RECOMMENDED METHODS FOR THE VISUAL DETERMINATION OF WHITE BLOOD CELL COUNT AND PLATELET COUNT

Prepared on behalf of the World Health Organization

by

The Expert Panel on Cytometry of the International Council for Standardization in Haematology*

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1. INTRODUCTION

Counting blood cells is an important aspect of diagnostic haematology. In many laboratories electronic particle counters are used for this purpose, but in smaller laboratories, and especially in developing countries, a method with visual counting using a microscope may be the only available technique. It may also be used as a back up for week-end and night duties in laboratories with electronic counters. Visual counting of red cells is not recommended because the number of cells which must be counted for a reliable measurement (see 4.2) is too demanding of time in a busy diagnostic laboratory; red cell indices (MCV and MCH) computed from an inaccurate visual red cell count are worthless for reliable diagnosis of different forms of anaemia. Anaemia may also be diagnosed reliably by haemoglobin measurement and/or packed cell volume (haematocrit) determination.

2. BLOOD SAMPLING

Blood sample may be taken from a capillary puncture of the heel [lateral or medial plantar surface] in young infants and a finger [palmar surface of the distal phalanx] or ear lobe in others. However, a venous sample is preferred. Capillary punctures must be deep enough to ensure a free flow of blood. If pressure has to be exerted to obtain a sufficient amount of blood from the puncture, an error will be introduced because of dilution of the blood with tissue fluid. A venous specimen may be collected into one of the salts of ethylene diamine tetra-acetic acid (EDTA); where possible K$_2$EDTA.2H$_2$O, 1.5-2.2 mg/ml blood, should be used. Before sampling from a venous specimen the blood must be mixed either by fully inverting the tube 8-10 times by hand or on a mechanical mixer for 2-3 minutes.

3. EQUIPMENT

3.1 Pipettes

For pipetting the blood, Sahli type capillary pipettes of 20 µl (0.02 ml) capacity are recommended. For dispensing the diluent, graduated 0.5- or 1-ml ‘to deliver’ pipettes or a syringe capable of delivering 0.4 ml accurately are suitable.

The diluent pipettes or syringes should be cleaned in detergent, followed by thorough rinsing with distilled or de-ionized water and dried. Capillary pipettes which have come into contact with blood should be soaked in 2.5% hypochlorite for 30-60 minutes, washed with water and then, prior to being used again, they should be cleaned in detergent, followed by thorough rinsing in distilled or de-ionized water and dried. It is also advisable to clean the pipettes once a week in a cleaning agent (e.g., 0.1mol/l HCl) to remove any protein film, followed by thorough rinsing with distilled or de-ionized water and drying.

Before using the pipettes examine them carefully for any damage, especially chipped ends which will invalidate their accuracy.
3.2 Counting chamber

Counting chambers consist of a depressed area with a ruled grid, to facilitate counting, on a glass slide; they are converted into volumetric chambers when overlaid by a special, thick, optically flat cover-glass. A thin cover glass will bow resulting in marked errors in the depth (and thus the volume) of the chamber and must never be used.

Care is needed to ensure the correct positioning of the special cover glass on top of the counting chamber. When the cover glass is correctly applied to the surface of a counting chamber, Newton's rings first form and then disappear showing that the relative position of cover glass and chamber is sufficiently even to ensure a constant depth of chamber throughout its entire area.

A large number of counting chambers has been described. Many of these have become obsolete, and the only one which is recommended is the improved Neubauer chamber (Fig.1). The grid contains four corner squares and a central square, each with an area of 1x1 mm (=1 mm²). With the cover-glass in position the depth is 0.1mm, so that each square has a volume of 0.1 µl. The total chamber area is 3 x 3 mm (= 9 mm²; 0.9 µl). The corner squares are sub-divided into 16 smaller squares of 0.25 x 0.25 mm; the central square is divided into 25 smaller squares, 0.2 x 0.2 mm, and each of these is further divided into 16 squares of 0.05x0.05mm. Thus 80 of the smallest squares provide an area of 0.2 mm² and volume of 0.02 ml.

4. Technique of Counting

4.1 Procedure

1. Wash the chamber and the cover glass in running tap water immediately after use to remove all residual organic matter. Before use, wash in alcohol and dry.

2. Place the chamber on a flat surface. Press the special cover glass down, checking that it is in the correct position by the presence of several Newton's rings that will appear on each side. If rings do not form, reclean the chamber and the cover glass. If rings still do not form, try again with another cover glass.

3. The blood, diluted in the appropriate fluid (see 5.1 and 6.1) is mixed on a mechanical mixer for 2 to 3 minutes, and taken up into a thin capillary tube.

4. Fill both sides of the chamber by allowing the diluted blood to flow under the upper edge of the cover glass in one smooth action. The chamber must be cleaned and the filling process repeated if any of the following occur:
   - The fluid overflows into the moat
   - The chamber area is not completely filled
   - Air bubbles occur anywhere in the chamber area
   - Any debris appears in the chamber area
5. Place the chamber into a petri dish which contains a small wad of damp blotting paper, filter paper or absorbent cotton wool. Leave undisturbed in a horizontal position, not exposed to direct sun or other heat source and free from drafts and vibrations for a sufficient time for the cells to settle - white cells 2 to 3 min., platelets at least 20 min.

