

## V79 COLONY FORMING ASSAY

Experiment Name :  $^3\text{HTdR}$  +0 or 10%DMSO (cluster, 100% labeling);

Exp. #: 1;

Experiment performed by : A. Bishayee

Date: 12/06/99

1. Set the rocker-roller at  $37^\circ\text{C}$  incubator with 5%  $\text{CO}_2$ , set the Coulter Counter, wash cells (from two  $150\text{ cm}^2$  flask, 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 transferring 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 : 4,012,000 cells/ml]
3. Transfer 1 ml of cell suspension into 14 12 ml tubes (Falcon plastic test tube,  $17\times 100$  mm) labeled 1-14 both on cap and wall
4. Keep the tubes in the roller for 3-4 h at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  Date/Time: 12/06/99; 3-00 pm
5. Prepare MEMB containing radioactivity in hood  
120  $\mu\text{l}$   $^3\text{HTdR}$  (Stock:  $\mu\text{Ci}/\mu\text{l}$  on 12/20 ) + 4.8 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: 12/06/99; 7-15 pm.

Tube #	<sup>3</sup> HTdR uCi/ml	Cells in MEMB (ml)	MEMB (ml)	MEMB+ <sup>3</sup> HTdR (ml) [ <sup>24</sup> uCi/ml I]
1	0	1.0	1.0	0
2	0	1.0	1.0	0
3	0.5	1.0	0.96	0.04
4	2	1.0	0.834	0.166
5	4	1.0	0.67	0.33
6	8	1.0	0.34	0.66
7	12	1.0	<del>0.34</del>	<del>0.66</del>
8	0	1.0	1.0	0
9	0	1.0	1.0	0
10	0.5	1.0	0.96	0.04
11	2	1.0	0.834	0.166
12	4	1.0	0.67	0.33
13	8	1.0	0.34	0.66
10	12	1.0	0	1

7. Return test tubes to roller for 12 h. Date/Time: 12/06/99; 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: 12/06/99; 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 with or without 10% DMSO
18. Centrifuge tubes for 5 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 with or without 10% DMSO, 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: 12/07/99; 11-00 a.m.
23. Transfer 30 ul supernatant in three sets of 6 ml scintillation vials containing 6 ml liquid scintillation cocktail (Ecolume) from 150 ul supernatant removed earlier (Step 10) and count them for radioactivity      Date/Time: 12/07/99; 1-00 p.m.
24. After 72 h, carefully remove the supernatant from the top, resuspend pellet in 200 ul wash MEMA and transfer the content to 14 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: 12/10/99; 10-00 a.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 transferring 100 µl to Coulter cup
34. Vortex tube, transfer 0.5 ml into dilution 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)

Tube #1-7: 0% DMSO  
 " # 8-14: 10% DMSO

Tube 1-7: 0% DMSO

Tube 8-14: 10% DMSO

37. Incubate petridishes for 1 week

38. Count vials for radioactivity

Date/Time : 12/10/99; 1-30 pm

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.

