

## 2. LYMPHOCYTE SEPARATION FROM FISH BLOOD

### Aim:

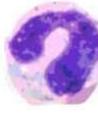
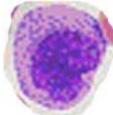
To separate lymphocytes from fish blood using density gradient media.

### Introduction:

The Lymphocyte Separation Medium (LSM) is an iso-osmotic, low viscosity medium containing polysucrose and sodium diatrizoate adjusted to a density of 1.0770 +/- 0.0010 g/ml. This medium offers a quick and reliable method for the simple isolation of vertebrate mononuclear cells and lymphocytes from de-fibrinated EDTA blood.

### Principle:

White blood cells (WBCs) or leukocytes are cells of the immune system involved in defending the body against both infectious disease and foreign materials. Five different and diverse types of leukocytes exist, but they are all produced and derived from lymphoid organs. Leukocytes are found throughout the body, including the blood and lymphatic system. There are several different types of white blood cells. They all have many things in common, but are all distinct in form and function. A major distinguishing feature of some leukocytes is the presence of granules; white blood cells are often characterized as granulocytes or agranulocytes as shown in the following table:

| Type          |            | Microscopic appearance  | Diagram  | Nucleus               |
|---------------|------------|---|--|-----------------------|
| Granulocytes  | Neutrophil |  |  | Multilobed            |
|               | Eosinophil |  |  | Bi-lobed              |
|               | Basophil   |  |  | Bi-lobed or tri-lobed |
| Agranulocytes | Lymphocyte |  |  | Eccentric             |
|               | Monocyte   |  |  | Kidney bean shaped    |

The first step in studying lymphocytes is to isolate them so that their behavior can be analyzed in vitro. Lymphocytes are present in blood, peritoneal exudates or lymphoid organs mixed with other cells. A pure population of lymphocytes can be obtained by various separation procedures. Isolation of lymphocytes is based on the adapted centrifugation method of Boyum in which diluted defibrinated blood is layered on a solution of sodium diatrizoate and polysucrose and centrifuged at low speeds for 30 minutes. Differential migration following centrifugation results in the formation of several cell layers. Mononuclear cells (lymphocytes and monocytes) and platelets are contained in the banded plasma-LSM interphase due to their density, and the pellet that is formed contains mostly erythrocytes and granulocytes, which have migrated through the gradient to the bottom of the tube. Most extraneous platelets are removed by low speed centrifugation during the washing steps. Lymphocytes or other mononuclear cells (monocytes or mesenchymal stromal cells) or granulocytes are recovered by aspirating the plasma layer and then removing the cells. Excess platelets, LSM and plasma can then be removed by cell washing with isotonic diluent buffer.

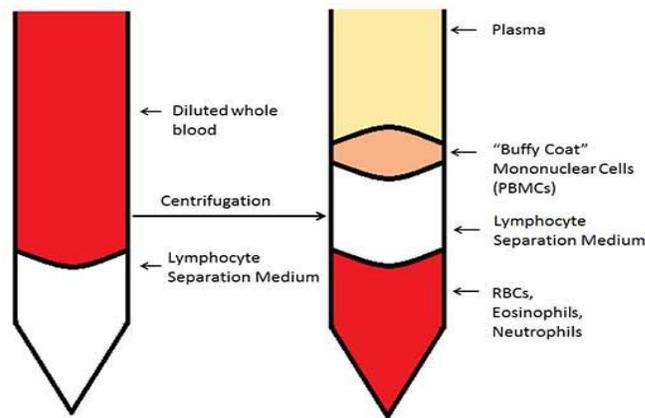


Fig1: After differential centrifugation lymphocytes and monocytes are found in the buffy coat.

