

Red blood cell (RBC) count

Red blood cell (RBC) count is typically ordered as a part of complete blood count (CBC) and may be used as a part of health checkup to screen for variety of conditions. It is an important test because the number of (RBCs) affects how much oxygen the tissues receive.

➤ Normal Value:

Males: 5.2 (4.5 to 5.9) M/mm³

Females: 4.6 (4.1 to 5.1) M/mm³

➤ Causes of High RBC count (polycythemia/Erythrocytosis):

1. Cardiac or pulmonary disease, living at high altitude; when the patient suffers from chronic hypoxia, the body tries to compensate by producing more red blood cells.
2. Tumors that produce excess erythropoietin e.g. hepatocellular carcinoma and renal cell carcinoma.
3. Smoking.
4. Dehydration; as the volume of fluid in the blood drops, the count of RBCs per volume of fluid artificially rises. (Relative polycythemia)
5. Polycythemia vera, a bone marrow disease that causes overproduction of RBC.

➤ Causes of Low RBC count (Anemia):

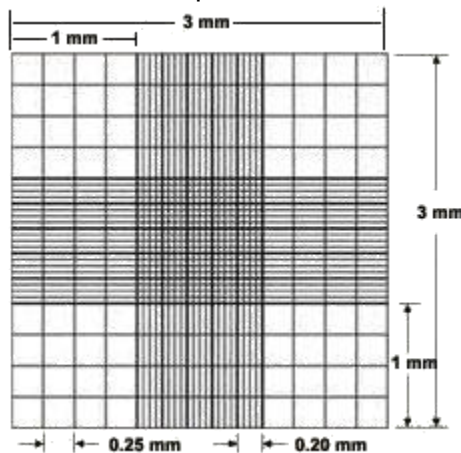
1. Internal or external bleeding
2. Red blood cell destruction, as in hemolytic anemia.
3. Nutritional deficiency such as iron deficiency or vitamin B12 or folate deficiency
4. Bone marrow disorder (aplastic anemia) or suppression by drugs, chemotherapy, irradiation.
5. Kidney failure, decreased production of erythropoietin.

EXPERIMENT 1 RBC COUNTING

1. By the usage of **Hemocytometer**; a special microscope slide on which precise grids have been etched within the counting chamber designed to hold an exact volume of diluted blood sample.
2. Clean it very well.
3. Place a coverslip over the counting area.
4. Dilute the blood sample to the ratio of 1:200, this is done by adding 199 units of solvent to 1 unit of blood.

NOTE: Dilution Factor = $\frac{\text{Final volume}}{\text{Volume of blood}}$

5. Make sure that the blood is thoroughly mixed before adding it to the diluting fluid and before loading.
6. Draw small amount with pipette and gently touch the junction of the coverslip and hemocytometer. The diluted blood will flow by capillary attraction to fill the chamber. Wait 3 minutes allowing RBC to settle.
7. First use the 10X lens to identify the center square (1 mm²), then use 40X lens to focus on the smaller squares (0.04 mm²).



8. Count cells in five small squares (0.04 mm², 4 corners and center) of the grid and obtain an average number.
9. If there are some cells touch the boundaries, count those on two boundaries and exclude the others.
10. The final result expresses the number of red blood cells per cubic millimeter of the original blood sample:
 - For example, you have found 120 cells.
 - The volume of small cube (0.2×0.2×0.1=0.004 mm³)
 - The average that you have counted (120) is the number of cells in 0.004 mm³, find how many cells in 1 μL by multiplying by (1/ 0.004 =250), So (120×250=30,0000).

- The dilution factor is 200, so the final step is multiplying the number that you have got (30,000) by the dilution factor. ($30,000 \times 200 = 6,000,000$ RBC/ mm^3)
- Note that $1\mu\text{L} = 1\text{ mm}^3$

White Blood Cell (WBC) count

This test is often included in the complete blood count (CBC), it is done to get an impression about the immune system since the Leukocytes (WBC) play an integral role, to get more informative results it is often combined with the differential count (Experiment 3).

- Normal Value: 4400 - 11,000 cells/ μL . In children, the normal WBC count varies somewhat with age.
- Causes of High WBC count (Leukocytosis):
 1. Any active inflammatory condition or infection.
 2. Malignancy such as Leukemia.
 3. Recent vigorous exercise, thermal burn, electric shock, surgery, or trauma.
 4. Certain medications e.g. glucocorticoids (neutrophilia)
 5. Dehydration.
- Causes of Low WBC count (Leukopenia):
 1. Bone marrow failure; aplastic anemia, fibrosis, metastatic cancer and infection.
 2. HIV.
 3. Autoimmune diseases.