30ml medium

USER: 6 ID:H3 HOWELL      PRESET TIME: 1.00      TUE 07 DEC 1999 12:55  
SAMPLE REPEAT: 1 CYCLE REPEAT: 1 SCR:N      RS232:N  
1 AGC:N GCF: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	9.00	66.67	1.00	2.30	86.0	
2	**	2	5.00	89.44	1.00	4.27	89.0	
3	**	3	9.00	66.67	1.00	6.30	89.0	
4	**	4	12.00	57.74	1.00	8.48	87.0	
5	**	5	10.00	63.25	1.00	10.82	88.0	
6	**	6	7.00	75.59	1.00	12.75	89.0	
7	**	7	3979.00	3.17	1.00	14.73	86.0	
8	**	8	5133.00	2.79	1.00	16.80	88.0	
9	**	9	4580.00	2.96	1.00	18.93	85.0	
10	**	10	20304.00	1.98	0.50	20.60	87.0	
11	**	11	19040.00	1.91	0.57	22.25	89.0	
12	**	12	19478.18	1.93	0.55	23.77	87.0	
13	**	13	41645.29	1.90	0.26	25.06	89.0	
14	**	14	11.00	60.30	1.00	27.14	84.0	→ Sample not added
15	**	15	49533.33	1.83	0.24	28.86	91.0	
16	**	16	70245.72	1.80	0.17	30.11	88.0	
17	**	17	86889.57	1.68	0.16	31.29	87.0	
18	**	18	97615.95	1.72	0.14	32.35	89.0	
19	**	1	141451.33	1.58	0.11	33.81	89.0	
20	**	2	146400.00	1.25	0.17	35.07	90.0	
21	**	3	157439.39	1.39	0.13	36.67	90.0	
22	**	4	7.00	75.59	1.00	38.59	91.0	
23	**	5	13.00	55.47	1.00	40.62	90.0	
24	**	6	11.00	60.30	1.00	42.93	89.0	
25	**	7	5.00	89.44	1.00	45.41	90.0	
26	**	8	10.00	63.25	1.00	47.39	88.0	
27	**	9	8.00	70.71	1.00	49.37	88.0	
28	**	10	6113.00	2.56	1.00	51.56	91.0	
29	**	11	5577.00	2.68	1.00	53.64	90.0	
30	**	12	6446.00	2.49	1.00	55.77	94.0	
31	**	13	18738.89	1.99	0.54	57.42	86.0	
32	**	14	25223.53	1.93	0.43	58.92	92.0	
33	**	15	24048.89	1.92	0.45	60.33	88.0	
34	**	16	51165.00	1.98	0.20	61.50	91.0	
35	**	17	54204.44	1.81	0.23	62.59	91.0	
36	**	18	57937.78	1.75	0.23	64.19	92.0	
37	**	1	97404.91	1.59	0.16	65.43	91.0	
38	**	2	104006.13	1.54	0.16	66.62	91.0	
39	**	3	108441.72	1.50	0.16	67.82	91.0	
40	**	4	149446.81	1.19	0.19	69.15	91.0	
41	**	5	174272.00	1.36	0.12	70.68	92.0	
42	**	6	172440.00	1.15	0.17	71.96	92.0	

TABLE-1

Expt. # : 1

Date/Time : 12/07/99, 1-00 pm.

Tube #	Medium count for 30 ul (cpm)	Avg. cpm	dpm [cpm/ <del>0.65</del> 0.56]	$\mu$ Ci/ml (A) on counting [dpm/66600]	$\mu$ Ci/ml (A <sub>0</sub> ) on addition [A <sub>0</sub> /e <sup>-<math>\lambda</math>t</sup> ]
1					
2					
3		4564	7868	0.118	
4		19607	33805	0.5075	
5		45589	78601	1.18	
6		84916	146407	2.19	
7		148430	255913	3.84	
8					
9					
10		6045	10422	0.1565	

11		22669	39085	0.5869	
12		54435	93853	1.409	
13		103283	178075	2.67	
14		165386	285148	4.28	

700 µl cells

SER: 6 ID: H3 HOWELL      PRESET TIME: 1.00      FRI 10 DEC 1999 13:32  
 SAMPLE REPEAT: 1 CYCLE REPEAT: 1 SCR: N      RS232: N  
 H#: 1 ABC: N DCF: N RCM: N  
 CHANNEL 1-LL: 0 UL: 400 ZSIGMA: 2.00 BKG SUB: 0.00 BKG ZSIG: 0.00 LSR: 0  
 DATA CALC: CPM, UNKNOWN REPLICATES: 1      NORM FACTOR: 1.00000  
 HALF LIFE(DAYS): N

