V79 COLONY FORMING ASSAY

Experiment Name : ^{3}HTdR toxicity (cluster, 100% labeling); **Exp. # :** 3;
Investigator: A. Bishayee **Date:** 12/17/98

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 ~4,000,000 cells/ml in MEMB [Actual count : 5,409,333 cells/ml)
3. Transfer 1 ml of cell suspension into ten 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:** 12/17/98; 3-00 p.m.
5. Prepare MEMB containing radioactivity in hood

   16 µl ^{3}HTdR (Stock : 1 µCi/µl on 11/12/98) + 3 ml MEMB
6. After 3-4 h, remove test tubes from roller and add MEMB with or without radioactivity according to Table below. **Date/Time:** 12/17/98; 7-00 p.m.

Tube #	^{3}HTdR uCi/ml	Cells in MEMB (ml)	MEMB (ml)	MEMB+ ^{3}HTdR (ml) [4uCi/ml]
1	0	1.0	1.0	0
2	0	1.0	1.0	0
3	0.01	1.0	0.995	0.005
4	0.05	1.0	0.975	0.025
5	0.1	1.0	0.950	0.050
6	0.2	1.0	0.900	0.100
7	0.5	1.0	0.750	0.250
8	0.75	1.0	0.625	0.375
9	1	1.0	0.500	0.500
10	2	1.0	0	1

7. Return test tubes to roller for 12 h . **Date/Time:** 12/17/98; 7-15 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:** 12/18/98; 9-00 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 10 ml wash MEMA
16. Centrifuge tubes for 10 min at 2000 rpm, 4°C
17. Decant supernatant, click tubes, vortex, resuspend in 7 ml of MEMA
18. Centrifuge tubes for 10 min at 2000 rpm, 4°C
19. Decant supernatant, click tubes,vortex, transfer the cell suspension in polypropylene microcentrifuge tubes with attached caps (Helena Plastics, 400 ul) using 200 ul pipet tips
20. Again add 200 ul ice cold MEMA, resuspend and transfer the cell suspensions in the same polypropylene microcentrifuge tubes (Total volume ~400 ul)
21. Centrifuge tubes for 5 min at 1000 rpm, 4°C
22. Transfer tubes at 10°C for 72 h. **Date/Time:** 12/18/98; 11-00 a.m.
23. 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:** 12/18/98; 10-15 a.m.
24. 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:** 12/21/98; 3-00 p.m.
25. Again add 200 ul wash MEMA in microcentrifuge tubes, resuspend and transfer the cell

suspensions in 12 ml tubes

26. Centrifuge the tubes for 10 min at 2000 rpm, 4°C ***(precooled centrifuge)***
27. 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.
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 10 ml wash MEMA

31. Centrifuge tubes for 10 min at 2000 rpm, 4°C
32. Decant supernatant, click tubes, vortex, resuspend in 2 ml wash MEMA, pass five times through 3 cc syringe with 21 gauge needle
33. Determine cell concentration by transfering 100 µl to Coulter cup
34. 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.
35. 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.
36. Transfer 200 µl of cell suspension (in triplicate) to 20 ml scintillation vial containing 6 ml cocktail (Aquasol)
37. Incubate petridishes for 1 week
38. Count vials for radioactivity **Date/Time :**
39. After 1 week, wash colonies 3 times with normal (1X) saline, and 2 times with methanol. Stain colonies with 0.05% crystal violet
40. Count colonies. There must be between 25 and 250 colonies for the dish to be a valid data point.

3HTdR toxicity
Expt#3
30µl medium

USER: 1 ID:H-3 TIME: 1.00 FRI 18 DEC 1998 10:16
SAMPLE REPEAT: 1 CYCLE SCR:N RS232:N
#: 1 AQC:N QCF:N RCM:N NITOR:N
CHANNEL 1-LL: 0 UL: 400 00 BKG SUB: 0.00 BKG 2SIG: 0.00 LSR: 0
DATA CALC: CPM, UNKNOWN REF 1 NORM FACTOR:Q 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	1.48	65.0	
2	**- 2	1	1M	13.00	55.47	1.00	3.12	60.0	
3	**- 3	1		8.00	70.71	1.00	4.76	60.0	
4	**- 4	1	2M	14.00	53.45	1.00	6.39	63.0	
5	**- 5	1	3M	256.00	12.50	1.00	7.98	61.0	
6	**- 6	1		268.00	12.22	1.00	9.58	64.0	
7	**- 7	1	4M	1137.00	5.93	1.00	11.22	64.0	
8	**- 8	1		1186.00	5.81	1.00	12.86	64.0	
9	**- 9	1	5M	2372.00	4.11	1.00	14.50	62.0	
10	**-10	1		2328.00	4.15	1.00	16.14	63.0	
11	**-11	1	6M	4613.00	2.94	1.00	17.78	64.0	
12	**-12	1		4838.00	2.88	1.00	19.37	62.0	
13	**- 1	1	7M	12188.24	1.96	0.85	20.89	63.0	
14	**- 2	1		11650.00	1.95	0.90	22.43	64.0	
15	**- 3	1	8M	18211.67	1.91	0.60	23.62	63.0	
16	**- 4	1		17436.67	1.96	0.60	24.85	64.0	
17	**- 5	1	9M	23620.00	1.94	0.45	25.93	63.0	
18	**- 6	1		23551.11	1.94	0.45	27.01	67.0	
19	**- 7	1	10M	48432.00	1.82	0.25	27.87	64.0	
20	**- 8	1		47916.00	1.83	0.25	28.75	64.0	

TABLE-1

Expt. # : 3 **Date/Time :** 12/18/98; 10-15 a.m.

