Hemo-cyto-meter:

Hemocytometer consists of:
1. Improved Neubuer chamber

2. Two pipettes
   a. WBC diluting pipette:
      Characterizes by:
      1. It has the numbers 0.5, 1, and 11
      2. It has a white bead
      3. White mouth piece
   b. RBC diluting pipette:
      Characterizes by:
      1. It has the numbers 0.5, 1, 101
      2. It has a red bead.
      3. Red mouth piece
Procedure:
1. With a safety bulb draw up to 0.5 marks on RBC's pipette blood and complete to 101 with Hayem's solution.
2. Mix for 2 - 3 minute
3. Charge hemacytometer:
   Load the counting chamber with diluted blood as follows:
   Discard the first 4-5 drops.
   Place tip of the pipette at edge of the central platform of hemacytometer slide and let a drop of diluted blood run between the hemacytometer slide and cover slip by capillarity.
4. Let the hemacytometer to stand on the bench for 3 - 5 minute so the cells are settled down.
5. Count and calculate:
   An erythrocytes count is performed with a Neubauer hemacytomcter as follows:
   a. Using 40 X magnification, count the erythrocytes in the four (4) corner squares and the one in the center of the large center square of the counting chamber.
   Count all cells that touch any of the upper and left lines, do not count any cell that touches a lower or right line.
   b. Make your calculations:
RBC's/mm³ = number of cells in 5 squares (80 small square) X VCF X DF
The dilution factor (DF) = total volume / sample volume
= 101-1/0.5=200
Volume correction factor (VCF) = 1 mm³ / total volume of the five medium squares
The total volumes of the five medium squares =
Volume of each small square x total number of small squares:
Volume of each small square = (width x length x height)
= 1/20 mm x 1/20 mm x depth 1/10 mm = 1/4000 mm³
Total number of small squares = 5 x 16 = 80 (each of the five medium squares has 16 small squares)
Then the total volumes of the five medium squares = 1/4000 x 80 = 1/50 mm³
So the volume correction factor = 1(1/50) = 50
Example:
If total number of RBC counted in the five medium squares is = 423
Then the number of RBC in 1 mm³ blood = 423 X 200 X 50 = 4,230,000
Hematocrit or Packed cell volume (PCV)

Procedure:
1. Draw well-mixed anti-coagulated blood into two micro-hematocrit tubes by capillary action avoiding air bubbles. The tubes should be filled about 3/4 full.
2. Wipe off excess blood with a Kim wipe or gauze.
3. Seal one end of each tube with a small amount of clay material at a 90° angle. Be sure the seal has a perfectly flat bottom.
4. Place the filled and sealed capillary tubes into the centrifuge. The sealed end should point toward the outside of the centrifuge. The duplicate samples should be placed opposite each other in order to balance the centrifuge. Record the position number of each specimen.
5. Securely fasten the flat lid on top of the capillary tubes.
6. Centrifuge for 5 minutes at a set speed (11000 rpm). This separates the RBCs from plasma and leaves a band of buffy coat consisting of WBCs and platelets
7. Allow the centrifuge to stop on its own. Do not use the hand brake.
8. After the centrifuge has stopped, open the top and remove the cover plate.
9. Promptly read the hemotocrit on the hematocrit reader. (Instructor will review directions on using the hematocrit reader).
Do not include the buffy coat layer.

\[ \text{Hct (\%)} = \frac{\text{Height of Packed Red Cells (mm) \times 100}}{\text{Height of Packed Red Cells and Plasma (mm)}} \]

Estimation of blood hemoglobin

**Practical part:**

**Sahli’s Method of Hemoglobin Determination:**

**Apparatus:**

- Sahli’s tube which is having red and yellow scales on two sides
- Red scale is percentage scale and yellow scale is gram percentage or g/100ml scale.
- Hemometer which is having two standards Sahli’s pipette
- Error percentage is 3\%.
Procedure:
In sahli’s tube, take N/10 hydrochloric acid: HCL which is (1/10th of the original HCL) up to 10th level of scale.
In sahli’s pipette, take 0.02ml (20microleter) blood.
Add blood from pipette into tube. HCL will cause the lysis of the blood cells and hemoglobin is released. Hemoglobin after combining with HCL, forms acid hematin which is of tan color (i.e. to change all forms of hemoglobin to acid hematin to standardize the color index)
Put tube in the hemometer and continuously add drops of distilled water and shake with the stirrer until color matches. Then, take the reading.
Drabkin’s method cyanide
The method of choice for hemoglobin determination is the cyanmethemoglobin method (This is a type of colorimetric method). The principle of this method is that when blood is mixed with a solution containing potassium ferricyanide and potassium cyanide, the potassium ferricyanide oxidizes iron to form methemoglobin. The potassium cyanide then combines with methemoglobin to form cyanmethemoglobin, which is a stable color pigment read photometrically at a wave length of 540nm.
Three advantages of the cyanmethemoglobin method are:
1. measures all forms of hemoglobin except sulfhemoglobin
2. can be easily standardized
3. cyanmethemoglobin reagent (also called Drabkin's solution) is very stable

