V79 COLONY FORMING ASSAY

Experiment Name : Cell separation by FACS (^{3}HTdR cluster, 50% labeling);
Exp. # : 1; **Investigator:** A. Bishayee **Date:** 04/12/99

1. Set the rocker-roller at 37^{0}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 : 1,994,666 cells/ml)
3. Transfer 1 ml of cell suspension into eight 12 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:** 04/12/99; 4-00 p.m.
5. Prepare MEMB containing radioactivity in hood
   16 µl ^{3}HTdR (Stock : 1 µCi/µl on 3/8/99) + 2 ml MEMB
6. After 3-4 h, remove ~~first set of 10~~ test tubes from roller and add MEMB with or without radioactivity according to Table below. **Date/Time:** 04/12/99; 7-15 p.m.

Tube #	^{3}HTdR uCi/ml	Cells in MEMB (ml)	MEMB (ml)	MEMB+ ^{3}HTdR (ml) [8uCi/ml]
1	0	1.0	1.0	0
2	0	1.0	1.0	0
3	0	1.0	1.0	0
4	2	1.0	0.5	0.5
5	0	1.0	1	0
6	2	1.0	0.5	0.5
7	0	1.0	1	0
8	2	1.0	0.5	0.5

7. Return test tubes to roller for 12 h . **Date/Time:** 04/12/99; 7-30 p.m.
8. Next day, while test tubes are in roller label 10 gamma-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:** 04/13/99; 9-30 a.m.
10. Remove buckets from centrifuge and carefully remove 150 µl of supernatant and place in prelabeled gamma-tube.
11. Decant supernatant, click tubes, vortex, resuspend in 10 ml ~~wash MEMA~~ PBS and transfer to 15 ml plastic tube
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. (100 µl)
18. Add 8 ml of PBS in each tube, vortex ~~and transfer the content to 15 ml plastic centrifuge tube~~ * Next time prepare 2x dye and add after step 17
18. Centrifuge tubes for 10 min at 2000 rpm, ~~4°C~~
19. Decant supernatant, click tubes,vortex
20. Add 2 ml of CFDA (0.05, 0.5 and 2.5 uM) in prewarmed PBS in tubes # 4, 6, and 8 respectively and PBS in the remaining tubes.
21. Incubate all tubes at 37°C for 15 min.
21. Centrifug 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 of one tube to the next one (1to 2; 3 to 4 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 ice 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~~ 10-5°C for 72 h. **Date/Time:** 04/13/99; 1-00 p.m.
31. After 72 h, carefully remove the supernatant from the top, resuspend pellet in 200 ul wash MEMA and transfer the content to ~~eight~~ four 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, ~~resuspend in 2 ml PBS, syringe and transfer~~ aliquots ~~for cell as well as radioactivity count~~

33. ~~Add 9 ml of PBS and transfer 1 ml~~ in 13x100 mm transperant plastic tube for FACS, wrap the tubes with aluminium foil.

33(a) Only the cells that labeled with 0.05μM CFDA were sorted

Preparation of 4% BSA - 0.1% NaN in 1X PBS ; 500 ml.

1. BSA = 20 g
2. 10% NaN = 5 ml
3. 1X PBS = ~ 500 ml

$10\% \times V_1 = 0.1\% \times 500$

$V_1 = \frac{0.1 \times 500}{10}$

$= 5$ ml.

04/16/99

34 After sorting, collect 6 50 ml tubes containing the sorted cells (non-labeled) not dyed)

35. centrifuge and pool them together

36. Final pellet suspended in 2 ml PBS.

37. Perform cell count by transferring 200μl.

38. Transfer 0.5 ml in a tube for sorting again

Expt # 1

04/12/99

Initial Count = 1066, 1019, 1026

Avg. cell count = 1037

Cell conc. = 4,148,000

We need 2,000,000 cells/ml × 10 = 20,000,000 cells

Vol. required = $\frac{20,000,000}{4,148,000}$ = 4.8

Take 4.8 ml cells + 5.2 ml MEMB = 10 ml

Final count = 486, 513, 497

Avg. Cell ~~conc~~ count = 498.6

cell conc. = 1,994,666 cells/ml

Before forming the pellet

USER: 6 ID:H3 HOWELL PRESET TIME: 1.00 TUE 13 APR 1999 16:18
SAMPLE REPEAT: 1 CYCLE REPEAT: 1 SCR:N RS232:N
H#: 1 AQC:N QCF:N RCM:N
CHANNEL 1-LL: 0 UL: 400 2SIGMA: 2.00 BKG SUB: 0.00 BKG 2SIG: 0.00 LSR: 0
DATA CALC: CPM, UNKNOWN REPLICATES: 1 NORM FACTOR:@ 1.00000
HALF LIFE(DAYS):N