EXPERIMENT 2 WBC COUNTING

The technique is similar to that used in the previous experiment, with the following EXCEPTIONS:

- The blood is diluted in a ratio of 1:20, so the dilution factor will be 20.
- The counting squares are four of the 1 mm^2 , the corners of the grid chamber.
- Volume of one of these cubes = $1 \times 1 \times 0.1 = 0.1\text{ mm}^3$
- Usage of 10X lens
- Calculation:
- WBC count = Average number of cells $\times 20 \times 10 = (X)\text{ cell/ mm}^3$

EXPERIMENT 3
DETERMINATION OF DIFFERENTIAL LEUKOCYTE COUNT

In the differential count the percentage of each type of WBC is determined. WBCs are divided into two types depending on the presence of cytoplasmic granules. Granulocytes (Neutrophils, Eosinophils and Basophils) and Agranulocytes (lymphocytes and monocytes).

Neutrophils	65% of total WBC polymorphonuclear cells (PMNs)
	Three-lobed purple nucleus, fine pink cytoplasmic granules. They are called neutrophil because their granules are not very amenable to staining with either acidic or basic dyes.
Eosinophils	2-4% of total WBC
	Bilobed blue-purple nucleus, coarse red-orange cytoplasmic granules.
Basophils	0.5% of total WBC
	Bilobed blue-black nucleus, Large deep blue cytoplasmic granules.
Lymphocytes	25% of total WBC
	Can be large or small. They have very large and spherical nucleus that occupies most of the cytoplasm. The cytoplasm has no granules.
Monocytes	1-7% of total WBC
	Large cells, They have large indented nuclei, often kidney-shaped and non-granular cytoplasm.

- **Absolute neutrophil count (ANC)** = $\text{WBC (cells/ } \mu\text{L)} \times \text{percent (PMNs+bands)} \div 100$
- **Neutrophilic leukocytosis;** Neutrophilic leukocytosis is defined as a total WBC above 11,000/ μL along with an absolute neutrophil count (ANC) greater than 7700/ μL .
- **Neutrophilia;** An elevated ANC greater than 7700/ μL in the presence of a total WBC count less than 11,000/ μL .
- Neutrophilic leukocytosis is commonly seen in infection, stress, smoking, pregnancy, and following exercise.
- **Lymphocytic leukocytosis** can be seen following infections such as infectious mononucleosis, mumps, rubella and pertussis or in lymphoproliferative disorders such as the acute and chronic lymphocytic leukemias.

- **Monocytic leukocytosis** can be seen in acute bacterial infection or tuberculosis.
- **Eosinophilic leukocytosis** can be seen in infection with helminthic parasites and in the allergic reactions.
- **Neutropenia;** is defined as an ANC <1500 cells/ μL , occurs due to decreased production of neutrophils from the bone marrow (nutritional deficiencies as vitamin B12, folate, copper/ myelodysplastic syndromes), Immune destruction of circulating neutrophils, which can be the result of a drug reaction or an autoimmune disorder.
- **Lymphocytopenia;** causes include infections such as human immunodeficiency virus (HIV), and chemotherapy or immunosuppressive therapy, including glucocorticoids.

EXPERIMENT 4 IDENTIFICATION OF RETICULOCYTE

Reticulocytes are the immediate precursor of fully formed RBCs, they contain a remnant of basophilic ribosomal RNA. The reticular network can be seen in reticulocytes only in the presence of certain supravital dyes, such as methylene blue. Reticulocytes can be appreciated on a standard blood smear stained with Wright-Giemsa as RBC with a blue tint, larger than mature RBC, with irregular borders and a lack of central pallor. Reticulocytes can be counted with more accuracy via automated blood counters after staining with a fluorescent dye, such as thiazole orange, which binds to the RNA of reticulocytes.

In normal subjects, reticulocytes comprise about 1 percent of the red cell population. An increased percentage of reticulocytes is seen in response to bleeding or hemolysis.

Absolute reticulocyte count – accurately reflecting the degree of reticulocytosis regardless of the degree of anemia. The normal absolute reticulocyte count is between 25,000 to 75,000/ μL (i.e. approximately 1 percent of an absolute RBC count of 5,000,000 cells/ μL).

