

# Original Research Article

## SEPARATION AND OPTIMIZATION OF A SUCROSE DENSITY GRADIENT CENTRIFUGATION PROTOCOL FOR ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)

### Abstract:

One step centrifugation procedure used commonly for separation of blood cells is the ficoll gradient centrifugation. In this method, after centrifugation, the peripheral blood mononuclear cells (PBMCs) are located on the top of the separation fluid, whereas other blood cells erythrocytes and granulocytes sediment to the bottom. In the present study pure lymphocyte suspension could be separated by using a one-step density gradient centrifugation of citrated blood with Sucrose. Sucrose was diluted into different concentrations using miliQ water (10%,20%,30%,40%,50%,60%,70%,80%,90%,100%,). 4 mL of diluted blood was layered on each sucrose solution and centrifuged for 45minutes at 1000 rpm. Clear separation of PBMCs could be observed in solution with 40% sucrose. The separated PBMCs were analyzed in heme analyzer which showed 75% lymphocytes, 23% monocytes and 2% of other cells.

**Keywords:** Sucrose; PBMCs; Gradient centrifugation; Blood

### Introduction:

Blood is made up of plasma and other types of cells including PBMCs. PBMCs are small white blood cells, varying from 7 to 8 micrometers in length. In the human body, WBCs are the only cells which can be transformed to proliferate. These cells originated from lymphatic tissue which is present ~~in~~ throughout the body [1].

WBCs protect the body from diseases, invasion of foreign bodies, tumors and infections. They are used for the immunological studies directed to histocompatibility antigens and cellular immunity; cytotoxicity studies; determining the proportion of T cells to B cells, and for trying to separate helper T cells and Suppressor T cells [1,2].

The separation of WBCs is carried out by density gradient centrifugation [3, 4, 5]. The separated blood components can subsequently be used for their respective clinical and scientific applications and investigations. At present to separate and isolate different parts of blood, commercially available ficoll is used for centrifugation which is highly costly [6, 7, 8]. In this background, the present study was undertaken to simplify and make it cost effective process.

**Comment [I1]:** "pure" depicts 100% of lymphocytes but your technique yields 75%?

**Comment [I2]:** Spacing?

**Comment [I3]:** Suggests tha plasma is a type of cells

**Comment [I4]:** Name all the cells comprised in blood to enrich the communication implied in the sentence

**Comment [I5]:** Does this description also cover monocytes?

**Comment [I6]:** What about monocytes

**Comment [I7]:** :

**Comment [I8]:** separating

## Materials and methods

The materials used in the study are as follows: 1X PBS Buffer, Sucrose, Human blood in Heparinized green top tubes, Centrifuge tubes, Centrifuge.

A non-reducing disaccharide, sucrose is composed of glucose and fructose linked by anomeric carbons. Commercially it is obtained from sugarcane plant and extensively used as a sweetener for food [9]. Molecular structure and formula for sucrose is  $C_{12}H_{22}O_{11}$  (Fig. 1).

## Method

### Isolation of PBMCs

2 ml of blood was collected from a normal healthy person in a sodium heparinized vacutainer. The blood was diluted with 1X PBS in 1:1 ratio. Different concentrations of sucrose solutions (10,20,30,40,50,60,70,80,90,100%) were prepared. 4 ml of diluted blood was layered on each sucrose solution. The solution was immediately centrifuged at 1000 rpm for 45 min in a cooling centrifuge (4°C). After centrifugation, the tube was removed carefully without disturbing the solution. four layers could be observed: top reddish clear supernatant, a middle opaque fluid containing the PBMC, clear solution of sucrose and bottom with RBCs. Carefully opaque PBMC layer was transferred to another centrifuge tube and 1X PBS was added until it became 8 ml. The solution was mixed well and centrifuged at 1000rpm for 10 min in a cooling centrifuge (4°C). Discard the supernatant and once again make up the solution to 8 ml with 1X PBS. Mix well and centrifuge at 1000rpm for 10 min in a cooling centrifuge (4°C) to remove platelets.

