Mammalian Physiology Practical Manual

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Practical 1: Determination of blood groups

The blood cells play a significant role in transportation, protection and regulation. Blood groups are mainly determined by the presence or absence of antigens and antibodies on the surface of our red blood cells or erythrocytes. The blood transfusion from the wrong blood group can be life-threatening. The four major blood groups are: A, B, AB and O.

Matarial and Reagents:

- Toothpicks
- Blood sample
- Alcohol Swabs
- Lancet
- Clean glass slide
- Sterile cotton balls
- Biohazard disposal container
- Monoclonal Antibodies (Anti-A, B, and D)

Procedure:

1) Take a clean glass slide and draw three circles on it.
2) Unpack the Monoclonal Antibodies (MAB) kit. In the first circle add Anti-A, to the second circle add Anti-B and to the third circle add Anti-D with the help of a dropper.
3) Keep the slide aside safely without disturbing.
4) Now wipe the ring finger with the alcohol swabs and rub gently near the fingertip, where the blood sample will be collected.
5) Prick the ring fingertip with the lancet and wipe off the first drop of the blood.
6) As blood starts oozing out, allow it to fall on the three circles of the glass slide by gently pressing the fingertip.
7) Apply pressure on the site where it was pricked and to stop blood flow. Use the cotton ball if required.
8) Mix the blood sample gently with the help of a toothpick and wait for a minute to observe the result.

Practical 2: Finding the bleeding and clotting time of blood

The bleeding and clotting time indicate the conditions of the blood clotting mechanisms. The latter gives information on the intrinsic clotting mechanisms while the former shows the mutual effects of blood and injured tissue.

Matarial and Reagents:

- Tissue paper

- Stopwatch or timer
- Antiseptic
- Bandage
- Parafilm
- Sterile needle

Procedure:

1) Clean the puncture site with an antiseptic to minimize the risk of infection.

2) Place a pressure cuff around your upper arm and inflate it.

3) Make two small cuts on your lower arm. These will be deep enough to cause slight bleeding. You might feel a slight scratch when they make the cuts, but the cuts are very shallow and shouldn't cause much pain.

4) Using a stopwatch or timer, wipe off the blood with a piece of filter paper every 30 seconds until the bleeding stops.

5) Record the time it takes for you to stop bleeding and then bandage the cuts. Normal bleeding time is between 2 to 3 minutes.

Clotting Time:

Continue step 1 to 3 of bleeding time.

4) Drop the first clear drop of blood onto the inert parafilm and start timer.

5) Draw a sterile needle through the drop in every 30 seconds

6) Stop the timer when the first fibrin fibre appears. Normal clotting time is between 5 to 10 minutes on paraffinated surfaces.

Practical 3: Counting of mammalian RBC

Hematology is the study of blood and the blood smear one of the most basic and yet most reliable ways of evaluating blood for multiple conditions of disease. We will use a droplet of blood to make a thin smear, dry it, fix and stain it and observe under a microscope. Cell fixation is done by placing the slide in methanol.

Materials:

- Droplet of blood from capillary bleed
- RBC diluting pipette
- 50 ml beaker for waste material

- RBC diluent 3.2 or 4% w/v Sodium Citrate (Na3C6H5O7)
- A 2.5% bleach mixture for cleaning
- 95% Alcohol for rinsing
- 99% Methanol for fixation
- Giemsa stain (1:20, vol/vol from stock)
- Sterile lancet
- Hemocytometer for RBC and WBC counting
- Microscope
- Tissue paper
- Gloves for use while staining

Procedure:

A. Making blood smear

1. Place a clean slide on a piece of tissue paper and write your unique initials in one corner, using the glass-marker pen.
2. Using a cotton swab dipped in 75% ethanol, clean the middle- or ring-finger with it. This both disinfects the surface as well as causes a slight increase in blood flow due to the evaporation and resultant compensatory blood-rush.
3. Carefully open the protective covering of a sterile Stab gently (vertical w.r.t. the length of the finger) the finger-tip and wait for a drop of blood to come up.
4. Make this drop fall ~1 cm from one of the short-edges of a clean slide.
5. Using the other slide, make contact at 30-40 degrees with the slide on which the blood droplet is. Drag the droplet towards the other end. As a result you should have a comet-like appearance of the blood smear.
6. The smear should have a 'comet' like appearance- thick initially and becoming very thin at the end. The comet tail area is the one we will observe under the microscope.

B. Staining the smear

1. Fix the smear in ~99% methanol 3-5 minutes by dipping in Coplin Jars.
2. Stain in Giemsa (methylene blue and eosin mixture) by dipping in Coplin Jar containing stain for a total time ~15 minutes.
3. Rinse the slide with tap water at room temperature (ensure rinsing doesn't wash away your sample). 4. Drain off the water by leaning it at ~45 degrees and leave it to air-dry.

C. Microscopy

Observe under 10 x magnifications to see that the cells are stained. Move to the 40x lens to see further details (be careful to prevent lens and slide collisions). Erythrocytes are the most numerous cells with a diameter of ~6-7 micrometers. Some larger macrocytes (d>9um) and smaller microcytes (d<6 um) have been seen. Purple diskettes around 3 um diameter are platelets. Leukocytes show nuclear stains purple in colour. The different granule patterns and nuclear morphologies allow a classification.

