V79 COLONY FORMING ASSAY

Experiment Name : ^{137}Cs toxicity (cluster) γ - irradiation
Experiment No.: BOG V79. 001
Date: 12/7/01

1. Seed a 225 cm^2 flask containing 50 ml MEMA with 3x10^6 cells 3 days before experiment.
2. Wash cells in 225 cm^2 flask with 20 ml PBS, trypsinize cells with 2ml Trypsin- EDTA, and resuspend in 7 ml MEMB; ← 3min, 37°C ← 10ml Pen. Str for 1ℓ PBS.
3. Aspirate cells and transfer to 14 ml 17x100mm Falcon tube and vigorously pass five times through 5 cc syringe with 21 gauge needle;
4. Perform cell count by transfering 100 µl in Coulter cup containing 20 ml Isotone-II (Coulter balanced electrolyte solution);
   Instrument settings: Lower Threshold (LT) – 5.0; Manometer – 100 µl.
   Perform cell count in triplicates. For example, ~~5353~~ 5442 5291. Average value – 5362.
   Cell concentration – 5362 x 400 x 5 = 10,7 x 10^6 cells/ml.
5. Dilute to ~4,000,000 cells/ml in MEMB (final volume 11 ml). For example, 4.1 ml stock + 6.9 ml MEMB gives 4 x 10^6 cells/ml. ← Repeat Coulter Counting - ? Transfer 1 ml of cell suspension into ten 14 ml tubes (Falcon 352059 polypropylene culture tube, 17x100 mm) labeled 1-10 both on cap and wall till 10 ml level
6. Roll the tubes on rocker-roller for 4 h at 37°C, 5% CO_2 **Date/Time:** 10ml
7. After ~4 h incubation period, remove tubes, and centrifuge at 2000 rpm RT for 10 min.
8. Decant supernatant, click tubes, vortex, resuspend in 10 ml MEMA
9. Centrifuge tubes for ~~10~~ min at 2000 rpm, RT leave ~ 200 µl
10. Decant supernatant, click tubes, vortex, transfer the cell suspension to 400 ul polypropylene microcentrifuge tubes with attached caps (VWR) using 200 ul pipetter
11. Again add 200 ul MEMA, resuspend and transfer the cell suspensions in the same polypropylene microcentrifuge tubes (Total volume ~400 ul)
12. Centrifuge tubes for 5 min at 1000 rpm, RT
13. Transfer tubes at 10.5°C for 72 h. **Date/Time:** Dec 12, 2001 / 22:00

14. After 72 h, place the tubes on the perforated plate of Rainin pipet tip box containing ice (to maintain ~ 10.5°C) Dec. 10 2001

15. The tubes were irradiated using Mark I irradiator (^{137}Cs gamma-ray), one tube at a time, while placing onto a separate Rainin pipet tip box containing ice as per the Table below

Tube #	Total Dose (Rad)	Dose rate (Rad/min)	Time (min)	Attenuat.	
1.2	1	0	0	0	0	3 dishes
2.2	2	0	0	0	0	3
~~3.2~~	~~3~~	~~100~~ 1500	101.4	~~0.98~~	~~X-10~~ X-5	
3.2	~~4~~ 3	200	101.4	~~1.97~~ 1.27	X-10 X-5	3
4.2	~~5~~ 4	300	101.4	~~2.95~~ 1.90	~~X-10~~ X-5	3
5.2	~~6~~ 5	400	101.4	~~3.94~~ 2.53	~~X-10~~ X-5	3
6.2 6.3	~~7~~ 6	500	101.4	~~4.93~~ 3.16	~~X-10~~ X-5	6
7.2 7.3	~~8~~ 7	750	169.6	~~4.42~~ ~~4.75~~ 1.99	X-5 X-2	6
8.2 8.3	~~9~~ 8	1000	169.6	~~5.89~~ ~~6.33~~ 2.66	X-5 X-2	6
9.3 9.4	~~10~~ 9	1250	169.6	~~7.37~~ ~~7.90~~ 3.33	X-5 X-2	6
10.3 10.4	10	1500	359	~~9.50~~	X-2	60

