Preparation of primary human lymphocytes

Cell numbers and expected yield

With a typical yield of $1 - 2 \times 10^6$ mononuclear cells per ml of blood, we usually obtain $5 \times 10^6$ to $1 \times 10^9$ mononuclear cells/ leukopack (50 ml leukocytes enriched from 450 ml whole blood). Assuming 70% PBLs, expected yield is $3.5 - 7 \times 10^8$.

Supplies required

Leukopack (Buffy coat) - We obtain pathogen-negative leukopacks from the Gulf Coast Blood Regional Blood Center.
Isolymph (Gallard & Schlesinger #759-001) or Ficoll-Paque (Amersham #17-1440-02)
Phosphate-buffed saline (PBS)
RPMI + endotoxin-free 10% fetal bovine serum (FBS) + pen/strep (= Complete RPMI)
50 ml conical tubes (polypropylene)
Fenwal sampling site coupler (punctures blood bag), Baxter Transfusion Therapy: 4C2405
Vacutainer needle (transfer from blood bag to draw tube), Fisher #02-665-20, 21 gauge, 1 inch
Serum tube, Fisher #02-683-81, 15 ml, 16 x 125 mm
RBC lysis buffer (155mM NH$_4$Cl, 10 mM KHCO$_3$, 0.1 mM Na$_2$EDTA) – filter sterilized.
Trypan blue (0.4%)

Note: All supplies must be sterile. Media should be warmed to 37°C prior to use. Isolymph, PBS and RBC lysis buffer are used at room temperature (R.T.).

Procedure

This procedure should be performed under sterile BL-2 conditions.

1. Carefully remove content of leukopack and transfer to conical tubes.
   Peel back opening and puncture tube with site coupler (beveled end).
   Carefully insert needle and collect blood into serum tubes.
   Transfer to 5 50 ml conical tubes (~10 ml/tube).

2. Dilute 1:2 with PBS.
   Add PBS to bring volume up 30 ml/tube.

3. In a separate set of five 50 ml conical tubes, add 20 ml Isolymph/Ficoll-Paque per tube.

4. Very gently, layer the diluted blood on top of the Isolymph/Ficoll-Paque. Do not allow layers to mix.
   Using a sterile pipet, transfer the blood to the tube containing the Isolymph/Ficoll-Paque.
   Position the tip of the pipet against the wall of tube just above the Isolymph/Ficoll-Paque.
   Pipet slowly as to not disturb the interface.

5. Centrifuge at 500g for 30 min. at 20°C - No brake.
6. Recover lymphocyte layer (buffy coat).
   *Using pipet, remove plasma down to about 1 cm above buffy coat (lymphocyte) layer.*
   *With clean pipet, collect the buffy coat and transfer to a 50 ml conical tube (collect ~5 ml/tube).*
   *Split layers between 2 clean conical tubes.*

7. Wash 1X with PBS.
   *Add PBS to 45 ml.*
   *Centrifuge at 400g for 10 min. at R.T. (Resume use of brake).*

8. Lyse red blood cells with RBC lysis buffer.
   *Gently resuspend pellet by adding 10 ml RBC lysis buffer (rake bottom of tube across rack before adding buffer to loosen cell pellet).*
   *Incubate 5 min. at R.T with occasional gentle mixing. Ensure pellet is completely resuspended.*
   *After incubation, add 30 ml PBS.*

9. Centrifuge at 200g for 5 min. at R.T. (If RBCs still visible, repeat lysis steps #8 & 9).

10. Wash 1X with PBS.
    *Gently resuspend pellet with 40 ml of PBS (remove any aggregates with Pasteur pipet).*
    *Centrifuge at 200g for 5 min. at R.T.*

11. Gently resuspend pellets from both tubes in a total of 40 ml PBS (combine pellets).

12. Count aliquot of cells to determine cell number and cell viability.
    *To Eppendorf tube containing 160 µl PBS + 200 µl trypan blue, add 40 µl cells (1:10 dilution).*
    *Place on hemacytometer and immediately count cells in 2 quadrants (longer incubation with dye can lead to cell death).*
    *Calculate total cell #: cell count /2 X 10^6 X 10 (D.F.) X 40 (vol.)*
    *Calculate % viable cells: (total # of viable cells per ml of aliquot x 100) / total # of cells per ml of aliquot*

13. Centrifuge at 200g for 5 min. at R.T.

14. Resuspend cells in complete RPMI at a concentration of 4 x 10^6 cells/ml. Plate ~60 ml in a large T-flask.

15. Allow cells to incubate for 1 hr. at 37°C, 5% CO₂.

16. Remove nonadherent cells and transfer to a fresh T-flask.

17. Allow cells to incubate for 1 hr. at 37°C, 5% CO₂.

18. Count cells. Adjust cell concentration to 2 x 10^6 cells/ml.

19. Add activators (e.g. PMA, PHA) if desired and plate in appropriate size dish (usu. 10 cm dish).