

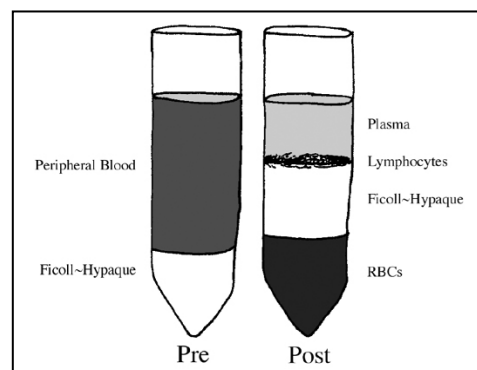
## Initiation and Care of Lymphoblast Cell Lines From Human Peripheral Blood

Note: This protocol assumes the investigator is beginning this with one full Yellow-Top (type A) BD Vacutainer tube of human blood (equals roughly 8 ml). As well, all conditions are sterile.

- Pre-warm your EBV virus and Cyclosporine-A in a 37° C water bath.
- Perform all steps, except centrifugation in a sterile TC hood using aseptic techniques.

### Ficoll gradient (Bottom Method)

- 1) In a sterile tissue culture hood, transfer blood from the vacutainer tube into a sterile 50 ml polypropylene conical centrifuge tube.
- 2) Bring volume to 30 ml with sterile incomplete 1X PBS.
- 3) Mix gently by pipetting up and down.
- 4) Very carefully underlay 10 to 14 ml of sterile, room-temperature ficoll beneath the diluted blood in the 50ml tube. Note: this will create a distinct layer beneath the blood to act as a “liquid filter”. The denser erythrocytes will sediment through the ficoll, whereas the less dense lymphocytes will be trapped above.
  - a. Do this by drawing 10-14 ml of ficoll into a sterile 10 ml serological pipet. Place the pipet all the way to the bottom of the 50 ml tube containing the blood, then withdrawing the pipet 2 or 3 mm to prevent obstructing the tip.
  - b. Slowly inject the ficoll into the bottom of the tube at a rate of about 12 ml/minute.
  - c. Slowly remove the near-empty pipet.



### Ficoll gradient (Top Method)

- 1) In a sterile tissue culture hood, transfer blood from the vacutainer tube into a sterile 50 ml polypropylene conical centrifuge tube.
- 2) Bring volume to 30 ml with sterile incomplete 1X PBS.
- 3) Mix gently by pipetting up and down.
- 4) In another sterile 50ml polypropylene conical centrifuge tube, carefully, so as to not get any on the sides of the tube, layer 10ml of ficoll on the bottom.
- 5) Holding the tube containing the ficoll at a 45° angle, slowly run the blood down the side of the tube so as to carefully layer it over the ficoll without mixing the interface. It's a good idea to have an experienced person show you this step. Make sure the pipette aide is set to the slowest speed and your hands are steady. As the tube fills, you can slowly return the ficoll tube to the upright position. The layers should appear as the diagram above, with 2 distinct layers.

**Separation**

- 6) Carefully place the tube(s) into a centrifuge (room temperature) and pellet at 2000 RPM for 25 minutes (Program #1). Note: if possible, it is recommended that you reduce the amount of braking on the centrifuge in order to maintain a clean interface.
- 7) After centrifugation, each sample should be assessed for the quality of the ficoll separation. A number system will be posted in a paper log at the time of separation, then the data uploaded into Labmatrix according to the following parameters:
  - 4** = no visible problems, 1-2 day old sample. Entered in Labmatrix as Apparent Quality-Best.
  - 3** = slightly imperfect separation of 1-2 day old samples (slight hemolyzation, “hazy” separation) **-OR-** 3 day old sample with no visible problems. Entered in Labmatrix as Apparent Quality-Acceptable.
  - 2** = visible problems with separation (clotting, bloody/hemolyzed, low volume to start < 4ml, few lymphocytes visible) **-OR-** 4 day old sample with no visible problems **-OR-** 3 day old with slightly imperfect separation. Entered in Labmatrix as Suspected Degradation-See Description. Enter details of the samples condition in the Notes section.
  - 1** = >5 day old (regardless of appearance of separation) **-OR-** lots of clotting, suspected temperature problems with blood sample **-OR-** chemo patient **-OR-** poor draw (lack of tube inversion). Entered in Labmatrix as Suspected Degradation-See Description. Enter details of the samples condition in the Notes section.
  - 0** = initiation abandoned. Enter as Rejected in Labmatrix, and describe details of problems in the Notes.
- 8) A) Using a 10 ml pipet, carefully aspirate and collect the lymphocytes located at the upper interface between the ficoll and the diluted plasma, and transfer to a clean 50 ml tube. Be careful to avoid aspirating any erythrocytes. Also, be sure to pay attention to the volume of sample in your pipet to avoid aspirating cells into your pipettor.  
  
B) Alternatively, you can “vacuum” up the cell layer with a plastic transfer pipette. This allows you to recover the cells with less residual plasma volume.
- 9) To the freshly collected lymphocytes, add enough 1X PBS to bring the total volume to 50 ml.
- 10) Pellet the cells by centrifuging at 600 x g for 10 minutes at room temperature.
- 11) Carefully pour off the supernatant into a waste container containing bleach.
- 12) Gently resuspend the pellet with 5 ml of EBV media and transfer to a sterile T25 flask. Add 2 ml of Cyclosporine-A to the flask containing the lymphocytes.
- 13) Store cultures in CO<sub>2</sub> incubator and care for in accordance with protocol GMB013: Care of Lymphoblast Cell Lines from Human Peripheral Blood.

**RPMI-1640 (incomplete)**

1X with L-glutamine: Gibco/Invitrogen: Cat. No. 11875-093 (500 ml bottle)

**RPMI-1640 (complete)**

500 ml of RPMI-1640

100 ml heat-inactivated FBS (20%) – Gibco/Invitrogen: Cat. No. 16140-071

6 ml antibiotic-antimycotic (1x) – Gibco/Invitrogen: Cat. No. 15240-062

1 ml Tylosin solution – Sigma-Aldrich: Cat. No. T3397

**Ficoll**

Ficoll-Paque PLUS: Amersham Biosciences: Cat. No. 17-1440-03 (500 ml bottle)

**EBV Media**

This is complete RPMI-1640 media that has been conditioned by B95.8 cells. The B95.8 cell line is a lymphoblastoid cell line from the marmoset monkey that expresses the human EBV virus. Please see protocol SB007 for preparation details.

**Cyclosporine-A**

Sigma-Aldrich: Cat. No. C1832

Stock aliquots of Cyclosporine-A should be made by dissolving 10 mg in 2.5 ml of ethanol to make a 4 mg/ml stock. Store 500 ul aliquots of 4 mg/ml stock at -20C.

Dilute in incomplete RPMI-1640 to 4 ug / ml (add 500 ul of the 4mg/ml ethanol stock to 500 ml of incomplete RPMI). When used in protocol yields slightly better than 1 ug final concentration.