* Dose rate X-5 157.95 rad/min

X-2 375.90

16. After irradiation, carefully remove the supernatant from the top, resuspend pellet in 200 µl wash MEMA and transfer the contents to ten 10 14 ml tubes (Falcon plastic test tube, 17x100 mm, labeled 1-10 both on cap and wall) containing 10 ml wash MEMA by using pasteur pipet

17. Again add 200 µl wash MEMA in microcentrifuge tubes, resuspend and transfer the cell suspensions to 14 ml tubes

18. Centrifuge the tubes for 7 min at 2000 rpm, 4°C previously sterilized

19. Labeling and preparation of dilution tubes and colony dishes
    - load ~~57~~ 60 mm petri dishes with 4 ml MEMA
    - load 39 T-tubes with 4.5 ml MEMA and label them [1.2, 1.3, 1.4, 1.5] (most diluted) [2.2, 2.3, 2.4, 2.5] (less diluted) ~~X.2, X.3~~, X.4, X.5 etc. (1:10 dilution)

20. ~~Decant supernatant, click tubes, vortex, resuspend in 10 ml wash MEMA~~

21. ~~Centrifuge tubes for 10 min at 2000 rpm, 4°C~~

22. Decant supernatant, click tubes, vortex, resuspend in 2 ml wash MEMA, pass five times

6.2 = tube 6 (2×10^2/ml dilution)
6.3 = tube 6 (2×10^3/ml dilution)

Note to (23).

5379 5074 5001

5000(400) = 2×10^6/ml

Monometer — 500 µl.

@

through 5 cc syringe with 21 gauge needle

23. Determine cell concentration by transferring 100 µl to Coulter cup (manometer – 50µl)
24. Vortex tube, transfer 0.5 ml (1×10⁶ cells) into dilution tube X.5, vortex tube X.5 and transfer 0.5 ml to tube X.4, vortex tube X.4 and transfer 0.5 ml to tube X.3 and vortex tube X.3 and transfer 0.5 ml to tube X.2. Keep tubes on ice.
25. Transfer 1 ml (4.0 ml) from dilution tubes into dishes labeled X.2, X.3, X.4 (in triplicate). Only X.2 should be seeded for control T-tubes.
26. Incubate petridishes for 1 week
27. After 1 week, wash colonies 3 times with normal (1X) saline, and 2 times with ethanol. Stain colonies with 0.0 5% crystal violet
28. Count colonies. There must be between 25 and 250 colonies for the flask to be a valid data point.

Note to (24)

×5 → 10^6 cells in 5 ml → 2×10^5 cells/ml. OK!

×4 → 2×10^4 cells/ml.