EXPERIMENT 5 DETERMINATION OF HEMOGLOBIN CONCENTRATION

In the clinical practice the Hb concentration is usually measured based on cyanmethemoglobin; in which blood is mixed in a solution containing potassium cyanide and potassium ferricyanide. The potassium ferricyanide converts Hb to methemoglobin which is converted to cyanmethemoglobin (HiCN) by potassium cyanide. The absorbance of the solution is then measured in a spectrophotometer or colorimeter.

Other method for measuring the hemoglobin concentration is by an instrument known as a hemoglobinometer (SAHLI method), which compares the color of light passing through a hemolyzed blood sample with a standard color. Blood is mixed with an acid solution so that hemoglobin is converted to brown-colored acid hematin. Which then diluted with water till the brown color matches that of the brown glass standard. The hemoglobin value is read directly from the scale.

Procedure (SAHLI method)

1. After ensuring the hemoglobin pipette and tube are dry.
2. add five drops of 0.1N HCl into the tube
3. Mix the EDTA sample gently and fill the pipette with 0.02ml blood
4. Wipe the external surface of the pipette to remove any excess blood.
5. Add the blood into the tube containing HCl. Wash out the contents of the hemoglobin pipette by drawing in and blowing out the acid few times so that the blood is mixed with the acid thoroughly.
6. Allow to stand undisturbed for 10min. (This is because, maximum conversion of hemoglobin to acid hematin, occurs in the first ten minutes)
7. Place the hemoglobinometer tube in the comparator and add distilled water to the solution drop by drop.
8. Stir with the glass rod till its color matches with that of the comparator glass.
9. While matching the color, the glass rod must be removed from the solution and held vertically in the tube.
10. The reading of the lower meniscus of the solution should be noted as the result.
11. Express the hemoglobin content as g/dl

Normal ranges for children vary with age and sex. The range for a normal hemoglobin level may differ from one medical practice to another.

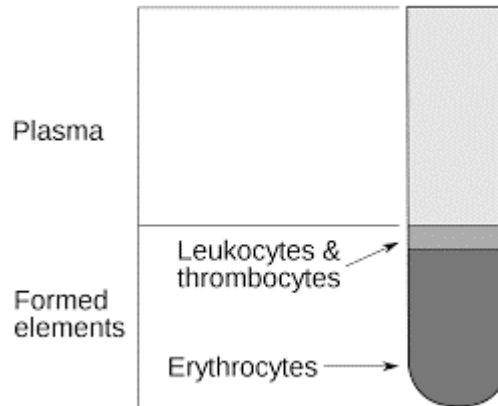
For men, 14 to 17 g/dl

For women, 12 to 15 g/dl

EXPERIMENT 6
Hematocrit (HCT) Packed cell volume (PCV)

HCT is the ratio of the volume of packed red cells to the total blood volume.

It is determined by centrifuging the blood in heparinized capillary tubes, the red blood cells become packed at the bottom of the tube, while the plasma is left at the top as a clear liquid.



$$\text{HCT} = \frac{\text{height of red cells (mm)}}{\text{height of red cells and plasma (mm)}} \times 100$$

Most labs use a hematocrit reader that reads the HCT value directly.

Normal range:

Males 43-49%

Females 36-45%

The HCT may fall to as low as 15% in severe anemia or rise to as high as 70% in polycythemia.

EXPERIMENT 7

Erythrocyte sedimentation rate (ESR)

The ESR is a simple non-specific screening test that indirectly measures the presence of inflammation in the body. It reflects the tendency of red blood cells to settle more rapidly in the face of some disease states, usually because of increases in plasma fibrinogen, immunoglobulins, and other acute-phase reaction proteins.

Method: When anticoagulated whole blood is allowed to stand in a narrow vertical tube for a period of time, the RBCs – under the influence of gravity - settle out from the plasma. The rate at which they settle is measured as the number of millimeters of clear plasma present at the top of the column after one hour (mm/hr).

ESR values increase markedly with age and are slightly higher among women than men. As a result, any single set of normal values will not be valid for the population at large. One can roughly correct ESR for age by using the following formulas:

The upper limit of the reference range= (age in years)/2 for men
(age in years + 10)/2 for women.

Or roughly; men are: <15mm/hr , women: <20mm/hr

The ESR can also be affected by changes that may be unrelated to inflammation, including changes in erythrocyte size, shape, and number; and by other technical factors (tilted ESR tube, high room temperature)

Some interferences which decrease ESR:

- Abnormally shaped RBC (sickle cells, spherocytosis, and polycythemia).
- Technical factors: low room temperature, delay in test performance (>2 hours), clotted blood sample, excess anticoagulant, bubbles in tube.