6. Scan the entire chamber area at low magnification to check that the cells are distributed evenly throughout the ruled grid.

7. Focus on the grid at a selected magnification (e.g. x 250 for white cells; x 400 for platelets) and count all the cells present in a sufficient number of squares to ensure an acceptable level of accuracy (see 4.2). Use a suitable convention to ensure that cells are not counted twice, e.g., count only cells that touch the top and right hand margins of a square, omitting from the count of that square any cell that touches its bottom and left hand margins (Fig.2). If during the counting period the chamber dries out, the preparation must be discarded. Evaporation can be delayed by setting up the microscope in a cool area away from direct sunshine. Avoid heating the chamber by exposing it to the microscope lamp light beam only during the counting.

4.2 Count variance

In all particle counting procedures there is an inherent error due to the fact that the particles are distributed in a random way (Poisson distribution). This variance (σ) is calculated as $\sqrt{n} \div n$ where $n$ is the total number of particles counted and variance is expressed as a percentage. For example, a count of 100 will have a variance of 10%; there is a 95% chance that the count could be any number between mean $n+2\sigma$ and $n-2\sigma$ in the adjacent squares, i.e. expanded uncertainty of 80 to 120. By contrast, when 2500 particles are counted, the variance is reduced to 2%; clearly, this amount of labour for each blood count is impractical for a routine test, and it is necessary to decide on a count that is sufficiently accurate to be clinically valid (Table 1)

<table>
<thead>
<tr>
<th>No of areas</th>
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<th>Expanded uncertainty</th>
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5. TOTAL WHITE BLOOD CELL (WBC) COUNT

5.1 Dilution procedure

The diluting fluid consists of 2% acetic acid lightly coloured with 1% crystal violet. When stored at about 20°C, the shelf life is at least one year.

For a 1:21 dilution of the blood, add 20 µl blood to 0.4 ml of diluting fluid. Mix for 2-3 minutes.

The red cells are lysed but the leucocytes remain intact. Before filling the chamber, inspect the fluid to ensure it is clear, unless the white cell count is markedly raised, in which case the solution will appear turbid, and a greater dilution should be used. Fill the chamber and leave the cells to settle for 3 - 5 minutes.

5.2 Counting

Place the chamber on the microscope stage, and, using the x 25 objective, count the number of cells seen in a sufficient number of 1 mm² areas to obtain at least 100 cells, observing the criteria for inclusion and exclusion of cells touching the borders.

5.3 Calculation

Cell count (/l): = N x (D/A) x 10 x 10⁶
where N = total number of cells counted, D = dilution of blood (1:21); A = total area counted (in mm²), 10 = factor to convert area to volume (in µl), assuming a chamber of 0.1 mm depth, and 10⁶ = factor to convert count per µl to count per litre.

Example:
If 200 cells were seen in the four large corner squares (= 4 mm²),
WBC = 200 x (21/4) x 10 x 10⁶ = 1050 x 10 x 10⁶ = 10.5 x 10⁹/l
Expanded uncertainty of the 200 cell count was 28; therefore the true WBC range was 9.0 - 12.0 x 10⁹/l.

6. PLATELET COUNT

6.1 Dilution Procedure

Platelet diluting fluid consists of 1% ammonium oxalate. This must be prepared using scrupulously clean glassware and glass distilled water. Not more than 500 ml should be prepared at a time. This solution must be filtered through a micropore filter and stored at 4°C; the shelf life is at least one year, but it must be discarded if it becomes turbid.

For a 1:21 dilution of the blood, 20 µl blood should be added to 0.4 ml of diluting fluid. Mix on a mechanical mixer for about 10 minutes. Then fill the chamber as described for
the WBC and leave the preparation for 20 minutes (but no longer than 30 minutes) for the platelets to settle, in a wet chamber (i.e., petri dish with a small wad of damp blotting paper, filter paper or absorbent cotton wool).

6.2 Counting

Place the chamber on the microscope stage, and, using the x40 objective, focus on the central square. The platelets appear as small (but not minute) refractile particles and this characteristic is greatly enhanced using phase-contrast microscopy.

A count of about 200 will suffice as this has a variance of 7%. When the count is normal this number of counts will be obtained in 80 of the smallest squares (=0.2 mm²). If the platelet count is low it may be necessary to reduce the dilution or to count a larger number of areas, remembering to take this into account in the calculation.

6.3 Calculation

Platelet count (/l): \[ N \times \frac{D}{A} \times 10 \times 10^6 \]

where \( N \) = total number of platelets counted, \( D \) = dilution of blood (1:21), \( A \) = total area counted (in mm²), 10 = factor to convert area to volume in (µl), assuming a chamber of 0.1 mm depth, and \( 10^6 \) = factor to convert count per µl to count per litre.

**Example:**
If 200 cells were seen in 80 small squares (= 0.2 mm²),
Count = 200 x (21/0.2) x 10 x 10^6 = 200 x 105 x10 x 10^6 = 210 x 10^9/l.
Expanded uncertainty of this count was ±28; therefore the true platelet count range was 180 – 239 x 10^9/l.

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