### The culture of lymphocytes:

Isolated PBMCs were cultured using 6 mL of RPMI1640 medium and incubated overnight at 5% CO<sub>2</sub> at 37°C. after incubation the tubes are centrifuged at 1000 rpm for 20 min and the supernatant was discarded. Pellet was mixed with 500uL of 1X PBS and 50uL of cell suspension was dropped on slides and observed under a microscope.

### Cell viability by the hematology analyzer

The isolated cells are analyzed using heme analyzer check for the number of blood cells in the suspension.

## Results

The results observed have been discussed along with the figures as follows.

Figure 2 shows various concentrations of sucrose solutions used for separation of PBMCs of the blood. 40% sucrose has given an excellent result with four layer separation.

**Comment [I9]:** Wrong way of starting a sentence with a number- "A 2ml volume of....."

**Comment [I10]:** Spacing?

**Comment [I11]:** Spacing

**Comment [I12]:** Up to 8 ml mark or volume

**Comment [I13]:** The supernatant was discarded

**Comment [I14]:** The subnatant dissolved to...

**Comment [I15]:** The solution was well mixed then centrifuged

**Comment [I16]:** What was the purpose of culturing?

**Comment [I17]:** were

**Comment [I18]:** Is this a solution or a suspension?

**Comment [I19]:** were

**Comment [I20]:** that checked the number of viable cells

**Comment [I21]:** The observed results are shown in figures 2 to 4

**Comment [I22]:** The 40 % sucrose concentration gave four-layered separation which was the best result

In figure 3, the four layers shown are: from above downwards other blood cells (platelets, plasma cells), PBMC, sucrose, and RBC. Isolated PBMCs was observed under heme analyzer for the quality check which showed 75% lymphocytes, 23% monocytes and 2% other cells.

**Comment [I23]:** Top to the bottom

To confirm whether the lymphocytes were separated by this method, isolated PBMCs were cultured using RPMI1640 and incubated overnight at 5% CO<sub>2</sub> at 37<sup>0</sup>C. Slides were prepared and observed under the microscope to confirm the viable cells (Fig. 4a, 4b).

### Discussion:

To isolate and purify biomolecules and cell structures, density gradient centrifugation is the common technique used. The principle behind this technique is that, in suspension molecules that are denser than the solvent will sediment, while the molecules which are less dense will float by layering liquids of decreased density on a density medium in a centrifuge tube. High-speed ultracentrifuge is used to accelerate this process to separate biomolecules within the density [10, 11, 12].

**Comment [I24]:** commest

**Comment [I25]:** Spacing ?

The commercially available density medium for the separation of layers is ficoll medium which is a polysaccharide. Ficoll gradient centrifugation method for separation of PBMCs is accurate and highly efficient, but it is very expensive. In the present method, commonly available sucrose is used to separate the blood cells. The method is simple and highly economic. Therefore this method is recommended even in small laboratories with low income for high-throughput sample preparation suitable for various immunological assays.

**Comment [I26]:** Spacing?

The resulting PBMC pellet can be used for any immunological and cytotoxicity assays and by this method we can store lymphocytes for a longer duration of time for any hematological studies or for related scientific research.

**Comment [I27]:** Lymphocytes can be stored

**Comment [I28]:** What method is being compared "longer" is comparative

### Conclusion:

Based on the above results we conclude that 40% sucrose is suitable for separation of blood cells or isolation of PBMCs by using low-cost sucrose. Further studies are needed for the development of new gradient media.