SAM	POS	CH		CPM	2SIG%	TIME	EL TIME	AVG H#	ERR	
1	**- 1	1	1c	10.00	63.25	1.00	1.70	66.0		
2	**- 2	1		7.00	75.59	1.00	3.53	68.0		
3	**- 3	1	2c	7.00	75.59	1.00	5.35	65.0		
4	**- 4	1		9.00	66.67	1.00	7.18	65.0		
5	**- 5	1	3c	9.00	66.67	1.00	9.05	66.0		
6	**- 6	1		7.00	75.59	1.00	10.88	54.0		
7	**- 7	1	4c	7410.00	2.32	1.00	12.76	64.0		
8	**- 8	1		7612.00	2.29	1.00	14.58	66.0		100 µl cells
9	**- 9	1	5c	11.00	60.30	1.00	16.42	67.0		
10	**-10	1		4.00	100.0	1.00	18.24	64.0		
11	**-11	1	6c	7445.00	2.32	1.00	20.11	61.0		
12	**-12	1		8503.00	2.17	1.00	21.94	67.0		
13	**- 1	1	7c	11.00	60.30	1.00	23.78	55.0		
14	**- 2	1		5.00	89.44	1.00	25.57	65.0		
15	**- 3	1	8c	5549.00	2.68	1.00	27.40	61.0		
16	**- 4	1		5837.00	2.62	1.00	29.27	66.0		
17	**- 5	1	1M	19.00	45.88	1.00	31.15	57.0		
18	**- 6	1		22.00	42.64	1.00	33.02	57.0		
19	**- 7	1	2M	15.00	51.64	1.00	34.91	55.0		
20	**- 8	1		22.00	42.64	1.00	36.74	57.0		
21	**- 9	1	3M	10.00	63.25	1.00	38.61	54.0		
22	**-10	1		9.00	66.67	1.00	40.44	59.0		30 µl medium
23	**-11	1	4M	74369.70	1.81	0.17	41.42	57.0		
24	**-12	1		78569.70	1.76	0.17	42.40	61.0		
25	**- 1	1	5M	10.00	63.25	1.00	44.38	57.0		
26	**- 2	1		12.00	57.74	1.00	46.22	59.0		
27	**- 3	1	6M	65874.28	1.86	0.18	47.25	59.0		
28	**- 4	1		68931.42	1.82	0.18	48.29	57.0		
29	**- 5	1	7M	20.00	44.72	1.00	50.17	59.0		
30	**- 6	1		5.00	89.44	1.00	51.99	57.0		
31	**- 7	1	8M	67030.30	1.90	0.17	52.97	64.0		
32	**- 8	1		69394.28	1.81	0.18	54.01	59.0		

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TABLE-1

Expt. # : 1 **Date/Time :** 04/13/99; 4-20 p.m.

Tube #	Medium count for 30 ul (cpm)	Avg. cpm	dpm [cpm/0.65]	µCi/ml (A_t) on counting [dpm/66600]	µCi/ml (A_o) on addition [$A_t/e^{-\lambda t}$]
1					
2					
3					
4		76469	117644	1·76	
5					
6		67402	103696	1·56	
7					
8		68212	104941	1·57	
9					
10					

TABLE-2

Expt. # : 1 100 µl **Date/Time :** 04/13/99; 4-20 pm

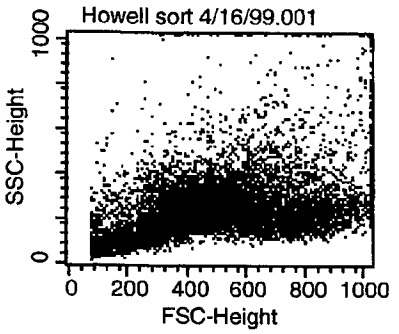
Tube #	Radioactivity for ~~200~~ ul cell suspension before forming the multicell-pellet (cpm)	Avg. cpm	dpm [cpm/0.65]	µ Ci/ml (A_t) on counting [dpm/~~444000~~ 222000]	µ Ci/ml (A_o) after 12 h incubation [$A_t/e^{-\lambda t}$]
1					
2					
3					
4		7511	11555	0·0521	
5					
6		7974	12267	0·0553	
7					
8		5693	8758	0·0395	
9					
10					

