FACS STUDY

Experiment Name : Cell separation by FACS (^{3}HTdR cluster, 50% labeling, ~~two~~ one dye conc.)

Exp. # : 1; **Experiment performed by:** A. Bishayee **Date:** 06/08/~~99~~ 06/05/00

1. Set the rocker-roller at 37^{0}C incubator with 5% CO_2, set the Coulter Counter, wash cells (from two 175 cm^2 flasks, subcultured 1:2, 24h before) with PBS, trypsinize cells, each resuspend in 9 ml MEMB, pool, pass five times through 3 cc syringe with 21 gauge needle, perform cell count by transfering 100 ul in Coulter cup containing 20 ml isotone (Coulter balanced electrolyte solution)
2. Dilute to ~2,000,000 cells/ml in MEMB [Actual count : cells/ml)
3. Transfer 1 ml of cell suspension into eight 14 ml tubes (Falcon plastic test tube, 17x100 mm) labeled 1-8 both on cap and wall
4. Keep the tubes in the roller for 3-4 h at 37°C, 5% CO_2 **Date/Time:**
5. Prepare MEMB containing radioactivity in hood

   100 µl ^{3}HTdR (Stock : 1 µCi/µl on) + 4.9 ml MEMB
6. After 3-4 h, remove tubes from roller and add MEMB with or without radioactivity according to Table below.

Date/Time:

Tube #	^{3}HTdR uCi/ml	Cells in MEMB (ml)	MEMB (ml)	MEMB+ ^{3}HTdR (ml) [20uCi/ml]	CFDA in PBS (0.05 uM) (ml)	CFDA in PBS (0.1 uM) (ml)	PBS (ml)
1	0	1.0	1.0	0	0	0	2
2	0	1.0	1.0	0	0	0	2
3	0	1.0	1.0	0	0	0	2
4	0	1.0	1.0	0	0	0	2
5	10	1.0	0	~~0.1~~ 1	2	0	
6	10	1.0	0	~~0.1~~ 1	2	0	
7	10	1.0	0	~~0.3~~ 1	0	2	
8	10	1.0	0	~~0.3~~ 1	0	2	

7. Return test tubes to roller for 12 h **Date/Time:**
8. Next day, while test tubes are in roller label 8 tubes (13 X 100 mm VWR glass test tube)
9. After ~12 h incubation period, remove tubes and centrifuge at 2000 rpm at 4°C for 10 min (***precooled centrifuge***). **Date/Time:**
10. Remove buckets from centrifuge and carefully remove 150 µl of supernatant and place in prelabeled tubes.
11. Decant supernatant, click tubes, vortex, resuspend in 10 ml wash MEMA
12. Centrifuge tubes for 10 min at 2000 rpm, 4°C
13. Decant supernatant, click tubes, vortex, resuspend in 10 ml wash MEMA
14. Centrifuge tubes for 10 min at 2000 rpm, 4°C
15. Decant supernatant, click tubes, vortex, resuspend in 10 ml wash MEMA
16. Centrifuge tubes for 10 min at 2000 rpm, 4°C
17. Decant supernatant, click tubes, vortex, resuspend in 2 ml of PBS, syringe and perform cell count as well as radioactivity count by transferring aliquots.
18. Add 8 ml of PBS in each tube, vortex and transfer the content to 15-ml plastic centrifuge tube
18. Centrifuge tubes for 10 min at 2000 rpm, 4°C
19. Decant supernatant, click tubes,vortex
20. Add 2 ml of 0.05 or 0.1 uM CFDA in prewarmed PBS as per the Table and only PBS in the remaining tubes.
21. Incubate all tubes at 37°C for 15 min.
21. Centrifuge tubes for 10 min at 2000 rpm, 4°C
22. Decant supernatant, click tubes, vortex, add 2 ml prewarmed MEMA
23. Incubate all tubes at 37°C for 30 min.
24. Centrifuge and decant the supernatant, suspend in 5 ml MEMA
25. Transfer the content from tubes 1-4 to 5-8 (1 to 4; 2 to 5...etc)
26. Centrifuge, decant the supernatant
27. Transfer the cell suspension in polypropylene microcentrifuge tubes with attached caps (Helena Plastics, 400 ul) using 200 ul pipet tips
28. Again add 200 ul cold MEMA, resuspend and transfer the cell suspensions in the same polypropylene microcentrifuge tubes (Total volume ~400 ul)
29. Centrifuge tubes for 5 min at 1000 rpm, 4°C
30. Transfer tubes at 10°C for 72 h. **Date/Time:**
31. After 72 h, carefully remove the supernatant from the top, resuspend pellet in 200 ul wash MEMA and transfer the content to eight 15 ml tubes containing 10 ml PBS by using pasteur pipet **Date/Time:**

32. Again add 200 ul PBS in microcentrifuge tubes, resuspend and transfer the cell suspensions in 15 ml tubes
33. Centrifuge the tubes for 10 min at 2000 rpm, 4°C ***(precooled centrifuge)***
32. Decant supernatant, click tubes, vortex, pooled cells from **two** tubes, centrifuge, decant the suoernatant, resuspend in 2 ml PBS with 0.005 mM EDTA, syringe and transfer aliquots (100 ul) for cell count and radioactivity count
33. Centrifuge, decant, resuspend in 1 ml PBS with 0.005 mM EDTA to have ~10,000,000 cells/ml for each tube and transfer ~1ml in Falcon 12x75 mm polystyrene 6 ml tube, wrap the tubes with aluminium foil, put in ice and transfer for FACS study.

06/09/00

Preparation of 0.1 µM COCF in PBS

① Take the component A and B, thaw them

② Add 90 µl of DMSO from component B to component A. (Final conc = 10 mM)

$S_1V_1 = S_2V_2$

$10,000 \times V_1 = 0.1 \times 8$

or $V_1 = \frac{0.1 \times 8}{10000}$

$= 0.$

③ Take 5 µl of 10

$S_1V_1 = S_2V_2$ 06/19/00

$10,000 \times V_1 = 10,000 \times 10\,\mu l$

10 µl + 10 µl ⇒ 10 µM

↓

1:10

300 µl → 3 µl

1 µM of COCF

~~646~~
703
663

~~757~~
683

2,732,000 cells

1715, 1714, 1745

1725

1725 x 4000 = 6,900,000 Cells/mL

2,200,000 cells/mL x (1) = $\frac{22000000}{6,900,000}$

= 3.2 mL

3.2 cells + 6.8 mL MEMB

= 511, 414,

06/09/00

MS = 50ul

1	157,	164,	133	in 2ml
2	155,	173,	156	
3.	154,	136,	152	
4	131,	123,	119	
5	161,	152,	166	
6	139,	176,	128	
7	132,	142,	155	
8	~~137~~,	168,	176, 155	

Cell count
789, 791, 759

690, 714, 679