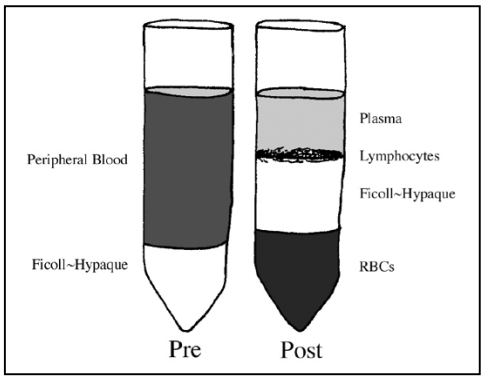
Initiation and Care of Lymphoblast Cell Lines From Human Peripheral Blood

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

- Pre-warm your EBV virus- conditioned media and Cyclosporine-A in a 37° C water bath.
- EBV virus-conditioned media must be contained in its own beaker within the water-bath.
- Perform all steps, except centrifugation in a sterile TC hood using aseptic techniques.

Ficoll Gradient

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 30ml with sterile1X PBS.
3) Mix gently by inversion.
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-Paque PLUS on the bottom.



5) Holding the tube containing the Ficoll-Paque PLUS at a 45^{o} angle, slowly run the blood down the side of the tube so as to carefully layer it over the top of the Ficoll-Paque PLUS, without mixing. 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 (Pre), with 2 distinct layers.

Separation

6) Carefully place the tube(s) into a centrifuge (room temperature) and spin at 2000 RPM for 25 minutes (Program #1). Note: if possible, it is recommended that you reduce the amount of braking on the centrifuge to "0" in order to maintain clean interfaces.
7) After centrifugation, each sample should be assessed for the quality of the ficoll gradient. Ideally, they should look like the diagram above (Post). 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) "Vacuum" up the cell layer with a plastic transfer-pipette being careful to avoid any ficoll or RBC contamination of the ficoll layer below. Transfer these cells to a clean 50ml conical centrifuge tube.
9) To the freshly collected lymphocytes, add enough 1X PBS to bring the total volume to 50ml.
10) Pellet the cells by centrifuging at 600 x g for 10 minutes at room temperature to wash.
11) Carefully pour off the supernatant into a waste container.*
12) Gently resuspend the pellet with 5ml of EBV media and transfer to a sterile T25 flask, properly labeled.

Decontaminate of any pipets used with the virus-conditioned media by placing them in the pipet chimney that contains 10% Clorox solution. Once the pipets have been soaking in the Clorox solution for at least 24 hours they are considered decontaminated and can be removed from the chimney, drained of liquid and disposed of in a biohazard box.

**Once you are done, decontaminate the collected liquid waste by adding 10% Clorox solution and allow to sit for 15 minutes. After 15 minutes, the decontaminated waste may be flushed down the sink with water.*

13) Add 2ml of Cyclosporine-A to the flask containing the lymphocytes.
14) Store cultures in CO_2 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)
500ml of RPMI-1640
100ml heat-inactivated FBS (20%)
6ml antibiotic-antimycotic (1x) – *Gibco/Invitrogen: Cat. No. 15240-062*
1ml 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 4mg/ml stock. Store 500ul aliquots of 4mg/ml stock at -20^{o}C.

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