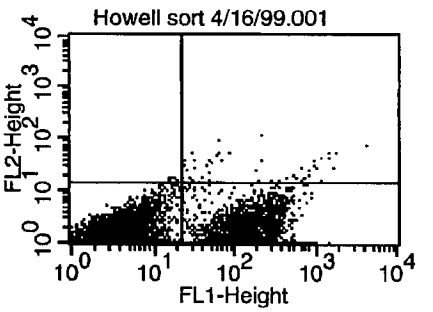
TABLE-3

Expt. # : 1 **Date/Time :** 04/13/99

Tube #	Coulter count for 100 ul cell suspension before having the multicellular cluster	Avg. count	Cells/ml [Avg. count x 4000]	pCi/cell [uCi/ml x10^6 Cells/ml]
1	186, 187, 189			
2	191, 188, 174			
3	284, 289, 297			
4	263, 256, 259	259	1037333	0.0502
5	352, 324, 360			
6	350, 362, 332	348	1392000	0.0397
7	320, 333, 342			
8	237, 232, 239	236	944000	0.0418
9				
10				

After 72h



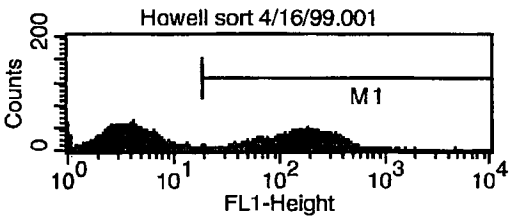


Quadrant Statistics

File: Howell sort 4/16/99.001
Sample ID:
Tube:
Acquisition Date: 16-Apr-99
Gated Events: 10000
X Parameter: FL1-H FL1-Height (Log)
Quad Location: 22, 14

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 10000
Y Parameter: FL2-H FL2-Height (Log)

Quad	Events	% Gated	% Total
UL	8	0.08	0.08
UR	49	0.49	0.49
LL	5174	51.74	51.74
LR	4769	47.69	47.69



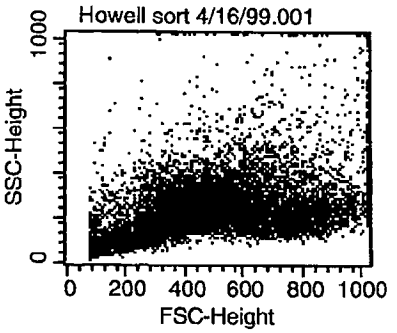
Histogram Statistics

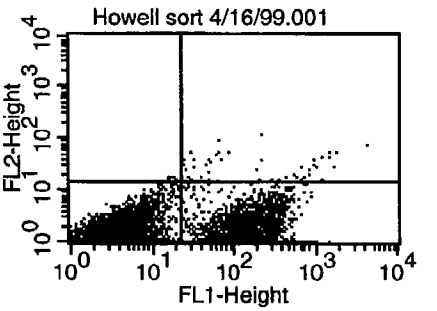
File: Howell sort 4/16/99.001
Sample ID: 50% (20)
Tube:
Acquisition Date: 16-Apr-99
Gated Events: 10000
X Parameter: FL1-H FL1-Height (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	10000	100.00	100.00	90.66	4
M1	18, 9910	4859	48.59	48.59	182.03	174

Mfloc 72k



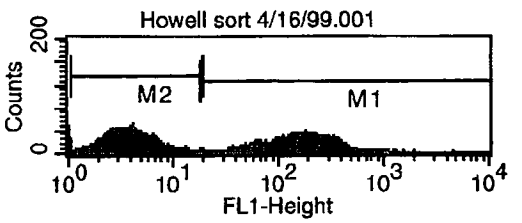


Quadrant Statistics

File: Howell sort 4/16/99.001
Sample ID:
Tube:
Acquisition Date: 16-Apr-99
Gated Events: 10000
X Parameter: FL1-H FL1-Height (Log)
Quad Location: 22, 14

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 10000
Y Parameter: FL2-H FL2-Height (Log)

Quad	Events	% Gated	% Total
UL	8	0.08	0.08
UR	49	0.49	0.49
LL	5174	51.74	51.74
LR	4769	47.69	47.69