D. Counting RBCs (using blood donated to you)

1. As before make a pin-prick using a fresh unused lancet on the index- or ringfinger. If you have been pricked before on that finger, use another finger. 2. Bring a cleaned RBC dilution pipette tip close to the droplet of oozing blood. Using the bulb allow (by capillarity and pressure) blood to enter up to the 0.5 mark. 3. Using a small tube of Sodium-Citrate solution, additionally aspirate this solution to reach the mark 101 (1:200 dilution). 4. Gently turn the dilution tube in your hand. 5. Introduce the diluted blood in the Hemocytometer. 6. Count as per the instructions in the finer grid.

Practical 4: Determination of TLC of mammalian RBC

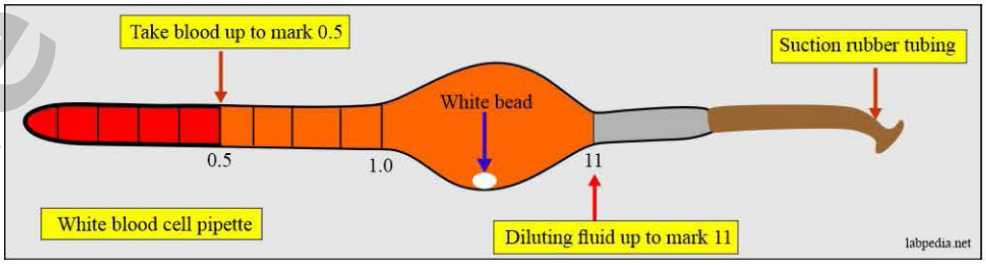
Principle

1. Blood is diluted with a fluid that causes the RBCs' hemolysis, but WBCs remain intact, and then these are counted in the Neubauer chamber.
2. Gentian violet lightly stains the leucocytes and allowing those to be counted.

Matarial and Reagents:

- Glacial acetic acid = 2 ml.
- Gentian violet (1%) = 1 ml.
- Distal water = 97 ml.
- This is basically a 2% solution of acetic acid.
  1) Add 2 ml of glacial acid (2 + 1), 1 mL gentian violet (1 %), and add 97 ml of distle Water to make up to 100 ml solution.
  2) Gentian violet is added until the color is pale blue-violet.
- TLC or WBC pipette
  This pipette (also called Thoma pipette) long stem is divided into two parts:

  1. The long stem is marked with 0.5 and 1.0
  2. While the short arm after the bulb is marked 11.
  3. Its central portion is a bulb or a globular shape with one white bead in it.
  4. Rubber tubing is attached to suck the blood.
  5. Ultimately the dilution of the blood to the TLC fluid is 1:20.



Procedure:

1. Take the TLC pipette, which has a white bead inside.
2. Fill the blood into the 0.5 marks and then add the TLC solution.
3. Fill the pipette with the TLC solution to point 11.
4. Remove the rubber tubing.
5. Seal both ends or hold in between two fingers.
6. OR can put this pipette on the mechanical device to shake it.
7. Shake for 1 minute or preferably for 2 minutes.
8. Shaking is important before filling the Neubauer chamber.
9. After thorough mixing, discard the first few drops and then gently fill the chamber until the platform is filled.
10. The capillary action will draw the fluid.
11. Allow the chamber on the microscope stage for 2 to 3 minutes till the cells are settled.

Tube method:

1. Take 0.02 mL blood and mix it with diluting fluid.
2. Take TLC dilution fluid 0.38 mL in a small tube and mix it with the blood. (1:20 dilution).
3. Mix them very well.
4. The dilution will be the same.
5. This tube method is more accurate than the Thoma pipette technique.

In case of low WBC count, fill the pipette to mark 1, and this will give dilution of 1:10, OR

1. Take 0.1 mL blood and 0.9 mL diluent solution (1:10 dilution).
2. In the case of high WBC count, then make higher dilution.

Practical 5: Determination of Hemoglobin in human blood (Cyanmethemoglobin method)

Principle: Blood is diluted in a solution containing potassium cyanide and potassium ferricyanide. The latter converts Hb to methemoglobin which is converted to cyanmethemoglobin (HiCN) by potassium cyanide. The absorbance of the solution is then measured in a spectrophotometer at a wavelength of 540nm or in a colorimeter using a yellow green filter.

Materials and Reagents:

- Hb pipette

- Spectrophotometer
- Drabkin's solution pH7.0-7.4 which contains
- Potassium cyanide 50 mg
- Potassium ferricyanide 200 mg
- Potassium dihydrogen phosphate 140 mg
- Nonionic detergent 1 ml Distilled water 1 L
- Absorption at 540nM

Procedure:

1) Take 5ml of Drabkin's solution in a test tube.
2) Mix the blood (Collected in EDTA solution) sample by gentle inversion and draw 0.02ml of blood into the Hb pipette. Wipe the outer surface of the pipette to remove excess blood.
3) Place the pipette into the tube containing Drabkin's solution and slowly expel the blood into the solution. Mix well and let it stand undisturbed for 5min.
4) Measure the absorbance of this solution at 540nm in a spectrophotometer after adjusting the OD by using Drabkin's solution as blank.
5) Calculate the hemoglobin concentration using a standard curve.