V79 COLONY FORMING ASSAY

Experiment Name : ^{3}HTdR toxicity (cluster, 100% labeling, 40 μM lindane); **Exp. # :** 1;
Investigator: A. Bishayee **Date:** 01/25/99

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 ~4,000,000 cells/ml in MEMB [Actual count : 4,241,333 cells/ml)
3. Transfer 1 ml of cell suspension into 20 12 ml tubes (Falcon plastic test tube, 17x100 mm) labeled 1-10 both on cap and wall
4. Keep the tubes in the roller for 3-4 h at 37°C, 5% CO_2 **Date/Time:** 01/25/99; 3-00 p.m
5. Prepare MEMB containing radioactivity in hood
   50 μl ^{3}HTdR (Stock : 1 μCi/μl on 11/12) + 4 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:** 01/25/99; 7-15 p.m.

Tube #	^{3}HTdR uCi/ml	Cells in MEMB (ml)	MEMB (ml)	MEMB+ ^{3}HTdR (ml) 12uCi/ml	MEMA (ml)	40 uM Lindane in MEMA (ml)
1	0	1.0	1	0	0.4	0
2	0	1.0	1	0	0.4	0
3	0	1.0	1	0	0	0.4
4	0	1.0	1	0	0	0.4
5	1	1.0	0.835	0.165	0.4	0
6	3	1.0	0.5	0.5	0.4	0
7	6	1.0	0	1	0.4	0
8	1	1.0	0.835	0.165	0	0.4
9	3	1.0	0.5	0.5	0	0.4
10	6	1.0	0	1	0	0.4

7. Return test tubes to roller for 12 h . **Date/Time:** 01/25/99; 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:** 01/26/99; 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
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 8 ml ~~wash~~ MEMA with or without 40 µM Lindane as per Table
16. Centrifuge tubes for 10 min at 2000 rpm, 4°C
17. Decant supernatant, click tubes, vortex, resuspend in 0.4 ml MEMA with or without Lindane as per the Table
18. Transfer the cell suspension in polypropylene microcentrifuge tubes with attached caps (Helena Plastics, 400 ul) using 200 ul pipet tips
19. Centrifuge tubes for 5 min at 1000 rpm, 4°C
20. Transfer tubes at 10°C for 72 h. **Date/Time:** 01/26/99; 11-45 a.m.
21. Transfer 30 ul supernatant in three sets of 20 ml scintillation vials containing 6 ml liquid scintillation cocktail (Aquasol) from 150 ul supernatant removed earlier (Step 10) and count them for radioactivity **Date/Time:** 01/26/99; ~~11-40 a.m~~. 1-00 p.m.
22. After 72 h, carefully remove the supernatant from the top, resuspend pellet in 200 ul wash MEMA and transfer the content to ten 12 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 **Date/Time:** 01/29/99; 11-00 a.m.
23. Again add 200 ul wash MEMA in microcentrifuge tubes, resuspend and transfer the cell suspensions in 12 ml tubes
24. Centrifuge the tubes for 10 min at 2000 rpm, 4°C (***precooled centrifuge***)
25. Labeling and preparation of dilution tubes and colony dishes
    - load 66, 60 mm petri dishes with 4 ml MEMA
    - load 40 sterile tubes with 4.5 ml MEMA and label them 1.2, 1.3, 1.4, 1.5; 2.2, 2.3, 2.4, 2.5; X.2, X.3, X.4, X.5 etc.
26. Decant supernatant, click tubes, vortex, resuspend in 10 ml wash MEMA
27. Centrifuge tubes for 10 min at 2000 rpm, 4°C
28. Decant supernatant, click tubes, vortex, resuspend in 10 ml wash MEMA
29. Centrifuge tubes for 10 min at 2000 rpm, 4°C
30. Decant supernatant, click tubes, vortex, resuspend in 2 ml wash MEMA, pass five times