Histogram Statistics

File: Howell sort 4/16/99.001
Sample ID:
Tube:
Acquisition Date: 16-Apr-99
Gated Events: 10000
X Parameter: FL1-H FL1-Height (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	10000	100.00	100.00	90.66	4
M1	18, 9910	4859	48.59	48.59	182.03	174
M2	1, 19	5100	51.00	51.00	4.38	4

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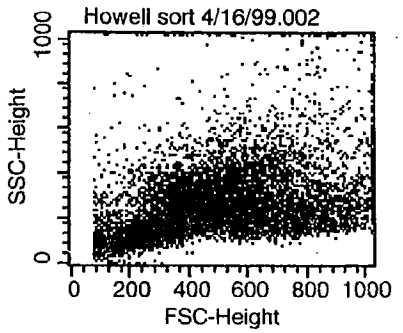
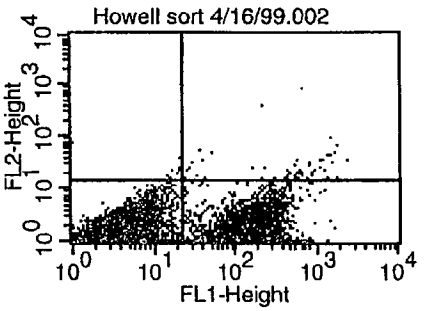
After 72h





Quadrant Statistics

File: Howell sort 4/16/99.002
Sample ID:
Tube:
Acquisition Date: 16-Apr-99
Gated Events: 100000
X Parameter: FL1-H FL1-Height (Log)
Quad Location: 22, 14

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 100000
Y Parameter: FL2-H FL2-Height (Log)

Quad	Events	% Gated	% Total
UL	83	0.08	0.08
UR	498	0.50	0.50
LL	50765	50.77	50.77
LR	48654	48.65	48.65

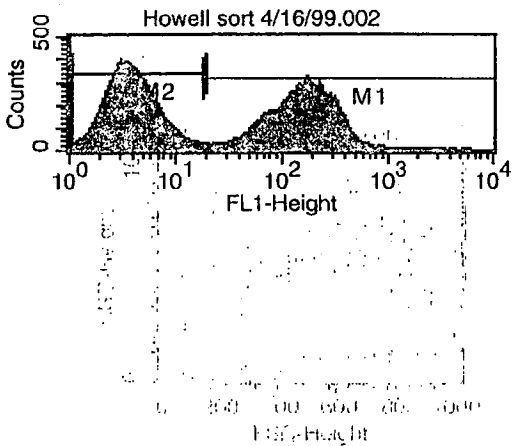


Fig 1

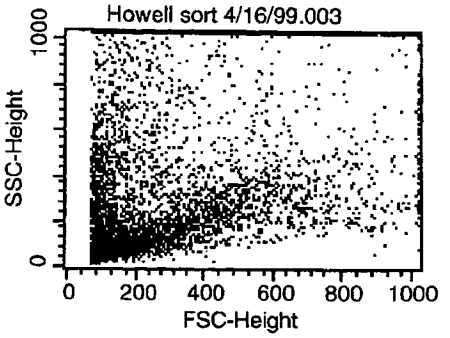
Histogram Statistics

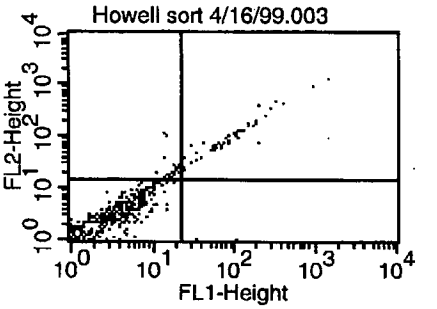
File: Howell sort 4/16/99.002
Sample ID:
Tube:
Acquisition Date: 16-Apr-99
Gated Events: 100000
X Parameter: FL1-H FL1-Height (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 100000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	100000	100.00	100.00	92.50	1
M1	18, 9910	49577	49.58	49.58	182.09	161
M2	1, 19	49867	49.87	49.87	4.50	2

