**PRINCIPLE**

Blood is mixed with a diluent that causes hemolysis of red cells. A hemocytometer is filled with the diluting fluid, and the platelets are counted under the microscope, preferably by using phase-contrast, if available.

**MATERIALS/EQUIPMENT**

- Flat-bottom, thin counting chamber (phase-contrast hemocytometer with Neubauer ruling)
- Phase-contrast microscope equipped with long-working-distance phase condenser, if available; otherwise an ordinary light microscope
- 20 µl pipette
- 2 ml graduated pipette
- 12 × 75 mm tube
- Mechanical mixer

**REAGENT**

Diluting fluid: 1% ammonium oxalate in distilled water. Store in the refrigerator, and always filter just before using.

**SPECIMEN**

If the blood sample is from a finger prick, the puncture must be clean and the blood free-flowing. Wipe away the first drop of blood. If the blood sample is from venous blood, it must be collected into a dry plastic (or siliconized glass) syringe with a short needle not smaller than 21 gauge. The needle must be removed before the blood is delivered into a plastic container with EDTA. The blood and anticoagulant must be mixed gently, to avoid frothing, without any delay.

**METHOD**

1. Pipette 0.38 ml of diluting fluid into a test tube.
2. Fill the 20 µl pipette to the mark and wipe off the outside of the pipette.
3 Expel the contents of the pipette into the diluting fluid, and wash out the pipette by drawing up the blood and expelling it into the tube a few times. Mix for at least 10 minutes by hand or, preferably, by mechanical mixer.

4 Fill the hemocytometer, as described below.

5 Cover the chamber with a petri dish for 10 to 20 minutes to allow the platelets to settle. Leave a piece of wet cotton or filter paper in the dish to prevent evaporation.

6 Using a microscope, count the platelets in the large 1 mm squares (= 0.1 µl). Count the platelets in as many squares as necessary to reach a count of at least 100. The platelets appear round or oval, and their internal granular structure and purple sheen allow them to be distinguished from debris. Ghosts of the red cells that have been lysed by the ammonium oxalate are seen in the background. If phase contrast is not available, an ordinary light microscope can be used, provided the condenser is racked down to provide a low intensity of light.

7 Calculate the number of platelets per litre of blood according to the formula below.

THE HEMOCYTOMETER

The hemocytometer counting chamber, with Neubauer or improved Neubauer ruling, is constructed so that the distance between the underside of the cover glass and the surface of the chamber is 0.1 mm. The surface of the chamber contains two specially ruled areas with dimensions as shown in Figure 10.1. The central 1 mm² has double or triple boundary lines. In the central areas are 25 squares in the improved Neubauer and 16 squares in the Neubauer ruling. Each square has an area of 0.04 mm² (0.2 x 0.2 mm). These squares are, in turn, divided into smaller squares, each 0.0025 mm² (0.05 x 0.05 mm). The outer quadrants of the ruled area are each 1 mm² and are divided into 16 squares.

CALCULATIONS

The formula for calculating the cell count is:

\[
\text{Count (cells/l)} = \frac{N \times D}{A \times 10 \times 10^6}
\]

Where

- \( N \) = total number of cells counted
- \( D \) = dilution
- \( A \) = total area counted (in mm²)
- \( 10 \) = factor to calculate volume in µl from area (in mm²) and depth of chamber (0.1 mm)
- \( 10^6 \) = factor to convert count/µl to count/l
Figure 10.1. Hemocytometer counting chamber (a) Neubauer and (b) Improved Neubauer
**SOURCES OF ERROR IN CELL COUNTING**

When capillary blood is used, a free-flowing drop must be obtained.

When anti-coagulated blood is used, the specimen must be carefully mixed by inverting the tube of blood at least 20 times before a sample is taken. Do not shake the tube, because shaking introduces foam, which makes accurate pipetting impossible. Tilt the well-mixed tube to an angle of 45° or slightly more, and pipette from the lip of the tube, following the same procedures as for capillary blood.

The blood-sampling pipettes must be clean and dry.

The pipette must be filled quickly, and the blood must be drawn accurately by using a pipette suction device attached to the pipette, filling up to the desired line. If the line is overshot slightly, the excess blood may be expelled by touching the lip of the pipette on a piece of filter paper or soft tissue. If the line is overshot, a fresh pipette must be used.

No air bubbles should be present in the blood column.

The outside of the pipette must be wiped free of blood (being careful not to pull blood from the tip) before it is introduced into the diluting fluid.

After the contents of the pipette have been discharged into the diluent, diluting fluid must then be drawn into the pipette with steady suction several times, to ensure that all the blood is discharged into the fluid.

The tube containing the diluted blood must be shaken gently for at least two minutes by hand or, preferably, in a mechanical shaker. After the tube has been shaken, the chamber is immediately filled by means of a Pasteur pipette or capillary tube.

The chamber is filled by capillary action, with the flow of fluid from the pipette or capillary regulated so that it fills quickly and smoothly. It must be filled completely, but fluid must not spill over into the moats. Allow the cells to settle in the counting area for 10 to 20 minutes, then proceed with the counting.

The hemocytometer chamber and glass cover must be clean and dry before they are used. Important errors can be introduced by fingerprints or an oily film.

A sufficient number of cells must be counted to reduce error due to chance distribution of cells. In practice, at least 100 cells should be counted. As a further check on correct distribution of cells in the chamber, the number of cells counted in each area (i.e. in the large squares) should not differ by more than 10%.

**CONTROLS**

Two dilutions must be made, and the mean of the two counts taken; the two counts should agree within 10%.
SOURCES OF ERROR IN PLATELET COUNTING

Blood obtained by a venipuncture is preferable to capillary blood, because platelets adhere to the wound and successive dilutions from a finger prick are not always reproducible.

The general errors of pipetting and hemocytometry are described above. In addition, special attention must be paid to ensuring that the counting chamber is scrupulously clean, since dirt and debris may be counted as platelets. Wash the chamber with soapy water, then rinse with distilled water, allow to drain dry, and wipe with lint-free tissue. Be sure that the cover slip is clean before using it.

The presence of platelet clumps precludes reliable counts. If the sample contains clumps, a fresh sample must be collected.

The ammonium oxalate diluent should be kept refrigerated and must be discarded if there is evidence of bacterial contamination.

The specimen must be counted within three hours of collection.