through 3 cc syringe with 21 gauge needle

31. Determine cell concentration by transfering 100 µl to Coulter cup
32. Vortex tube, transfer 0.5 ml into dilusion tube X.5, vortex tube X.5, transfer 0.5 ml into dilution tube X.4, vortex tube X.4 and transfer 0.5 ml to tube X.3, vortex tube X.3 and transfer 0.5 ml to tube X.2 and vortex. Keep tubes on ice.
33. Transfer 1 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.
34. Transfer 200 µl of cell suspension (in triplicate) to 20 ml scintillation vial containing 6 ml cocktail (Aquasol)
35. Incubate petridishes for 1 week
36. Count vials for radioactivity **Date/Time :** 01/29/99; 12-15 p.m.
37. After 1 week, wash colonies 3 times with normal (1X) saline, and 2 times with methanol. Stain colonies with 0.05% crystal violet
38. Count colonies. There must be between 25 and 250 colonies for the dish to be a valid data point.

Expt #1 01/25/99

Initial cell count = 1545, 1633, 1624
Avg. cell count = 1600
Cell conc. = 6,402,666 cells/ml

We need 4,000,000 cells/ml × 11 ml = $\frac{44000000}{6402666}$
= 6.9 ml

Take ~7ml cells + 4ml MEMB = 11ml

Final count = 1060, 1057, 1064
Avg count = 1060
Cell conc = 4,241,333 cells/ml

Preparation of 40µM Lindane in MEMA : Prepare 50 ml

5

Lindane (Hexachlorocyclohexane) MW = 290.8

40µM. Lindane :

1000 ml 1M = 290.8 g

1000 ml 1000 mM = 290.8 g

1000 ml 1 mM = $\frac{290.8}{1000}$ g

1000 ml 1 µM = $\frac{290.8}{1000 \times 1000}$ g

1000 ml 40 µM = $\frac{290.8}{1000 \times 1000} \times 40$ g

40 µM = $\frac{290.8 \times 40}{1000 \times 1000}$ g

= $\frac{11632}{1000000}$

= 0.00116 g

1 ml — 5 mg

5 mg — 1 ml = 0.0116 g

1 — $\frac{1}{5}$

1.16 ≈ 11.6 mg

Prepare 5mg/ml Lindane

10 ml 40 µM = 0.116 mg

10 ml 100 µM = 0.29 mg

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weigh ~ 10 mg of Lindane
Dissolve in chloroform to have 5 mg/ml

Take 0.118 ml of 5 mg/ml Lindane,
add 50 ml of MEMA & 10% FCS, L-glu, PS etc

wt of paper =	2.78841	= 0.20851
wt of paper + Lindane =	2.79909	0.23051
		0.0229
	= 22 mg	

Chloroform added = $\frac{22}{5}$ = 4.4 ml

30 µl medium

Rad Safety

[illegible]

[illegible] WED 27 JAN 1979 14.04
SAMPLE REPEAT: [illegible] CYCLE REPEAT: [illegible] [illegible]
[illegible]
CHANNEL [illegible]: 0 U: 400 2SIGMA: 2.00 BKG SUB: 0.00 BKG 2SIG: 0.00 LSR:
DATA CALC. DPM, UNKNOWN REPLICATES: 1 NORM FACTOR: 1.00000
HALF LIFE (DAYS): N

