentifiable forms. Only if the disrupted cell is still clearly identifiable (e.g., an eosinophil), it should be included in the differential count. Classify unidentifiable disrupted cells (smudges or baskets as “other” and include a comment on the laboratory report.
6. If the percentage of disrupted cells is high (> 10%) due to technical impacts, new slides are recommended. If disrupted cells are presented in the new slides, they should be classified as “other” and be commented.

## Counting procedure

Counting of the blood cells in the microscope is performed according to the following:

7. The examination of the blood film should be performed according to the “battlement pattern”, that is, reviewing in the area with monocellular layer (red blood cells seen one by one) and in a consecutive way as shown in Fig. 4.

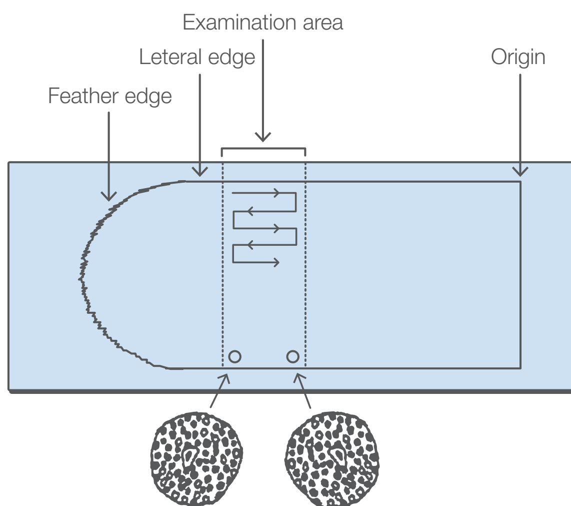


**Fig. 4.** Examination using the “battlement” track.

- The differential count is performed on at least 200 cells on each prepared slide by two independent examiners where one examiner uses slide A and the other one uses slide B. The data must be tracible to each slide and each examiner. In total, 400 cells are counted. If the blood is leukopenic, process additional slides in parallel. The number of counted cells should always be noted.
- The microscopy results are primarily calculated as percent of the total counted WBC cells. Convert the percent values to absolute values by using the WBC result given by the hematology analyzer.
- Use the Comp-U-Diff to sort the cells and to automatically get the absolute and percentage values (see Section Comp-U-Diff Counter).
- A qualitative classification of the leucocytes includes review of the nuclei, the cytoplasm and granules.
- Count any nucleated erythroid cells present and express the result as the number per 100 WBC counted.
- Transcribe the results from each differential count into the database program, Sample Browser.

## Comp-U-Diff counter

While counting the blood cells under the microscope, data entry of the differential counts is made in a workstation, called Comp-U-Diff counter. Counting using the Comp-U-Diff is performed according to the following steps:

1. Go to Menu by pressing the MENU button on the Comp-U-Diff counter.
2. Select *1.0 White Cell Counting.*
3. Enter the Sample ID.
4. Enter the WBC count reported by the reference analyzer or, if you have not the access to the reference analysis result, enter 10.0 (the decimal is the F-button).
5. Enter 200 for total counted cells.
6. Press ENTER.
7. Start counting and sort the cells according to Fig. 5.
8. When counting is finished press ENTER. The results from the Comp-U-Diff can be seen on the display and will be printed.
9. Review the counting and sign the printout with date and signature and save the original printouts together with the specific study.

| Comp-U-Diff buttons | Description |
|---|---|
| | **0 = Neutro**; Neutrophil granulocyte<br>**1 = Lymph**; Lymphocyte<br>**2 = Mono**; Monocyte<br>**3 = PROLYM**; Abnormal/immature lymphocytes<br>**4 = Eosino**; Eosinophil granulocyte<br>**5 = Baso**; Basophil granulocyte<br>**6 = BLAST**; blasts of all kinds (myeloblast, monoblast and lymphoblast)<br>**7 = Var. Lym**; “atypical” activated lymphocytes<br>**8 = BAND;** Band neutrophil granulocyte<br>**9 = META**; Metamyelocyt<br>**A = MY**; Myelocyt<br>**B = PRO**; Promyelocyt<br>**C = RET**; Reticulocyt, not included in the WBC differential count<br>**D = Others**; Unidentifiable disrupted cells, smudges, baskets<br>**E = NRBC**; Nuclei Red Blood Cell, not included in the WBC differential count<br>**F = MEGA**; Megacariocyt, only micromegacariocytes can be present in peripherical blood smear and are not included in the WBC differential count. The F-button is also used as a comma character |

**Fig. 5.** The Comp-U-Diff buttons and their meaning.

## Reporting the results

The results of the WBC differential count are expressed as a fraction (i.e., percentage of all the WBC counted). Erythroblasts and micromegakaryocytes are reported in number/100 leucocytes and are not including in the sum of the classified leucocytes.

## Quality assurance

Assessment shall be made according to ICSH recommendations regarding the standardized routine method for morphological classification and assessment of cells in blood smears. Those recommendations are that the human WBC classification should be included in an external quality program such as the Swedish Equalis.

## Error sources

The differential count of the blood cells can be affected by the following sources:

- Blood sampling procedure
- The age of blood sample (blood smear < 4 h from blood sampling and staining within 1 h according to Equalis' recommendation).
- Blood smear technical errors as shown in Fig. 3.
- Reproducible staining, stain technical errors, incubation times.
- Identification of the blood smear
- The skills of the examiner

## Qualitative morphological assessment (evaluation)

For the blood cell types present in the blood and their different characteristic appearances under normal or pathological conditions, see ICSH recommendations for standardization of nomenclature and grading of peripheral blood cell morphological features.

## Reference


1. Capillary blood sample collection
2. CellaVision DC-1 digital cell morphology assessment. Boule Diagnostics, 31737, Edition 1 (2019).



**boule.com**



CellaVision is a registered trademark of CellaVision AB.
COMP-U-DIFF is a trademark of Modulus Data Systems Inc.
© 2019 Boule Diagnostics AB



TR27437
Boule Diagnostics AB, Domnarvsgatan 4, SE-163 53 Spånga, Sweden

WPB34068-2 12/2019