Erythrocyte sedimentation Rate (ESR)

Practical part:
Procedure:
EDTA or any suitable anticoagulant is prepared in a test tube. Obtain 2ml of blood from a superficial vein.
Gently transfer the blood into the test tube; rotate carefully allowing a good mix of the blood with anticoagulant.
Do not shake, as this would cause bubbles to form in the blood and anticoagulant mixture.
Put the Westergren pipette in the tube that contains anti-coagulated blood, the blood is shucked carefully in the Westergren pipette up to zero mark. Fix the Westergren pipette vertically on a sedimentation pipette rack avoiding leakage of blood. Allow the pipette to remain undisturbed for exactly 1 hr.
At the end of that time, the upper portion of the blood column will appear clear because erythrocytes will have settled from that region. Read from the graduated scale on the pipette the number of millimeters that the erythrocytes have settled below the zero mark. Record the erythrocyte sedimentation rate in millimeters per hour (mm/hr).
Caution:
No air bubble should be trapped in the blood placed in Westergren pipette.
The Westergren pipette should be absolutely vertical. Slight inclination gives false reading of accelerated ESR and also disturbs the sedimentation forces of cells. Blood should at no stage show any signs of hemolysis or coagulation. Blood should be used within 2 hours of collection.

**Blood Typing**

**Practical part:**
1. Obtain a drop of blood from a finger prick in each of the circles of the disposable blood group slide.
2. Add one drop of Anti A (blue) and a drop of Anti B (yellow) and a drop of Anti D sera in the proper circles on the disposable blood group slide.
3. Using clean (un-contaminated) glass rod to mix the blood with antiserum.
4. Tilt the slide from side to side occasionally, after 2 minutes read for agglutination.

Leukocytes (White Blood Cells) count

Practical part:
1. Swab the tip of a little-used finger with 70% ethanol.

2. Lance with quick, firm jab to the side of the pad of the finger, wipe away first blood.

3. Using dilution pipit with the WHITE mixer, draw up to the 0.5 mark. Do not allow blood to congeal in pipette.

4. Immediately draw diluting fluid to the "11" mark while rotating the pipet between the thumb and forefinger to mix the specimen and diluent. Hold the pipet upright to prevent air bubbles in the bulb.
5. Mix the contents of the pipette for 3-5 minutes to ensure even distribution of cells. Expel unmixed and relatively cell-free fluid from the capillary portion of the pipette (usually 4 drops).

6. Place the forefinger over the top (short end) of the pipet, hold the pipet at a 45 angle, and touch the pipet tip to the junction of the cover glass and the counting chamber. Allow the mixture to flow under the cover glass until the chamber is completely charged. Similarly, fill the opposite chamber of the hemacytometer.
NOTE: If the mixture overflows into the moat or air bubbles occur, clean and dry the chambers, remix the contents of the pipette, and refill both chambers.

7. Allow the cells to settle for about 3 minutes. Under low-power magnification and reduced light, focus on the ruled area and observe for even distribution of cells.

8. Count the white cells in the four 1 sq mm corner areas corresponding to those marked A, B, C, and D in each of two chambers.

9. Count all the white cells lying within the square and those touching the upper and right-hand center lines. The white cells that touch the left-hand and bottom lines are not to be counted. In each of the four areas, conduct the count as indicated by the "snake-like" line. A variation of more than 10 cells between any of the four areas counted or a variation of more than 20 cells between sides of the hemacytometer indicate uneven distribution and require that the procedure be repeated.

**Calculation:**

(1) Routinely, blood is drawn to the 0.5 mark and diluted to the 11 mark with WBC diluting fluid. All the blood is washed into the bulb of the pipet (which has a volume of 10). Therefore, 0.5 volumes of blood are contained in 10 volumes of diluting fluid. The resulting dilution is 1:20. (These figures are arbitrary and refer strictly to dilution and not to specific volumetric measurements.)

