Ernst Lab
2007
C.Jude

G_0/G_1 analysis of KLS cells

Bone marrow

1. Remove Bones, clean and crush in ice cold HBSS 2% FBS in a mortar and pestle.

2. Filter cells through 40µm filter to remove bone fragments, pellet cells at 380 x g at 4º C for 4 minutes.

3. Resuspend in 1mL ACK red cell lysis, 5 minutes room temperature, stop with 5mL HBSS+, pellet cells at 380 x g at 4º C for 4 minutes.

4. Resuspend in 5mL HBSS+, count 1:20 in trypan blue.

5. Stain cells using 1µl Lineage mix/ 10^6 cells.

Lineage Mix

(Caltag except IL-7Rα eBiosciences)

CD3	CD4	CD8	GR-1	B220	CD19	IL-7Rα	TER119	MAC-1
1	2	1	2	2	1	2	1	2

6. Wash cells 2x with 1mL HBSS+, pellet cells at 380 x g at 4º C for 4 minutes.

7. Resuspend cells in 2mL HBSS+ in a FACS tube, then add 100µl Dynal beads. Dynal beads should be adjusted according to manufacturers recommendations. Nutate in a covered FACS tube at 4º C for 25 minutes.

8. Deplete lineage$^+$ cells by incubating FACS tube in Promega MagneSphere® Technology Magnetic Separation Stand (Z5333) for 2 minutes. Remove lineage$^{neg/low}$ cells with pasteur pipet.

9. Wash cells with 1mL HBSS+, pellet cells at 380 x g at 4º C for 4 minutes, resuspend in 1 mL HBSS+, count 1:10 in trypan blue.

   Expect 10-20% of starting cell number post depletion.

10. Restain cells using 1µl Lineage mix/ 10^6 cells.

11. Wash cells 2x with 1mL HBSS+, pellet cells at 380 x g at 4º C for 4 minutes

12. Stain with 2µl / 10^7 cells Tricolor anti Rat (Caltag) for 20 minutes on ice.

13. Wash cells 2x with 1mL HBSS+, pellet cells at 380 x g at 4º C for 4 minutes.

14. Resuspend in 150µl HBSS+, add 50µl Rat Ig to block for 5 minutes in the dark.

15. Add 10µl Sca-1/FITC, cKit/APC (BD Pharmingen), CD48/PE (eBioscience) mix /10^7 cells, incubate for 20 minutes on ice in the dark.

16. Wash cells 2x with 1mL HBSS+, pellet cells at 380 x g 4º C for 4 minutes.

17. Resuspend in 1mL HBSS+ with 1mg/mL

Sort controls
2 x 10^6 each sample

1. Unstained
2. CD44/FITC
3. B220/APC
4. FcR/PE
5. Lin mix+Tricolor
6. cKit/APC+Sca-1/FITC+CD48/PE+PI
7. Lin mix+Tricolor+Sca-1/FITC+CD48/PE+PI
8. Lin mix+Tricolor+cKit/APC+CD48/PE+PI
9. Lin mix+Tricolor+Sca-1/FITC+cKit/APC+PI
10. Lin mix+Tricolor+cKit/APC+Sca-1/FITC+CD48/PE+PI

18. Stain sort controls along with sort samples.

19. Use 2.5µl lineage mix and 1µl other antibodies except 0.25µl CD48 per control.

20. Sort KSL $CD48^+$ and $CD48^-$ cells for each sample.

21. Spin at 1480 RPM for 5 min (441 x g).

22. Incubate in 300µl Hoechst 10µg/ml and 1µl/ml (verapamil 100mM Molecular Probes) in Hoeschst staining buffer for 45 minutes at 37˚ C.

23. Add 6µl (Pyronin Y 100µg/ml) incubate for an additional 15 minutes at 37˚C.

24. Wash cells 2x with 1mL HBSS+, pellet cells at 441 x g at 4º C for 4 minutes.

25. Resuspend in 300µl HBSS+

G_0/G_1 controls

BM Hoechst + Pyonin Y
BM Pyronin Y
BM Hoechst
BM unstained
Thymus unstained
Thymus Pyronin Y
Thymus Hoechst
Thymus Hoechst + Pyronin Y

Hoechst staining buffer
45 ml HBSS
5 ml FBS
50mg dextrose/ glucose
1µl 1M HEPES