**Materials required:**

| Sl No. | Materials                               | Remarks  |
|--------|---|--|
| 1      | Lymphocyte Separation Medium (LSM) 1077 | All the materials were provided in HiPer® Lymphocyte Separation Kit. |
| 2      | Centrifuge Tube (15 ml)                 |  |
| 3      | Polypropylene Tube (0.5 ml)             |  |
| 4      | Diluent Buffer                          |  |
| 5      | Giemsa's Stain                          |  |
| 6      | Cedar wood Oil                          |  |
| 7      | Trypan Blue (0.5%)                      |  |
| 8      | Methanol                                |  |
| 9      | EDTA/Heparin coated collection tube     |  |
| 10     | Cotton                                  |  |
| 11     | Pipettes                                |  |
| 12     | Hemocytometer                           |  |
| 13     | Microscopic slides & coverslips         |  |
| 14     | Micropipettes & Tips                    |  |

**Procedure:**

**Lymphocyte Layer Separation and viability count:**

1. Collect 4 ml of blood in the EDTA coated collection tube, using sterile syringe and needle.
2. Mix immediately by inverting or vigorously shaking the tube for EDTA to be uniformly distributed.
3. Dilute the blood by adding 4 ml of diluent buffer.
4. Take 2.5 ml of LSM 1077 in a new 15 ml centrifuge tube. Overlay the LSM with 7.5 ml of diluted blood.
5. Centrifuge at 2300 rpm for 30 minutes in a fixed angle rotor.
6. Using a clean glass pasteur pipette carefully remove the lymphocyte layer in a new collection tube.
7. Add 5 ml of diluent buffer to the lymphocyte layer. Mix by gentle pipetting and centrifuge at 1900 rpm for 10 minutes. (This step helps to reduce the number of platelets).
8. Discard the supernatant obtained from above step.
9. Resuspend the pellet in 500 µl of diluent buffer.
10. Take 10 µl from above step in new collection tube and add 40 µl of diluent buffer and 50 µl of Trypan Blue.
11. Place a coverslip on the Neubauer chamber of the haemocytometer.
12. Cover one side of the Neubauer chamber of the haemocytometer with the sample.
13. Observe and count the live and dead cells under 45X magnification in a light microscope.

**Differential Staining:**

1. Spin the tube from step 9 at 2300 rpm for 10 minutes.
2. Discard the entire supernatant leaving around 50 µl in the tube.
3. Resuspend the pellet in the same solution and make a smear on a microscopic slide.
4. Air dry the slide for 5-10 minutes.
5. Add 5-10 ml of methanol to cover the slide. This step helps in fixing the cells onto the slide.
6. Discard the methanol carefully.
7. Add 15 ml of staining solution and incubate for 30 minutes at room temperature.
8. Observe under oil emulsion lens and look for the lymphocytes and monocytes.

**Observation and Result:**

The viable and dead cells were counted under the microscope (in four WBC chambers of the haemocytometer) after trypan blue staining and the following data were recorded:

| Viable cells (cells without dye) | Dead cells (cells with dye) |
|----------------------------------|-----------------------------|
| 8                                | 1                           |

Calculate the number of total cells as follows:

Area of one WBC chamber = L X H (1 mm X 1 mm)

$$\begin{aligned}
 &= 1 \text{ mm}^2 \\
 \text{Depth of Haemocytometer is } &0.1 \text{ mm} \\
 \text{Hence Volume of 1 WBC chamber} &= \text{Area X Depth} \\
 &= 1 \text{ mm X } 0.1 \text{ mm} \\
 &= 0.1 \text{ mm}^3
 \end{aligned}$$

4 WBC chambers were counted so, volume of 4 WBC chambers is  $4 \times 0.1 = 0.4 \text{ mm}^3$

$$\begin{aligned}
 \text{Total no. of cells (cells/ml)} &= \frac{\text{Total cells}}{\text{Volume of WBC chamber}} \times 1000 \times \text{Dilution Factor} \\
 &= \frac{9}{0.4 \text{ mm}^3} \times 1000 \times 5 \\
 &= 1.12 \times 10^5 \text{ cells / ml}
 \end{aligned}$$

Percentage of isolated lymphocytes, monocytes and total granulocytes were counted under the microscope after performing the differential staining and recorded in the following table:

| No. of total cells | No. of viable cells | Lymphocytes (%) | Monocytes (%) | Granulocytes (%) |
|--------------------|---------------------|-----------------|---------------|------------------|
| 9                  | 8                   | 65              | 2             | 33               |

**Observation and Result:**