Tube #	Medium count for 10 ul (cpm)	Avg. cpm	dpm [cpm/0.65]	µ Ci/ml (A_t) on counting [dpm/~~22200~~ 66600]	µ Ci/ml (A_o) on addition [$A_t/e^{-\lambda t}$]
1	See the attached				
2	sheet				
3		262	403	0·006	
4		1161	1786	0·026	
5		2350	3615	0·054	
6		4725	7270	0·109	
7		11919	18336	0·275	
8		17823	27420	0·411	
9		23585	36285	0·544	
10		48174	74113	1·112	

3H TdR toxicity (100% labeled)   Expt #3

200ul cells

USER: 1 ID:H-3   PRESET TIME: 1.00   TUE 22 DEC 1998 10:44
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
1c	1	**- 1	1	20.00	44.72	1.00	1.47	87.0	
	2	**- 2	1	11.00	60.30	1.00	3.12	80.0	
2c	3	**- 3	1	13.00	55.47	1.00	4.77	82.0	
	4	**- 4	1	52.00	27.74	1.00	6.41	82.0	
3c	5	**- 5	1	309.00	11.38	1.00	8.00	75.0	
	6	**- 6	1	346.00	10.75	1.00	9.58	82.0	
4c	7	**- 7	1	1171.00	5.84	1.00	11.23	82.0	
	8	**- 8	1	1446.00	5.26	1.00	12.87	83.0	
5c	9	**- 9	1	2554.00	3.96	1.00	14.51	81.0	
	10	**-10	1	2586.00	3.93	1.00	16.16	80.0	
6c	11	**-11	1	5280.00	2.75	1.00	17.81	83.0	
	12	**-12	1	5793.00	2.63	1.00	19.45	85.0	
7c	13	**- 1	1	14293.33	1.93	0.75	20.92	83.0	
	14	**- 2	1	17391.67	1.96	0.60	22.16	89.0	
8c	15	**- 3	1	22042.00	1.91	0.50	23.29	88.0	
	16	**- 4	1	21320.00	1.94	0.50	24.42	84.0	
9c	17	**- 5	1	28310.00	1.88	0.40	25.46	89.0	
	18	**- 6	1	29148.57	1.98	0.35	26.43	91.0	
10c	19	**- 7	1	56200.00	1.89	0.20	27.25	93.0	
	20	**- 8	1	57240.00	1.87	0.20	28.07	94.0	

TABLE-2

Expt. # : 3 **Date/Time :** 12/22/98; 10-44 a.m

Tube #	Radioactivity for 200 ul cell suspension (cpm)	Avg. cpm	dpm [cpm/0.65]	µ Ci/ml (A_t) on counting [dpm/444000]	µ Ci/ml (A_o) after 12 h incubation [$A_t/e^{-\lambda t}$]
1	See the attached				
2	Sheet				
3		327	503	0·00113	
4		1308	2013	0·00453	
5		2570	3953	0·00890	
6		5536	8517	0·0191	
7		15842	24372	0·0548	
8		21681	33355	0·0751	
9		28729	44198	0·0995	
10		56720	87261	0·1965	

TABLE-3

Expt. # : 3 **Date/Time :** 12/21/98; 4:00 p.m

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	770, 793, 789	784	3136000	–
2	721, 756, 724	732	2930666	–
3	751, 777, 757	761	3046666	0.00037
4	721, 694, 690	701	2806666	0.00161
5	743, 754, 768	755	3020000	0.00294
6	759, 766, 724	749	2998666	0.00636
7	753, 740, 713	735	2941333	0.0186
8	750, 754, 775	759	3038666	0.0247
9	707, 687, 674	689	2757333	0.036
10	620, 625, 620	621	2486666	0.079

TABLE-4

Expt # : 3 **Date :**

Tube.dilution	Colony 1	Colony 2	Colony 3	Avg Colony	SF
1·2	143	159	138	139·3	
2·2	126	129	141		
3·2	140	135	129	134·6	0·9665
4·2	132	129	124	128·3	0·9210
5·2	106	113	119	112·6	0·8086
6·2	100	92	85	92	0·6602
7·2	50	43	36	43	0·3086
8·2	20	17	19	18·66	0·1339
9·3	79	70	62	7·0	0·0504
10·3	49	38	30	0·39	0·0028

Figure C Expt #2