×3 → 2×10^3 cells/ml

×2 → 200 cells/ml

TABLE-3

Expt. # : **Date/Time :**

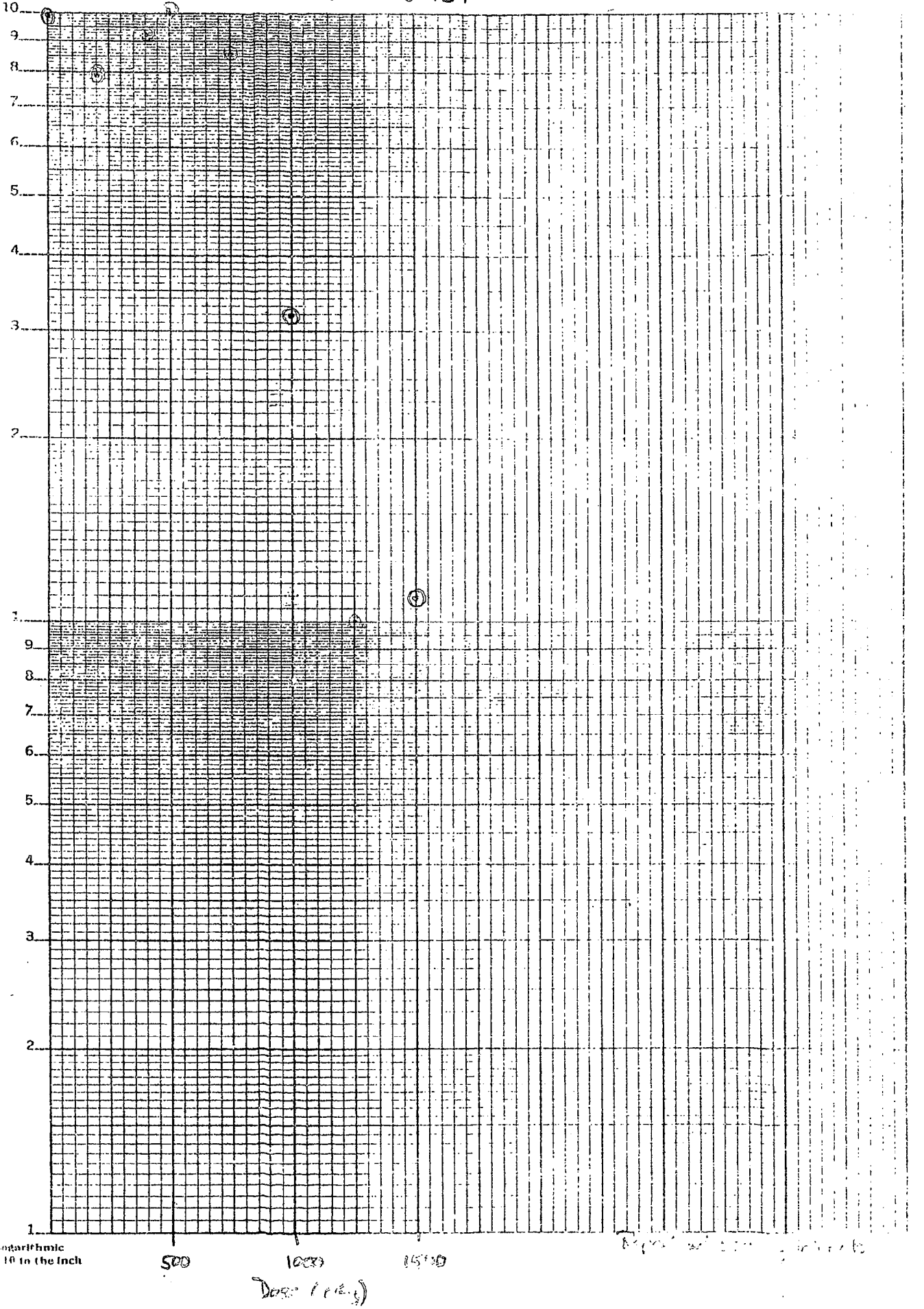
Tube #	Coulter count for 100 ul cell suspension 500 µl marcaine 20 ml Isotone II	Avg. count	Cells/ml [Avg. count x 4000]	pCi/cell [uCi/ml x10^6 Cells/ml]
1	5379 5074 5001			
2	4820 4843 4898			
3	4830 4939 4866			
4	5488 5562 5577			
5	5255 5229 5452			
6	5881 5881 5850			
7	6288 6288 6277			
8	5703 5603 5520			
9	5151 5198 5125			
10	5162 5229 5172			

Dec 26. 2001

Colonies

Tube #			SF	Dose (cGy)
1.2	124, 99, 109	105.7	1	0
2.2	91, 98, 113			
3.2	75, 92, 84	83.6	0.79	200
4.2	125, 106, 121	117.3	1.11	300
5.2	97, 96, 101	98	0.93	300
6.2	101, 103, 124	109.3	1.03	300
7.2	91, 93, 84	91	0.86	300
8.2	46		? 0.44 > .32	100
8.3	210		? 0.20	
9.4	↑ 400		?	125
10.3	128		? 0.12	

Cluster C8-137



SF

Semi-Logarithmic
Cycles x 10 to the Inch

Dose (rads)

B002760

TABLE-4

Expt # : **Date :**

Tube.dilution	Colony 1	Colony 2	Colony 3	Avg Colony	SF