White paper

Slide preparation for WBC differential by manual microscopy

Boule hematology analyzers generate alarms/flags that indicate abnormalities in classification of the white blood cells (WBC) differential count. In that case, a manual microscopic morphological count is the recommended reference method, and should be performed by qualified examiners. In order to make an accurate manual count, a slide must be prepared. This prepared includes both the blood film preparation and the staining technique.

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

Material

• Lens paper tissues 2 Blood stain glass cuvettes

• 20 uL micropipette, without any additives

• Clean, dry, and dust-free object slides of good quality, 25 × 75 mm, 0.8 × 1.2 mm thick.

• Spreader slide with polished edges or mechanical spreader

• Pencil to write the ID, date and specimen on the slide

• Fume cupboard

• Timer

• Test tube rack, to drain the slides

• Microscope with 10× to 40× objective for overview and 100× objective for morphological estimation and differential count.

• Differential cell counter

Reagents

• 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

• Absolute alcohol 95%–99% for lens cleaning

Preparation and staining procedures

Specimen collection

Venous blood is collected in tubes with K_2EDTA/K_3EDTA anticoagulantia. Alternatively, blood collected by capillary puncture may be used, however, always make sure to use the same source of blood for both the manual smear and the automated hematology analyzer.

Blood film preparation

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

Perform the following:

1. Mix the blood sample in the tube by 20 complete inversions, by hand.

2. Aspirate the blood sample with a micropipette.

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. 1C).

Boule

7. Air-dry the smear thoroughly and quickly through waving it in the air. 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.

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.

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

Romanowsky Staining

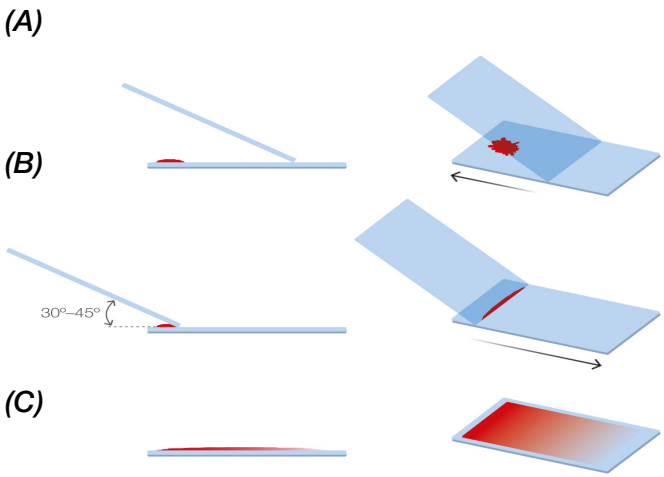

Fig. 1. Preparation of a blood smear.

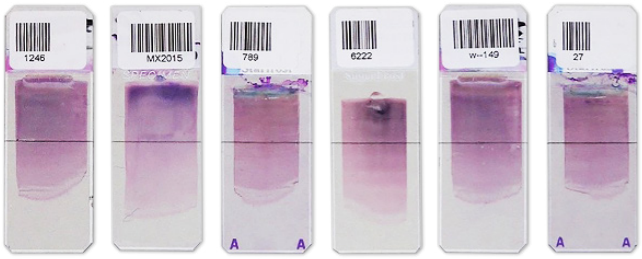

Fig. 2. Examples of acceptable blood smears (used with kind permission from CellaVision).

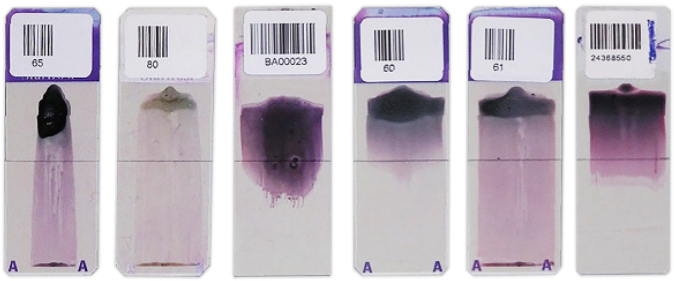

Fig. 3. Examples of unacceptable blood smears (used with kind permission from CellaVision).

Stain the slides, preferably within one hour after preparation of the smear.

Perform the following:

1. Pour May-Grünewald staining into one glass cuvette and Giemsa staining diluted with distilled water 1:20 in to another glass cuvette.

2. Prepare a glass beaker filled with distilled water to rinse the slides.

3. Place the dried blood smear slides into the May-Grünewald cuvette for eight minutes.

4. Rinse the blood smear slides carefully with distilled water.

5. Place the blood smears slides into the Giemsa cuvette for 15 minutes.

6. Rinse the blood smear slides carefully with distilled water. It is important to rinse all the expendable staining color to get a clear blood smear slide.

7. Let the smears completely dry in an upright position.

Examination of the 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

• Adjust the microscope before counting according to Köhler's principal.

• 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. The red blood cells shall be in a mono-layer, next to each other.

• Use a 100× oil immersion objective to resolve cytoplasmic granules and neutrophilic filaments.

• The leucocytes should be well preserved. Except for certain forms associated with pathological states (e.g., chronic lymphocytic leukemia), less than 2% of the leucocytes should be disrupted or in 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.

• If the percentage of disrupted cells is high (> 10%) due to technical impacts, new slides are recommended.

Counting procedure

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

• 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 traceble 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.

• A qualitative classification of the leucocytes includes review of the nuclei, the cytoplasm and granules.

• Count any nucleated erythroid cells (nRBC) present and express the result as the number per 100 WBC counted.

• 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.

• Use a Differential Counter to sort the cells and to automatically get the absolute and percentage values.

• 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.

• Transcribe the results from each differential count into a database program.

Error sources

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

• Blood sampling procedure

• The age of the blood sample

• Blood smear technical errors as shown in Fig. 3.

• Incubation times.

• Identification of the blood smear

• The skills of the examiner

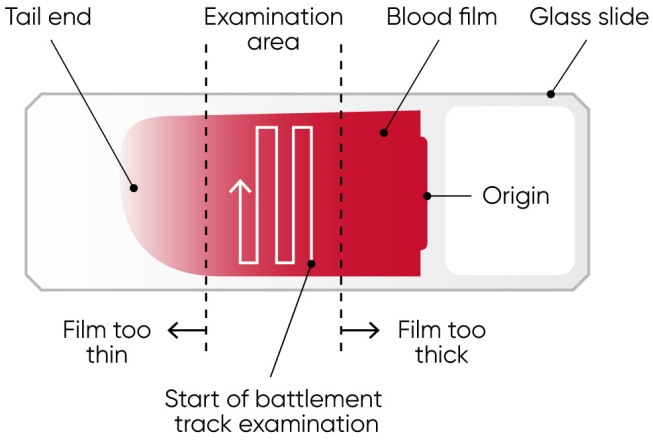

Fig. 4. Examination using the "battlement" track.

Qualitative morphological assessment (evaluation)

See ICSH recommendations for standardization of nomenclature and grading of peripheral blood cell morphological features.

References

1. CellaVision DC-1 digital cell morphology assessment. Boule Diagnostics, 31737, Edition 1 (2019).

boule.com

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