SAM	POS	CH	DPM	2SIG%	TIME	EL TIME	AVG H#
1	**- 1	1	10.00	63.25	1.00	1.65	64.0
2	**- 2	1	11.00	60.30	1.00	3.47	62.0
3	**- 3	1	10.00	63.25	1.00	5.30	66.0
4	**- 4	1	8.00	70.71	1.00	7.08	64.0
5	**- 5	1	8.00	70.71	1.00	8.87	65.0
6	**- 6	1	13.00	57.74	1.00	10.69	63.0
7	**- 7	1	8.00	70.71	1.00	12.47	[illegible]
8	**- 8	1	5.00	89.44	1.00	14.25	62.0
9	**- 9	1	24750.00	1.89	0.43	15.47	63.0
10	**-10	1	22534.84	1.95	0.47	16.75	63.0
11	**-11	1	71490.91	1.84	0.17	17.73	63.0
12	**-12	1	66893.33	2.00	0.15	18.63	65.0
13	**- 1	1	132752.22	1.63	0.11	19.64	65.0
14	**- 2	1	148955.75	1.54	0.11	20.59	64.0
15	**- 3	1	22548.54	1.95	0.47	21.88	64.0
16	**- 4	1	24439.76	1.97	0.41	23.11	65.0
17	**- 5	1	67830.30	1.89	0.17	24.09	64.0
18	**- 6	1	76200.00	1.81	0.15	25.01	67.0
19	**- 7	1	131077.53	[illegible]	0.11	25.95	65.0
20	**- 8	1	151470.00	1.53	0.10	26.82	66.0

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our counter

Expt #1
30 µl medium

USER: 1 ID:H-3 PRESET TIME: 1.00 TUE 26 JAN 1999 12:56
SAMPLE REPEAT: 1 CYCLE REPEAT: 1 SCR:N RS232:N
H#: 1 AQC:N QCF:N RCM:N 2 PHASE MONITOR: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:Q 1.00000
HALF LIFE(DAYS):N

SAM	POS	CH		CPM	2SIG%	TIME	EL TIME	AVG H#	ERR
1	**- 1	1	1M	12.00	57.74	1.00	1.48	63.0	
2	**- 2	1		29.00	37.14	1.00	3.12	59.0	
3	**- 3	1	2M	19.00	45.88	1.00	4.77	66.0	
4	**- 4	1		11.00	60.30	1.00	6.40	63.0	
5	**- 5	1	3M	14.00	53.45	1.00	8.04	64.0	
6	**- 6	1		10.00	63.25	1.00	9.68	64.0	
7	**- 7	1	4M	9.00	66.67	1.00	11.32	64.0	
8	**- 8	1		31.00	35.92	1.00	12.96	62.0	
9	**- 9	1		24851.11	1.89	0.45	13.99	63.0	
10	**-10	1	5M	25122.50	2.00	0.40	15.02	64.0	
11	**-11	1		71940.00	1.93	0.15	15.78	66.0	
12	**-12	1	6M	66686.66	2.00	0.15	16.51	63.0	
13	**- 1	1		136160.00	1.40	0.15	17.32	64.0	
14	**- 2	1	7M	48150.00	1.64	0.10	18.05	64.0	
15	**- 3	1		23028.89	1.96	0.45	19.13	65.0	
16	**- 4	1	8M	24677.78	1.90	0.45	20.19	65.0	
17	**- 5	1		68846.66	1.97	0.15	20.92	63.0	
18	**- 6	1	9M	76680.00	1.86	0.15	21.70	66.0	
19	**- 7	1		135540.00	1.72	0.10	22.43	64.0	
20	**- 8	1	10M	153010.00	1.62	0.10	23.15	66.0	

TABLE-1

Expt. # : 1 **Date/Time :** 01/26/99; 1-00 pm.

Tube #	Medium count for 30 ul (cpm)	Avg. cpm	dpm [cpm/0.65]	μ Ci/ml (A_t) on counting [dpm/66600]	μ Ci/ml (A_o) on addition [$A_t/e^{-\lambda t}$]
1					
2					
3					
4					
5		249865	38440	0·577	
6		69313	106635	1·601	
7		142155	218700	3·283	
8		23852	36696	0·551	
9		72763	111943	1·681	
10		144275	221961	3·333	

200µl Cells

USER: 1 ID:H-3 PRESET TIME: 1.00 FRI 29 JAN 1993 12:05
SAMPLE REPEAT: 1 CYCLE REPEAT: 1 SCR:N RS232:N
H#: 1 AQC:N QCF:N RCM:N 2 PHASE MONITOR: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:Q 1.00000
HALF LIFE(DAYS):N