After 72h



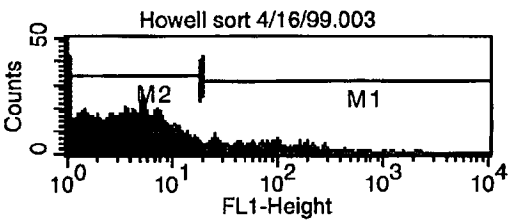


Quadrant Statistics

File: Howell sort 4/16/99.003
Sample ID: sorted
Tube:
Acquisition Date: 16-Apr-99
Gated Events: 5295
X Parameter: FL1-H FL1-Height (Log)
Quad Location: 22, 14

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 5295
Y Parameter: FL2-H FL2-Height (Log)

Quad	Events	% Gated	% Total
UL	227	4.29	4.29
UR	566	10.69	10.69
LL	4493	84.85	84.85
LR	9	0.17	0.17



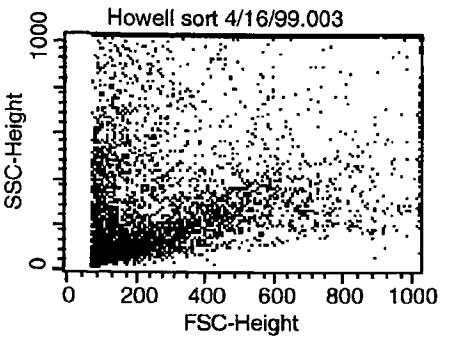
Histogram Statistics

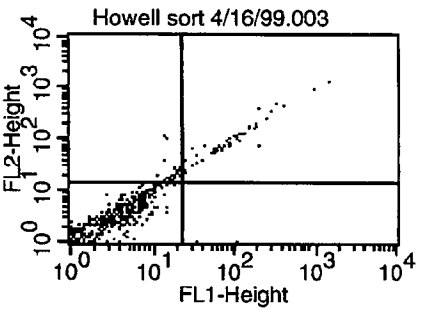
File: Howell sort 4/16/99.003
Sample ID: sorted
Tube:
Acquisition Date: 16-Apr-99
Gated Events: 5295
X Parameter: FL1-H FL1-Height (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 5295

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	5295	100.00	100.00	17.14	1
M1	18, 9910	638	12.05	12.05	116.27	24
M2	1, 19	3114	58.81	58.81	4.92	5

After 72h



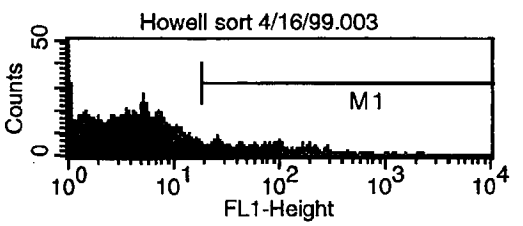


Quadrant Statistics

File: Howell sort 4/16/99.003
Sample ID: sorted
Tube:
Acquisition Date: 16-Apr-99
Gated Events: 5295
X Parameter: FL1-H FL1-Height (Log)
Quad Location: 22, 14

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 5295
Y Parameter: FL2-H FL2-Height (Log)

Quad	Events	% Gated	% Total
UL	227	4.29	4.29
UR	566	10.69	10.69
LL	4493	84.85	84.85
LR	9	0.17	0.17



Histogram Statistics

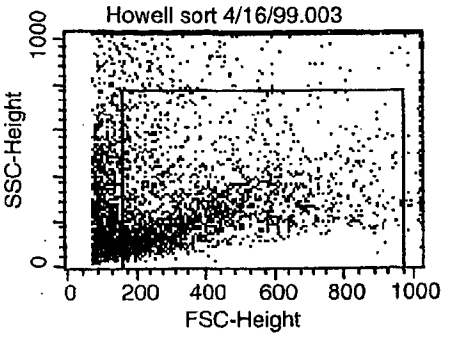
File: Howell sort 4/16/99.003
Sample ID: sorted
Tube:
Acquisition Date: 16-Apr-99
Gated Events: 5295
X Parameter: FL1-H FL1-Height (Log)

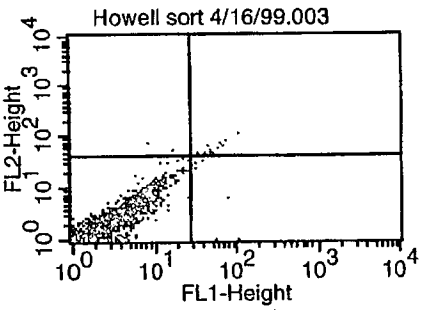
Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 5295

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	5295	100.00	100.00	17.14	1
M1	18, 9910	638	12.05	12.05	116.27	24