**Comment [I29]:** What gap are /is the new medium expected to close?

**References:**

1. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand. J. Clin. Lab. Invest. Suppl.* 97:77-89.
2. Boyum, A. 1976. Isolation of lymphocytes, granulocytes, and macrophages. *Scand. J. Clin. Lab. Invest. Suppl.* 5:9-15.
3. Boyum, A. 1977. Separation of lymphocytes, lymphocyte subgroups, and monocytes: a review. *Lymphology.* 10-2:71-6.
4. Boyum, A. 1984. Separation of lymphocytes, granulocytes and monocytes from human blood using iodinated density gradient media. *Methods Enzymol.* 108:88-102.
5. Boyum, A. et al. 2002. Separation of Human Lymphocytes from Citrated Blood by Density Gradient (Nycoprep) Centrifugation: Monocyte Depletion Depending upon Activation of Membrane Potassium Channels. *Scand. J. Immunol.* 56-1:76-84.
6. Koistinen, P. 1987. Human peripheral blood and bone marrow cell separation using density gradient centrifugation on Lymphoprep and Percoll in hematological diseases. *Scand. J. Clin. Lab. Invest.* 47-7:709-14.
7. Rola-Pleszczynski, M. and W.H. Churchill. 1978. Purification of human monocytes by continuous gradient sedimentation in ficoll. *J. Immunol. Methods.* 20:255-62.
8. McKeating D, Markey GM, Alexander HD, Morris TCM. Separation of lymphocytes from peripheral blood: an 'm-visitation. *J Clin Pathol* 1985; 38:595.
9. "Foods highest in Sucrose". Self Nutritiondata. Condé Nast. Archived from the original on 2015-07-19.
10. Brakke, Myron K. (April 1951). "Density Gradient Centrifugation: A New Separation Technique". *J. Am. Chem. Soc.* 73 (4): 1847–1848. doi:10.1021/ja01148a508.
11. Oster, Gerald; Yamamoto, Masahide (June 1963). "Density Gradient Techniques". *Chem. Rev.* 63 (3): 257–268. doi:10.1021/cr60223a003.
12. JoVE Science Education Database. Biochemistry. Density Gradient Ultracentrifugation. JoVE, Cambridge, MA, (2018).

**Comment [130]:** Spacing

Figure 1: Structure of sucrose

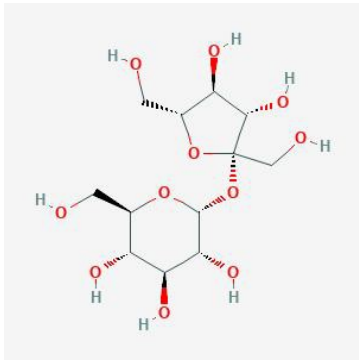


Figure 2: Various concentrations of sucrose solutions from 10% to 100%

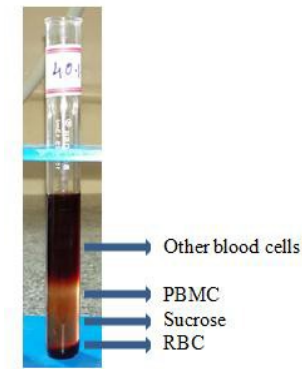
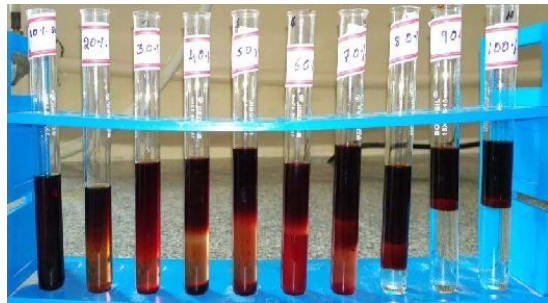


Figure 3: 40% sucrose solution showing 4 different layers of blood cells.

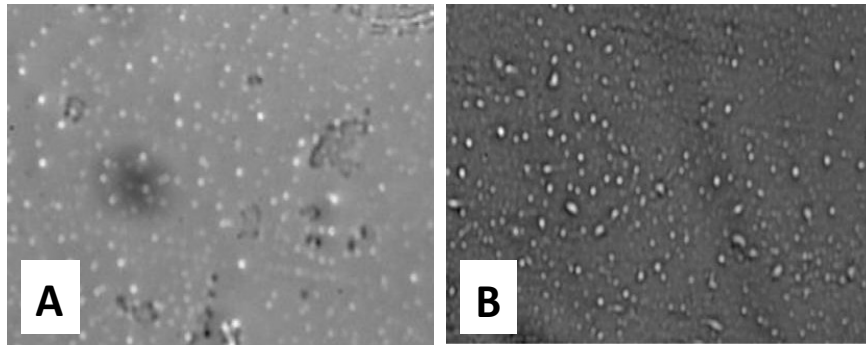


Figure 4: Microscopic evaluation showing isolated lymphocytes/ PBMCs

**Comment [I31]:** Spacing?