

SEPARATION OF LYMPHOCYTES USING FICOLL-HYPAQUE

A. Preparation of reagents

I. Ficoll-Hypaque

- a. Carefully weight out 23.89 g Ficoll (Pharmacia, Uppsala, Sweden).
- b. Add exactly 226 ml of distilled water and mix on a magnetic stirrer until Ficoll has dissolved.
- c. Add exactly 50 ml of 75% Hypaque (Winthrop Labs). Hypaque M – Sodium + Meglumine Diatrizoates 10 vials 50mL each
Note: Hypaque should be a clear solution. If crystals have formed, warm the bottle at 37°C until crystals dissolve.
- d. Add exactly 100 ml of distilled water and mix for 1 hour at room temp on magnetic stirrer.
Notes: 1. The final density of the Ficoll-Hypaque solution should be 1.076-1.078.
2. Store at 4t but warm to room temp prior to use.
3. Can be stored refrigerated for at least a month.

II. Phosphate-buffered saline pH 7.2-7.4

Combine in a 500 ml flask:

- a. 1.3 ml 1M KH_2PO_4 (1 ml $\text{KH}_2\text{PO}_4 = 136.09 \text{ g/l}$).
- b. 10.4 ml 0.5M $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ($0.5\text{M Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O} = 358.15 / 2 \text{ gm/l}$
or for anhydrous Na_2HPO_4 use 70.98 g/l)
- c. 50 ml of 10 times concentrated saline (8.5%).
- d. Fill to 500 ml with distilled H_2O and mix.
- e. Warm to room temp for use.

III. Dulbecco's PBS (for washing cells, pH ~ 7.4 - 7.5)

- a. Prepare the following stock solutions:

Solution 1	NaCL	40.45g
	KCL	1.45g
	KH_2PO_4	1.45g
	$\text{Na}_2 \text{HP04}$	5.79g
	water to 400 ml	

Solution 2	CaCl_2	0.95g with water to 50 ml
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Solution 3.	$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.95 g water to 50 ml
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- b. To a 1 liter volumetric, add the following aliquots in the following order:

10 ml	#2
110 ml	#3
900 ml	distilled H_2O (mix)
80 ml	#1

There should be no precipitate.

B. Separation of lymphocytes

- I. Take 10 ml of venous blood in a heparinized tube or syringe. Mix gently.
- II. Do total white cell count on an aliquot by diluting 1/10 in 2% acetic acid.
- III. Mix one part of heparinized blood with one part of PBS.
- IV. Put 10 ml of Ficoll-Hypaque in 40 ml centrifuge tube.
- V. Carefully layer 20 ml (or less) of the blood-PBS mixture onto Ficoll-Hypaque.
- VI. Centrifuge at 400g or at 1600 rpm in our centrifuge at 20°C for 35 min.
- VII. Three layers will be obtained of the centrifugation:
 - a. upper layer containing PBS
 - b. middle layer containing lymphocytes.
 - c. lower layer containing Ficoll-Hypaque, granulocytes and erythrocytes.Using Pasteur pipette withdraw the middle lymphocyte layer. Most of the cells will be between lower boundary of top layer and upper boundary of middle layer.
- VIII. Place lymphocyte suspension in 40 ml centrifuge tube; add ~ 25 ml of cold D's PBS and mix.
Centrifuge at 1500 rpm for 5 min.
- IX. Resuspend cells in about 30 ml of D's PBS + 5% FCS. Centrifuge at 1500 rpm, 5 minutes.
- X. Wash again as in #9; then, resuspend final pellet in 0.5 ml buffer or 0.5 ml buffer per every 10 ml blood used initially. Normal blood will yield $15-25 \times 10^6$ /ml prepared lymphocytes when resuspended in this manner.