EXPERIMENT 8

Osmotic Fragility Test

Introduction:

The osmotic fragility test (OFT) is used to measure erythrocyte resistance to hemolysis while being exposed to varying levels of dilution of a saline solution. The susceptibility of osmotic lysis of erythrocytes is a function of surface area to volume ratio.

0.9% NaCl solution is said to be **isotonic**: when blood cells reside in such a medium, the intracellular and extracellular fluids are in osmotic equilibrium across the cell membrane, and there is no net influx or efflux of water.

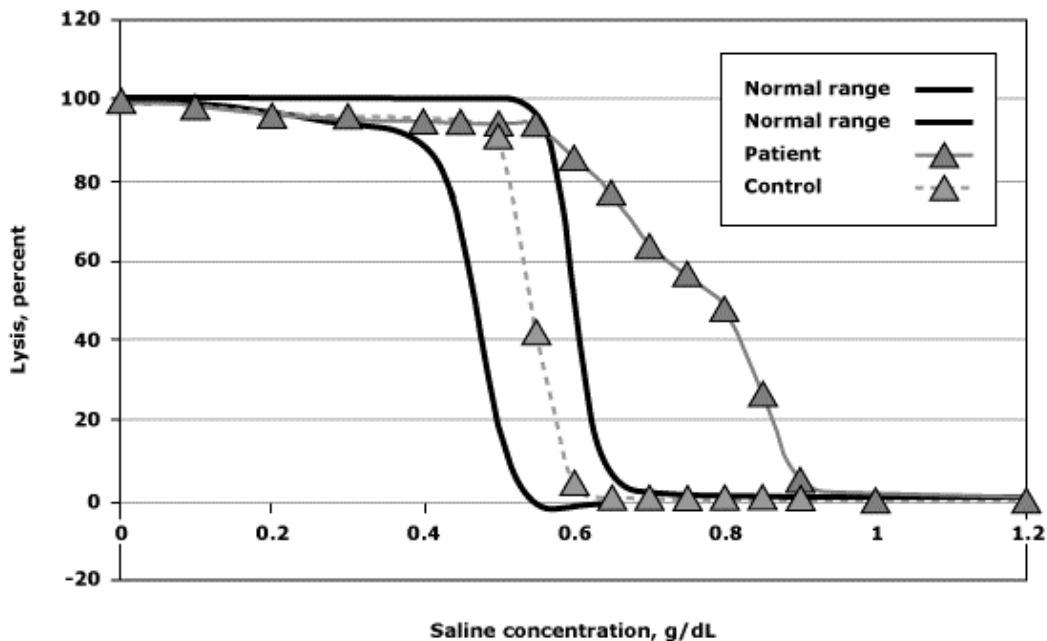
When subjected to *hypertonic* media (e.g. 1.8% NaCl), the cells lose their normal biconcave shape, undergoing collapse (leading to **crenation**) due to the rapid osmotic efflux of water.

On the other hand, in a *hypotonic* environment (e.g. 0.4% NaCl or distilled water), an influx of water occurs: the cells swell, the integrity of their membranes is disrupted, allowing the escape of their hemoglobin (**hemolysis**) which dissolves in the external medium.

Procedure:

- Put 20 labelled centrifuge tubes in a rack.
- Add 10 ml of different NaCl concentrations, starting with 0.85% till 0.25%.
- Add one drop of blood to each tube.
- Shake it well and put them in the centrifuge.
- For normal RBCs; from 0.85% to 0.45% there is no hemolysis.
- At the concentration of 0.4% and less, you can notice that the hemolysis has begun and the solution became red in color, but there are some settled RBCs in the tube.
- At the concentration of 0.33%, the solution is clear red and there are no settled RBCs (complete hemolysis).
- You can also use pipette to transfer enough of the supernatant fluid from each tube into spectrophotometer cuvettes.
- Calculate the percentage of hemolysis for each solution. Then plot the results against the NaCl concentrations, yielding an osmotic fragility curve that is then compared to obtained normal control values.

The osmotic fragility test in hereditary spherocytosis



This figure shows results of an incubated osmotic fragility test performed on red cells from an adult patient with hereditary spherocytosis, showing markedly increased osmotic fragility. Note that the patient's red cells were >60 percent hemolyzed at a saline concentration (0.7 g/dL) that did not cause any osmotic lysis in normal, incubated red cells.

Provided by Dr. Rebecca Connor.

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Conditions associated with increased osmotic fragility (sooner hemolysis) include the following:

- Spherocytosis
- Severe burns

Because spherocytes have already reduced surface area to volume ratio they lyse in less hypotonic solution than normal-shaped, biconcave RBC and have increased osmotic fragility.