SAM	POS	CH	CPM	2SIG%	TIME	EL TIME	AVG H#	ERR
			11.00*					
1	**- 1	1	~~499.00~~	8.95	1.00	1.48	87.0	
2	**- 2	1	19.00	45.88	1.00	3.07	81.0	
3	**- 3	1	13.00	55.47	1.00	4.71	81.0	
4	**- 4	1	8.00	70.71	1.00	6.35	82.0	
5	**- 5	1	11.00	60.30	1.00	8.00	94.0	
6	**- 6	1	5.00	89.44	1.00	9.63	55.0	
7	**- 7	1	14.00	53.45	1.00	11.27	84.0	
8	**- 8	1	6.00	81.65	1.00	12.86	84.0	
9	**- 9	1	19212.73	1.95	0.55	14.04	77.0	
10	**-10	1	17745.00	1.94	0.60	15.28	75.0	
11	**-11	1	53205.00	1.94	0.20	16.11	79.0	
12	**-12	1	49572.00	1.80	0.25	16.98	87.0	
13	**- 1	1	92393.33	1.70	0.15	17.84	84.0	
14	**- 2	1	10910.00	1.90	0.10	18.57	83.0	
15	**- 3	1	18690.91	1.97	0.55	19.75	88.0	
16	**- 4	1	18088.33	1.92	0.60	20.98	85.0	
17	**- 5	1	55535.00	1.90	0.20	21.76	87.0	
18	**- 6	1	55815.00	1.89	0.20	22.53	88.0	
19	**- 7	1	105060.00	1.95	0.10	23.25	89.0	
20	**- 8	1	12779.99	1.54	0.15	23.97	95.0	

* counted second time

TABLE-2

Expt. # :)

Date/Time : 01/29/99 ; 12-00 noon

Tube #	Radioactivity for 200 ul cell suspension (cpm)	Avg. cpm	dpm [cpm/0.65]	µCi/ml (A_t) on counting [dpm/444000]	µCi/ml (A_o) after 12 h incubation [$A_t/e^{-\lambda t}$]
1	See the attached				
2	sheet				
3					
4					
5		18478	28428	0·064	
6		513885	79059	0·1781	
7		101651	156386	0·3522	
8		18389	28290	0·0637	
9		55675	85653	0·1929	
10		108919	167568	0·3774	

