V79 COLONY FORMING ASSAY

Experiment Name : Cell separation by FACS (using EDTA) **Exp. # :** 2;
Experiment performed by : A. Bishayee **Date:** 08/23/99

1. Set the rocker-roller at 37°C incubator with 5% CO_2, set the Coulter Counter, wash cells (from two 150 cm^2 flusk, 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 8 tubes (Falcon plastic test tube, 17x100 mm) labeled 1-8 both on cap and wall
4. Keep the tubes in the roller for 16 h at 37°C, 5% CO_2 **Date/Time:** 08/23/99 ; 4-00p.m.
5. Centrifuge tubes for 10 min at 2000 rpm, 4°C Date/Time : 08/24/99; 9-15a.m.
6. Decant supernatant, click tubes, vortex, resuspend in Add 8 ml of PBS in each tube, vortex and transfer the content to 15-ml plastic centrifuge tube
7. Centrifuge tubes for 10 min at 2000 rpm, 4°C
8. Decant supernatant, click tubes,vortex
9. Add 2 ml of 0.05 uM CFDA in prewarmed PBS in tubes # 2, 4, and 6 respectively and PBS in the remaining tubes.
10. Incubate all tubes at 37°C for 15 min.
11. Centrifug tubes for 10 min at 2000 rpm, 4°C
12. Decant supernatant, click tubes, vortex, add 2 ml prewarmed MEMA
13. Incubate all tubes at 37°C for 30 min.
14. Centrifuge and decant the supernatant, suspend in 5 ml MEMA
15. Transfer the content of one tube to the next one (1to 2; 3 to 4 etc.)
16. Centrifuge, decant the supernatant
17. Transfer the cell suspension in polypropylene microcentrifuge tubes with attached caps (Helena Plastics, 400 ul) using 200 ul pipet tips
18. Again add 200 ul ice cold MEMA, resuspend and transfer the cell suspensions in the same polypropylene microcentrifuge tubes (Total volume ~400 ul)
19. Centrifuge tubes for 5 min at 1000 rpm, 4°C
20. Transfer tubes at 10°C for 72 h. **Date/Time:** 08/24/99; 2-00 p.m.
21. 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: 08/27/99; 12-00 noon

22. Again add 200 ul PBS in microcentrifuge tubes, resuspend and transfer the cell suspensions in 15 ml tubes
23. Centrifuge the tubes for 10 min at 2000 rpm, 4°C ***(precooled centrifuge)***
24. Decant supernatant, click tubes, vortex, resuspend in 2 ml PBS with or without 0.005 or 0.05 mM EDTA, syringe and transfer aliquots for cell count
25. Transfer 2x1ml aliquot from each tube in 13x100 mm polyvinyl plastic tube for FACS, wrap the tubes with aluminium foil and keep them at 10.5°C before performing FACS

cell count was low
Cells from all tubes pooled and sorted
After sorting dye-negative cells were collected and cell count was performed.

V79 COLONY FORMING ASSAY

Experiment Name : Cell separation by FACS (using EDTA) ~~and SF~~ **Exp. # :** 1;
Experiment performed by : A. Bishayee **Date:** 08/15/99

1. Set the rocker-roller at 37°C incubator with 5% CO_2, set the Coulter Counter, wash cells (from two 150 cm^2 flusk, 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 8 tubes (Falcon plastic test tube, 17x100 mm) labeled 1-8 both on cap and wall
4. Keep the tubes in the roller for 16 h at 37°C, 5% CO_2 **Date/Time:** 08/15/99; 4-00 P.M.
5. Centrifuge tubes for 10 min at 2000 rpm, 4°C Date/Time: 08/16/99; 9-30 a.m.

Next day.

6. Decant supernatant, click tubes, vortex, resuspend in Add 8 ml of PBS in each tube, vortex and transfer the content to 15-ml plastic centrifuge tube
7. Centrifuge tubes for 10 min at 2000 rpm, 4°C
8. Decant supernatant, click tubes, vortex
9. Add 2 ml of 0.05 uM CFDA in prewarmed PBS in tubes # 2, 4, 6 and 8 respectively and PBS in the remaining tubes.
10. Incubate all tubes at 37°C for 15 min.
11. Centrifug tubes for 10 min at 2000 rpm, 4°C
12. Decant supernatant, click tubes, vortex, add 2 ml prewarmed MEMA
13. Incubate all tubes at 37°C for 30 min.
14. Centrifuge and decant the supernatant, suspend in 5 ml MEMA
15. Transfer the content of one tube to the next one (1to 2; 3 to 4 etc.)
16. Centrifuge, decant the supernatant
17. Transfer the cell suspension in polypropylene microcentrifuge tubes with attached caps (Helena Plastics, 400 ul) using 200 ul pipet tips
18. Again add 200 ul ice cold MEMA, resuspend and transfer the cell suspensions in the same polypropylene microcentrifuge tubes (Total volume ~400 ul)
19. Centrifuge tubes for 5 min at 1000 rpm, 4°C
20. Transfer tubes at 10°C for 72 h. **Date/Time:** 08/16/99; 3-00 P.M.
21. 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: 08/19/99 ; 11-00 a.m.

22. Again add 200 ul PBS in microcentrifuge tubes, resuspend and transfer the cell suspensions in 15 ml tubes
23. Centrifuge the tubes for 10 min at 2000 rpm, 4°C ***precooled centrifuge***
24. Decant supernatant, click tubes, vortex, resuspend in 2 ml PBS with or without 0.005, 0.05 or 0.5 mM EDTA, syringe and transfer aliquots for cell count
25. Transfer 2x1ml aliquot from each tube in 13x100 mm polyvinyl plastic tube for FACS, wrap the tubes with aluminium foil and keep them at 10.5°C before performing FACS

Initial count = 1125, 1195, 1154
Avg. count = 1158
Cell conc. = 4,632,000 cells/ml

We need 2,000,000 cells/ml × 9 = 18,000,000 cells

Vol. required = $\frac{18,000,000}{4,632,000}$

= 3.9 ml

Take 3.9 ml cells + 5.1 ml MEHB = 9 ml

Final count = 503, 513, 512
Avg. count = 509
Cell conc. = 2,037,333 cells/ml

08/19/99

Cell Count After 72h

Final pellet suspended in 2 ml PBS with or without 0.005, 0.05 and 0.5 mM EDTA

Tube #	Count	cells/ml
1	236, 226, 228	920,000
2	257, 259, 267	1,044,000
3	264, 265, 260	1,052,000
4	249, 240, 248	~~9.8~~ 982,666

i) Sorting rate was below the FACS capability (63,000 cells in 9 min)

ii) Pool cells from tubes 1-3, centrifuge, resuspend in 0.5 ml PBS $\bar{c}$ 0.05 mM EDTA
Perform cell count : ~~[illegible]~~ 1000, 993, 1010
Cell conc. = 4004000 cells/ml

2) ~~[illegible]~~ The cells were sorted again. This time rapid sorting was achieved.

Results

i) 0.5 mM EDTA ~~or~~ was not suitable for cell sorting

ii) 10^7/ml cell count was required ~~for~~ to achieve optimum sorting rate