SAM	POS	CH	CPM	ZSIG%	TIME	EL TIME	AVG H#	ERR
1	**	1	8.00	70.71	1.00	1.89	107.0	
2	**	2	8.00	70.71	1.00	4.08	106.0	
3	**	3	11.00	60.30	1.00	6.07	110.0	
4	**	4	3.00	115.5	1.00	8.09	109.0	
5	**	5	6.00	81.65	1.00	10.02	109.0	
6	**	6	13.00	55.47	1.00	12.29	109.0	
7	**	7	6963.00	2.40	1.00	14.28	108.0	
8	**	8	7036.00	2.38	1.00	16.26	108.0	
9	**	9	6941.00	2.40	1.00	18.29	109.0	
10	**	10	21742.00	1.92	0.50	19.76	110.0	
11	**	11	21376.00	1.93	0.50	21.43	111.0	
12	**	12	24744.58	1.97	0.41	22.87	110.0	
13	**	13	61160.00	1.81	0.20	24.03	110.0	
14	**	14	61935.00	1.80	0.20	25.20	108.0	
15	**	15	57215.00	1.87	0.20	26.37	112.0	
16	**	16	104448.59	1.89	0.11	27.71	108.0	
17	**	17	107859.99	1.57	0.15	28.83	110.0	
18	**	18	103599.99	1.60	0.15	29.96	109.0	
19	**	1	168275.36	1.31	0.14	31.08	109.0	
20	**	2	154380.36	1.26	0.16	32.28	109.0	
21	**	3	140582.86	1.28	0.17	33.55	110.0	
22	**	4	1813.00	4.70	1.00	35.78	108.0	
23	**	5	2119.00	4.34	1.00	37.77	110.0	
24	**	6	2411.00	4.07	1.00	39.69	109.0	
25	**	7	184.00	14.74	1.00	41.71	109.0	
26	**	8	211.00	13.77	1.00	43.68	110.0	
27	**	9	155.00	16.06	1.00	45.87	107.0	
28	**	10	6984.00	2.39	1.00	47.90	110.0	
29	**	11	6255.00	2.53	1.00	49.93	110.0	
30	**	12	6877.00	2.41	1.00	51.97	108.0	
31	**	13	25572.50	1.98	0.40	53.34	108.0	
32	**	14	28227.50	1.88	0.40	54.92	110.0	
33	**	15	30385.71	1.94	0.35	56.23	110.0	
34	**	16	47800.00	1.93	0.23	57.52	111.0	
35	**	17	47880.00	1.83	0.25	58.74	111.0	
36	**	18	47533.33	1.87	0.24	59.90	109.0	
37	**	1	85993.33	1.76	0.15	61.07	108.0	
38	**	2	83717.39	1.86	0.14	62.14	109.0	
39	**	3	84993.33	1.77	0.15	63.27	111.0	
40	**	4	124339.99	1.46	0.15	64.40	109.0	
41	**	5	123251.43	1.36	0.17	65.66	110.0	
42	**	6	126048.00	1.59	0.12	66.66	109.0	
43	**	1	13.00	55.47	1.00	67.01	86.0	
44	**	2	17.00	48.51	1.00	71.23	90.0	
45	**	3	12.00	57.74	1.00	73.46	83.0	

possibly contaminated with radioactivity

TABLE-2

Expt. # : 1

Date/Time : 12/10/99, 1-30 pm.

Tube #	Radioactivity for 200 ul cell suspension (cpm)	Avg. cpm	dpm [cpm/0.65] 0.58	$\mu\text{Ci/ml (A}_t\text{)}$ on counting [dpm/444000]	$\mu\text{Ci/ml (A}_0\text{)}$ after 12 h incubation [A <sub>t</sub> /e <sup>-λt</sup> ]
1					
2					
3		6980	1284	0.0271	
4		22620	39001	0.0878	
5		60103	103626	0.2334	
6		105302	181555	0.4089	
7		154412	266228	0.5996	
8					
9					
10		6705	11560	0.026	

11 28052 48366 0.1089

12 47737 82306 0.1854

13 84901 146381 0.3297

14 124546 214734 0.4836



TABLE-3

Date/Time : 12/10/99

Expt. # : |

Tube #	Coulter count for 100 ul cell suspension	Avg. count	Cells/ml [Avg. count x 4000]	pCi/cell [uCi/ml x 10 <sup>6</sup> / Cells/ml]	KBa/ cluster [pCi/cell x 148]
1	655, 644, 660	648			
2	711, 722, 745	726			
3	672, 666, 659	665	2662666	0.0102	1.51
4	782, 799, 786	789	3156000	0.0278	4.11
5	881, 862, 872	871	3486666	0.0669	9.91
6	835, 852, 821	836	3344000	0.1222	18.09
7	811, 829, 835	825	3300000	0.1817	26.89
8	756, 742, 769	755			
9	701, 695, 714	703			
10	649, 661, 639	649	2598666	0.01000	1.48
11.	852, 829, 840	840	3361333	0.0323	4.79
12.	633, 615, 609	619	2476000	0.0748	11.08
13.	719, 709, 715	714	2857333	0.1154	17.08
14.	695, 727, 711	711	2844600	0.1700	25.17

TABLE-4

Expt # : \

Date : 12/17/99

Tube.dilution	Colony 1	Colony 2	Colony 3	Avg Colony	SF
1.2	148	159	162	} 160.5	
2.2	165	172	157		
3.2	123	129	136	129.3	0.8058
4.2	58 129	68 118	79 137	68.3 12.8	0.4257 0.079
5.3 6.4	81	90	72	0.81	0.005
7.4	12	21	16	0.16	0.0010
8.2	135	149	151	} 140.33	
9.2	141	129	137		
10.2	123	117	112	117.3	0.8361
11.2	73	82	92	82.3	0.5867

12.2	23	26	20	23	0.1639
13.3	39	50	42	4.36	0.031
14.4	99	112	88	0.99	0.0071