TABLE-3

Expt. # : 1 **Date/Time :** 01/29/99

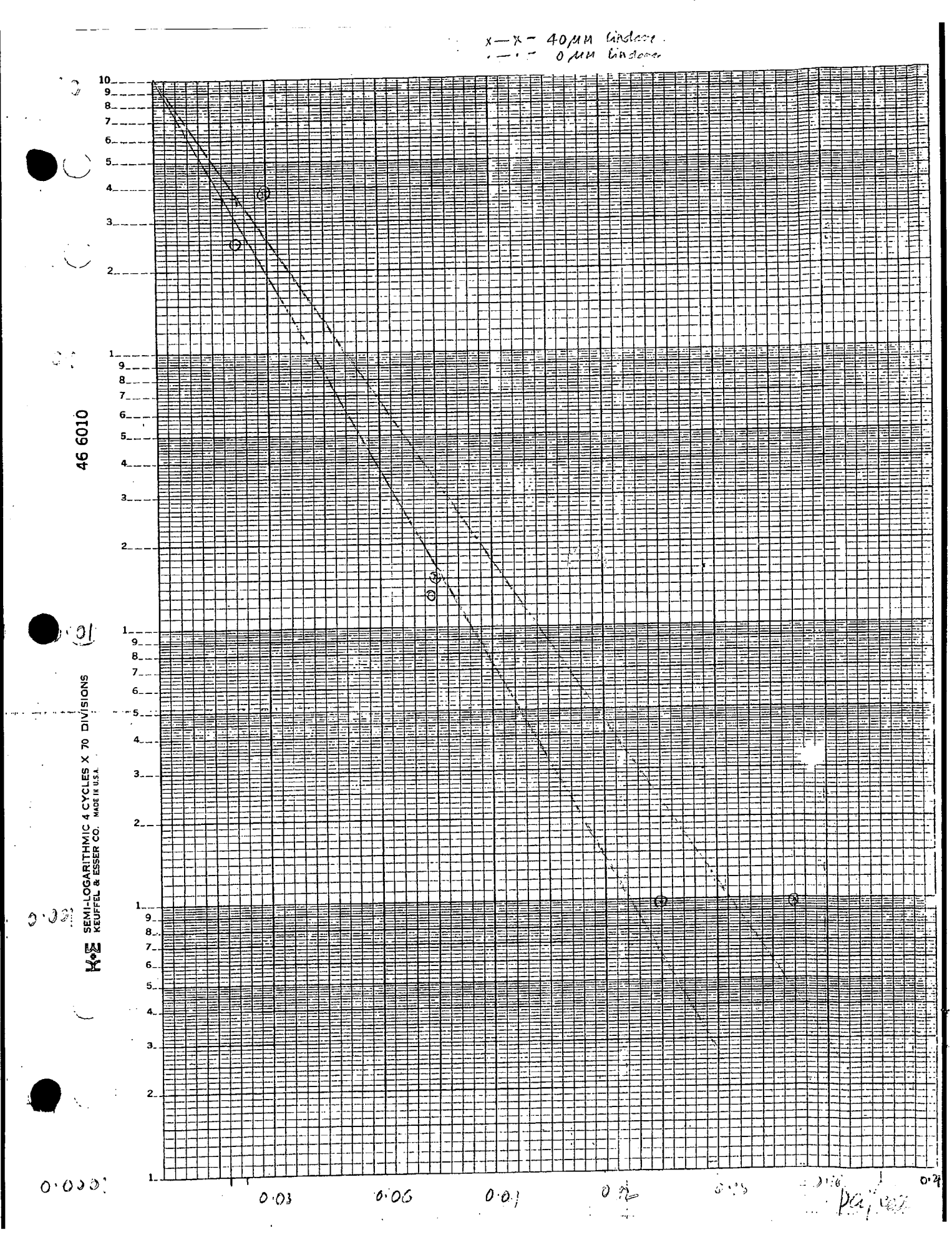
Tube #	Coulter count for 100 ul cell suspension	Avg. count	Cells/ml [Avg. count x 4000]	pCi/cell [uCi/ml x10^6 Cells/ml]
1	664, 644, 696			
2	716, 726, 721			
3	651, 675, 664			
4	643, 681, 636			
5	700, 690, 717	702	2809333	0·0228
6	618, 618, 657	631	2524000	0·0706
7	669, 691, 653	669	2677333	0·1315
8	536, 505, 557	532	2130666	0·0299
9	642, 626, 633	633	2534666	0·0761
10	541, 558, 569	556	2224000	0·1697

TABLE-4 (1)

Expt # : 2/5/99 **Date :** 2/5/99

Tube.dilution	Colony 1	Colony 2	Colony 3	Avg Colony	SF
1·2	150	135	139	137.33	
2·2	129	140	131		
3·2	107	100	98	105.33	0·767
4·2	14	109	107		
5·2	35	40	29	34·66	0·2524
6·3	18	20	16	1·8	0·0131
7·4	13	13	14	0·13	0·00097
8·2	40	47	32	39·6	0·3765
9·4	146	157	167	156	0·0149
10·4	14	16	15	0·15	0·00094

x—x— 40μm Lindane
·—·—· 0μm Lindane



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~~Long bill~~

~~908-647-7984~~

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