(2) The depth of the counting chamber is 0.1 mm and the area counted is 4 sq mm (4 squares are counted, each with an area of 1.0 sq mm therefore, 4 x 1.0 sq mm = a total of 4 sq mm). The volume counted is: area x depth = volume.

Four sq mm x 0.1 mm = 0.4 cu mm

(3) The volume correction factor =1 / 0.4 = 2.5

If there are n cells in 0.4 mm$^3$ diluted blood, then the number of cells in 1 mm$^3$ blood

= n x dilution factor x volume correction factor

= n x 20 x 2.5

= n x 50

**Example:**

If the total number of WBCs counted in the four large corner square is = 120.

Then the number of WBCs in 1 mm$^3$ blood =120 x 50 = 6000 cell.
Leukocytes (WBC) differential count

Practical part:
The WBC differential begins with preparation and staining of a blood smear.

First step: preparing the blood smear.

Step 1. Placing a small drop of venous blood on a glass microscope slide, using a glass capillary pipette. A wooden applicator stick can also be used for this purpose.
Step 2. A spreader slide has been positioned at an angle and slowly drawn toward the drop of blood.

Step 3. The spreader slide has been brought in contact with the drop of blood and is being drawn away. Note layer of blood at the edge of the spreader slide.

Step 4. The spreader slide is further pulled out, leaving a thin layer of blood behind.

Step 5. The blood smear is nearly complete.
Note:

1- A satisfactory blood smear is evenly distributed; the red cells, being close together but not over lapping. Check that by examining the smear under the microscope before staining it.

2- The good blood smear:
   a- It is neither thin nor thick (rainbow appearance)
   b- It should not touch the sides of the slide
   c- It should form a tail.

Second step:

Staining of the smear:

Do not stain the film until you have made a satisfactory blood smear.

1- Holding the blood slide by edges of one end, lower it into a staining dish containing quick-stain (Wright and Giemsa stains) and leaves it in the stain for 5 sec).

2- Transfer the slide to a second dish containing distilled water and let it remain in the water for 5-10 sec.

3- Rinse the slide quickly with tap water by allowing the stream of water to strike the edge of the slide and flow across the blood smear; the water should not strike the blood smear directly.
4- Following complete dryness of the slide, examine it under a microscope using an oil immersion lens. Place a drop of immersion oil on a region of the slide where the blood film is thin (on the end of the slide opposite that to which the drop of blood was applied).
5- Identify as many types of blood cells on the slide as you can.
Estimation of clotting mechanism

Clotting mechanism:

Practical Part:
Blood coagulation tests:

Bleeding Time:
The bleeding time is the time taken from the puncture of the blood vessel to the stoppage of bleeding. The bleeding time test is a useful tool to test for platelet plug formation and capillary integrity. The bleeding time is more important than Clotting time.

Principle:
Bleeding time assays are used to evaluate the vascular (effectiveness of vasoconstriction) and platelet responses that are associated with hemostasis. When a sharp-pointed knife is used to pierce the tip of the finger or lobe of the ear, bleeding ordinarily lasts for 1 to 6 minutes. The time depends largely on

1. the depth of the wound
2. the degree of hyperemia in the finger or ear lobe at the time of the test.

Lack of any one of several of the clotting factors can prolong the bleeding time, but it is especially prolonged by lack of platelets.

Method:
Bleeding time assay is referred to as the Duke method and in this assay bleeding should cease within 1–5 minutes.

A less invasive bleeding time assay involves the use of a lancet or special needle and a 3–4mm deep prick is made on the fingertip or earlobe.

Bleeding times by Duke’s method:
1. Clean and sterilize either the lobe of the ear or the tip of the little finger of your subject with 70% alcohol.
2. Allow the alcohol to dry and make a wound about 4mm deep in the ear lobe or the finger using a sharp lancet.
3. Remove the blood from the wound every 30 seconds on a clean piece of filter paper, using a different area of the paper each time.
4. Continue until the bleeding stops.
5. Count the spots of blood on the filter paper divided by 2 and that will give you the bleeding time in minutes. The usual time is about 2-6 minutes.

Bleeding times by Ivy method:
1. Clean the anterior surface of the forearm with spirit.
2. The blood pressure cuff is placed on the upper arm and inflated to 40 mmHg.
3. A lancet or scalpel blade is used to make a shallow incision that is 1 millimeter deep on the anterior of the forearm.
4. The time from when the incision is made until all bleeding has stopped is measured and is called the bleeding time. Every 30 seconds, filter paper or a paper towel is used to draw off the blood. Normal BT by this method is 3-6 minutes.