B011756

After FACS Sorting





Quadrant Statistics

File: Howell sort 4/16/99.003
Sample ID: sorted
Tube:
Acquisition Date: 16-Apr-99
Gated Events: 2334
X Parameter: FL1-H FL1-Height (Log)
Quad Location: 28, 43

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G1
Total Events: 5295
Y Parameter: FL2-H FL2-Height (Log)

Quad	Events	% Gated	% Total
UL	4	0.17	0.08
UR	28	1.20	0.53
LL	2281	97.73	43.08
LR	21	0.90	0.40

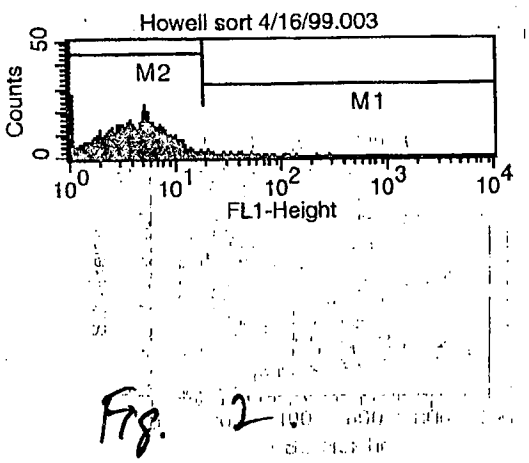


Fig. 2

Histogram Statistics

File: Howell sort 4/16/99.003
Sample ID: sorted
Tube:
Acquisition Date: 16-Apr-99
Gated Events: 2334
X Parameter: FL1-H FL1-Height (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G1
Total Events: 5295

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	2334	100.00	44.08	5.57	1
M1	18, 9910	77	3.30	1.45	45.56	19
M2	1, 18	2257	96.70	42.63	4.20	1

USER: 6 ID:H3 HOWELL PRESET TIME: 1.00 MON 19 APR 1999 11:51
SAMPLE REPEAT: 1 CYCLE REPEAT: 1 SCR:N RS232:N
H[redacted] 1 AQC:N QCF:N RCM:N
CHANNEL 1-LL: 0 UL: 400 2SIGMA: 2.00 BKG SUB: 0.00 BKG 2SIG: 0.00 LSR: 0
DATA CALC: CPM, UNKNOWN REPLICATES: 1 NORM FACTOR:0 1.00000
HALF LIFE(DAYS):N

SAM	POS	CH	CPM	2SIG%	TIME	EL TIME	AVG H#	ERR	
1	**- 1	1	8.00	70.71	1.00	1.60	66.0		200µl PBS
2	**- 2	1	18.00	47.14	1.00	3.38	76.0		
3	**- 3	1	13.00	55.47	1.00	5.16	61.0		200µl cells
4	**- 4	1	12.00	57.74	1.00	6.94	64.0		

47.00 → 1 ml cells.

4/16/99

Cell count : 200µl cells in PBS

Coulter count = 183, 176, 170

Cell conc. = 176 x 4000

= 70, 533 cells/ml

# of cells in 200µl = 14,1,066 cells

4/19/99

Check cpm in sorted cells

50% labeling ^{3}HTdR, dyed these cells with Tracker dye
Sorted & kept non-dyed, non-^{3}HTdR

97% pure on dye-negative

^{3}HTdR cells have 0.042 pCi/cell $\left(\frac{0.037 \text{ dps}}{\text{pCi}}\right)\left(\frac{60 \text{ s}}{\text{m}}\right)$

$= 0.0932$ dpm/cell $\times 2 = 0.186$ dpm/^{3}H cell
$= 0.11$ cpm/^{3}H cell

Sort cells @ 200Hl cells contains 14,107 cells

If pure ^{3}H labeled cells expect

0.11 cpm (14107) = 1580 cpm

If pure ~~carbon~~ non-^{3}H cells expect
background

If 3% labeled, expect

0.11 cpm (0.03)(14107) = 46.5 cpm − backgrd.
46.5 − 13 = 33 cpm reg

Actually got 13 cpm − backgrd.

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47 cpm − 13 cpm = 34 cpm in 1ml of cells

Activity per cell $= \frac{34 \text{ cpm}/60(0.65)}{5(14{,}107)} = 1.24 \times 10^{-5}$ Bq $\left(\frac{1 \text{ pCi}}{0.037 \text{ Bq}}\right) = 0.000335$ pCi/cell