MANUAL LEUKOCYTE COUNT

Principle

Whole blood is diluted with a 3% acetic acid solution, which hemolyzes mature erythrocytes and facilitates leukocyte counting. The standard dilution for leukocyte counts is 1:20. This dilution is prepared using the leukocyte Unopette system.¹ The dilution is mixed well and incubated to permit lysis of the erythrocytes. Following the incubation period, the dilution is mounted on a hemacytometer. The cells are allowed to settle and then are counted in specific areas of the hemacytometer chamber under the microscope. The number of leukocytes are calculated per µL (x 10⁹/L) of blood.

Reagents and Equipment

1. Two leukocyte Unopette reservoirs; each containing 0.475 mL of diluent:
   - Glacial acetic acid 28.6 mL
   - QS with distilled water to 1 liter
2. Two Unopette capillary pipets, 25 µL
3. Hemacytometer with cover glass
4. Petri dish with filter paper
5. Hand counter
6. Microscope

Quality Control

Commercial quality control materials with established control limits should be run periodically. The frequency is determined by each laboratory's workload. For instance, quality control material may be run at the beginning of each eight-hour shift.

Specimen

Whole blood, anticoagulated with EDTA, or free-flowing capillary blood may be used.

Procedure

1. Prepare two leukocyte Unopettes as follows:
   a. Using the protective shield on the capillary pipet, puncture the diaphragm as follows:
      1) Place reservoir on a flat surface. Grasping the reservoir in one hand, take the pipet assembly in the other hand and push the tip of the pipet shield firmly through the diaphragm in the neck of the reservoir, then remove.
b. Remove the shield from the pipet assembly with a twist and fill the capillary pipet with whole blood. Transfer the whole blood to reservoir as follows:

1) Wipe excess blood from the outside of the capillary pipet, making certain that no blood is removed from the capillary bore.

2) Squeeze the reservoir slightly to force out some air. Maintain pressure on the reservoir.

3) Cover opening of overflow chamber of the pipet with your index finger and seat the pipet securely in the reservoir neck.

4) Release pressure on the reservoir. Then remove your finger from the pipet opening. Negative pressure will draw the blood into the diluent.

5) Squeeze the reservoir gently two or three times to rinse the capillary bore, forcing diluent into, but not out of, the overflow chamber, releasing pressure each time to return the mixture to the reservoir.

6) Place your index finger over the pipet opening and gently invert several times to thoroughly mix the blood with diluent.

7) Let stand for 10 minutes to allow erythrocytes to hemolyze.

2. Clean the hemacytometer and cover glass by flooding them with 70% alcohol. Dry thoroughly with gauze or tissue; do not allow the alcohol to dry on the hemacytometer. Be sure to remove all lint. Place the cover glass in position over the ruled area.

3. Following incubation, mix diluted blood thoroughly by inverting reservoir to resuspend cells. Charge hemacytometer as follows:

   a. Convert to dropper assembly by withdrawing the pipet from the reservoir and reseating it securely in its reverse position.

   b. Clean the capillary bore by inverting the reservoir, gently squeeze the sides, and discard the first three or four drops.

   c. Place the pipet tip on the edge of the ruled area of the counting chamber. Carefully charge the hemacytometer with diluted blood by gently squeezing the sides of the reservoir to expel the contents until the chamber is properly filled.

   d. Using the same procedure, charge the other side of the hemacytometer with the second Unopette reservoir.

   e. Place the hemacytometer on moistened filter paper in a Petri dish, and allow to stand five minutes to permit the cells to settle.
4. Carefully place the hemacytometer on the microscope stage. Perform cell count as follows:

a. With the low-power (10x) objective, locate the ruled area. With proper light adjustment, the leukocytes should appear as dark dots.

b. Begin the counting procedure in the upper left square marked "W" (1 mm$^2$) as seen in Figure 7-7. Use the fine adjustment knob to focus up and down as needed to identify the leukocytes.

c. Using a hand counter, count all the leukocytes in the upper-left large square as follows:

1) Count the leukocytes in the first row of squares going from left to right, then from right to left in the second row; follow this pattern until all four rows are counted.

2) Within each square, count all leukocytes touching the top and left-hand borders. Do not count any cells touching the bottom or right-hand borders.

d. Repeat this counting procedure for the three other corner squares marked "W. Record the counts for each large (1 mm$^2$) square.

e. Count the leukocytes in the four corner squares ("W") on the opposite side of the hemacytometer. The difference between the highest and lowest count for the eight squares should not exceed 15 cells.

Calculations

1. The calculation formula for hemacytometer cell counts determines the number of cells within 1 μL (1 mm$^3$) of blood (Figure 7-9). To make this determination, the total number of cells counted must be corrected for the initial dilution of blood and the volume of diluted blood used. The standard dilution of blood for leukocyte counts is 1:20; therefore the dilution factor is 20. The volume of diluted blood used is based on the area and depth of the counting area. The area counted is 4 mm$^2$ and the depth is 0.1 mm; therefore the volume factor is 0.4 mm$^3$.

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\text{Total number of cells counted} \times \text{dilution factor} \times \frac{1}{\text{volume factor}} = \text{cells/mm}^3
\]

For example if 150 cells were counted in the four corner squares the WBC count is:

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150 \times 20 \times \frac{1}{0.4} = 7,500 \text{ cells/mm}^3 \text{ or } 7.5 \times 10^9/L
\]

2. Average leukocyte counts from the duplicate pipets and report result (x 10$^9$/L or /mm$^3$).
Reference Intervals

Adult 4.0-11 x 10^9/L
Newborn 9-30 x 10^9/L (18.1 x 10^9/L mean)

Comments

1. Leukocyte counts should be performed within three hours following dilution in Unopette reservoir.  
2. The moistened filter paper retards evaporation of diluted specimen while cells settle on the hemacytometer.
3. Technical sources of error in hemacytometer cell counts are given in Web Table 7-4.
4. Physiologic sources of error in hemacytometer cell counts
   a. Errors resulting in false low count:
      1) Difficulty in obtaining capillary blood – cells become diluted with plasma
      2) Excessive tissue trauma during collection of blood resulting in cell clumping
5. The presence of nucleated erythrocytes results in a falsely elevated leukocyte count, since they are indistinguishable from leukocytes at 100x magnification and are preserved in 3% acetic acid. A leukocyte differential must be performed to distinguish between nucleated erythrocytes and leukocytes. The number nucleated erythrocytes per 100 leukocytes is noted. The correction formula (Web Figure 8-2) is applied to the leukocyte count when five or more nucleated erythrocytes are observed during the 100-cell differential.

References