Interpretation:
The bleeding time is affected (prolonged) by any defect in platelet function, by vascular disorders, and in von Willebrand disease but is not affected by other coagulation factors.
Disorders that are commonly associated with an increased bleeding time include:
1. Thrombocytopenia,
2. Inherited plasma defect (e.g. disseminated intravascular coagulation (DIC) and factor V)
Abnormal bleeding times are also found in patients with Cushing syndrome, severe liver disease, leukemia, and bone marrow failure.

- Vascular abnormality
- Drug (e.g. aspirin and anti-histamine)
- Thrombo-asthenia (platelet number is normal but function is abnormal)

**Clotting time:**
Clotting Time is the time taken from the puncture of the blood vessel to the formation of a fibrin thread.

**Principle:**
Whole blood, when removed from the vascular system and exposed to a foreign surface, will form a solid clot.
The time required for a sample of blood to coagulate in vitro under standard conditions is called "clotting time".
The whole blood clotting time is a rough measure of all intrinsic clotting factors in the absence of tissue factors.
Variations are wide and the test sensitivity is limited.
This procedure has been replaced in most laboratories with the APTT.
It is not sensitive to platelet abnormalities.
A prolonged clotting time immediately indicates impaired coagulation, but a normal clotting time does not exclude many serious clotting defects.

**A. Capillary Glass Tube Method:**

**Method:**

1. Clean the finger and sterilize it with 70% alcohol.
2. Obtain a capillary tube labeled “coagulation tubes” or “non-heparinized” tubes.
3. Prick your third fingertip and allow a large drop of blood to collect.
4. Hold the capillary tube horizontally, with one end in the drop of blood, and fill it quickly. Note the time at which the tube is filled.
5. Each 30 sec thereafter, break off about 1 cm of the tube (a paper towel can be used to shield the tube) and gently pull the piece away from the rest of the tube.
6. Continue breaking the tube and pulling away the broken segment until you see fibers or strands in the blood between the broken segment and the rest of the tube.
The strand or fibers are evidence of clotting and, when they are observed, the time should again be noted. The interval between the time that the tube was filled and the time that coagulation was observed is known as the clotting time or coagulation time.

Normal clotting time by this method: 3-8 minutes

B. Lee & White method:

Method:

The one most widely used is to collect blood in a chemically clean 8 mm diameter glass tube and then to tip the tube back and forth about every 30 seconds until the blood has clotted.

By this method, the normal clotting time is 6 to 12 minutes. Procedures using multiple test tubes have also been devised for determining clotting time more accurately.

Unfortunately, the clotting time varies widely, depending on the method used for measuring it, so it is no longer used in many clinics. Instead, measurements of the clotting factors themselves are made, using sophisticated chemical procedures.

Interpretation:

The clotting time is prolonged if there is deficiency in one of the coagulation factor of the intrinsic pathway.

Causes of prolonged clotting time:
1. Vitamin K deficiency as obstructive jaundice & prolonged use of antibiotics that will kill intestinal flora
2. Liver cell failure
3. Hemophilia: factor II, VIII, IX, X and XI deficiency
4. Patients receiving dicumarol or heparin (anticoagulant drugs).

Thrombocytopenia bleeding time increased, clotting time normal.

Hemophilia bleeding time normal, clotting time increased.

**Capillary Fragility Test:**

Capillary Fragility Test (also called the Hess test or Rumpel-Leede test or tourniquet test) is a medical test used to assess mechanical capillary fragility by raising pressure within them.

**Method:**

1. A circle of 5 cm diameter is marked on the forearm.
2. Any purple spots present in the circle are marked with blue ink.
3. Pressure is applied to the forearm with a blood pressure cuff inflated to between systolic and diastolic blood pressure for 15 minutes.
4. After removing the cuff, the number of petechial in the area under pressure is counted. Normally less than 15 petechiae are seen. 15 or more petechiae indicate capillary fragility,

**Interpretation:**
Positive Capillary Fragility Test is seen in
1. Poor platelet function or number (thrombocytopenia)
2. Scurvy
3. Dengue fever
Interfering factors with this test are women who are premenstrual, postmenstrual and not taking hormones, or those with sun damaged skin, since all will have increased capillary fragility.