Eosin-5-maleimide binding test — a flow cytometric test based on interaction between the dye eosin-5-maleimide and band 3 protein has been proposed as a rapid diagnostic test for hereditary spherocytosis.

The following conditions are associated with decreased fragility:

- Thalassemia
- Iron deficiency anemia
- Sickle cell anemia

EXPERIMENT 9

Blood Typing

Introduction:

The human RBC has around 30 antigens on its membrane. In blood typing they are called agglutinogens. These agglutinogens react with complementary agglutinins (antibody) in a process known as agglutination. Most of the agglutination reactions that occur in transfusion are caused by two antigen-antibody systems ABO and Rh. Therefore blood transfusion cannot be performed indiscriminately between the different types. If incompatibility occurs, the immune system will attack the new blood cells and destroy them.

ABO system:

Antigens and the non-complementary antibodies of the ABO system are genetically predetermined in contrast to the other plasma antibodies that are synthesized in response to specific antigens.

Blood type	Antigen	Antibody	Note
A	A	Anti-B	
B	B	Anti-A	
AB	A , B	Neither	Universal Recipient
O	Neither	Anti-A, Anti-B	Universal Donor

Rh system:

Rh antigens, named for the rhesus monkey in which they were first discovered by Landsteiner and Wiener. There are a few Rh antigens (commonest one is called D). Red cells expressing the Rh antigens are called Rh positive. Red cells which do not express this surface antigen are Rh negative (about 15% of the human population).

In contrast to ABO system there is no preformed Anti-D in the Rh-ve individual.

Rh incompatibility between mother and fetus; in such a case, the antibody-mediated cytotoxicity mechanism involved threatens the well-being of the fetus.

During birth, a leakage of the baby's red blood cells often occurs into the mother's circulation. If the baby is Rh positive (inheriting the trait from its father) and the mother is Rh negative, these red cells will cause the mother to manufacture antibodies against the Rh antigen. The antibodies (IgG class) do not cause problems for that first born, but can cross the placenta and attack the red cells of a subsequent Rh+ fetus. The red cells are destroyed, leading to anemia and jaundice. The disease - erythroblastosis fetalis or hemolytic disease of the newborn- may result in fetal death.

Determination of Blood Group Procedure:

1. Obtain a clean microscopic slide.
2. Lance the tip of your finger, place three separate drops of blood on the slide.
3. Add one drop of Anti-A to the first drop, Anti-B to the second drop, and Anti-D to the third drop.
4. Mix well, using separate wooden sticks.
5. The results are read directly from the slide. The subject is blood group A if agglutination occurred with the Anti-A test serum; group B if agglutination occurred with the Anti-B test serum; group AB if agglutination occurred with both test serums, and O if there was no agglutination in either case.
6. If agglutination occur in the Rh drop, the subject is considered as Rh+ve.
7. The strength of agglutination reaction is not the same in all people, in some case it may be necessary to examine the drops under microscope.

EXPERIMENT 9

Bleeding Time

A bleeding time evaluation is used to measure the primary phase of hemostasis, which involves platelet adherence to injured capillaries and then platelet activation and aggregation. The bleeding time can be abnormal when the platelet count is low or the platelets are dysfunctional. Causes of abnormal bleeding time can be hereditary or acquired.

Disadvantages: Insensitive, Invasive & high inter-operator variability.

Normal range: 3-5 minutes

1. Clean the tip of your finger with 70% alcohol.
2. Puncture the finger with a lancet to make a wound that is 3 mm deep and start the stopwatch.
3. At the 30 seconds interval wipe the blood drop by the filter paper.
4. Repeat the procedure until no more blood is absorbed on the filter paper.
5. Record the time.

EXPERIMENT 10

Clotting Time

Measurement of the time that is required for a sample of blood to coagulate in vitro under standard conditions. Clotting time depends on the availability of coagulation factors. It is prolonged in conditions like hemophilia, vitamin K deficiency, liver diseases, and overdose of anticoagulants (warfarin).

Normal value 3 to 8 minutes.

1. Clean the tip of your finger then puncture it to obtain large drop of blood.
2. Draw the blood into two nonheparinized capillary.
3. Start the stopwatch.
4. After 2 minutes, start breaking the capillary tube at 1 cm distance to see whether a thread of coagulated blood is formed between the two broken ends.
5. Stop the watch and calculate the time from average of the two capillary tubes.

References:

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