V79 COLONY FORMING ASSAY, Mutation, comet assay

Experiment Name : ^{3}HTdR + 0 or 10% DMSO (cluster, 100% labeling); **Exp.#** 1

Experiment performed by : A. Bishayee **Date:** 02/03/00

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 flasks, 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 : 3,976,000 cells/ml)
3. Transfer 1 ml of cell suspension into 10 14-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:** 02/03/00 2-30 pm
5. Prepare MEMB containing radioactivity in hood
   40 µl ^{3}HTdR (Stock : 1 µCi/µl on 01/31/00) + 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:** 02/03/00; 7-00pm

Tube #	^{3}HTdR uCi/ml	Cells in MEMB (ml)	MEMB (ml)	MEMB+ ^{3}HTdR [8uCi/ml] (ml)
1	0	1.0	1.0	0
2	0.5	1.0	0.875	0.125
3	1	1.0	0.75	0.25
4	2	1.0	0.5	0.5
5	4	1.0	0	1
6	0	1.0	1.0	0
7	0.5	1.0	0.875	0.125
8	1	1.0	0.75	0.25
9	2	1.0	0.5	0.5
10	4	1.0	0	1

MEMB = 4.96 ml
^{3}H = 0.04 ml
5.00 ml

7. Return test tubes to roller for 12 h. **Date/Time:** 02/03/00; 7-30 pm.
8. Next day, while test tubes are in roller label 10 gamma-tubes (12 X 75 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:** 02/04/00; 9-30 a.m.
10. Remove buckets from centrifuge and carefully remove 150 µl of supernatant from each tube 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 8 ml of MEMA with (Tube# 6-10) or without (Tube#1-5) 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:** 02/04/00; 11-00 a.m.
23. Transfer 30 ul supernatant from from 150 ul supernatant removed earlier (Step 10) in triplicate in 6 ml scintillation vials containing 6 ml liquid scintillation cocktail (Ecolume, ICN) and count them for radioactivity **Date/Time:**
24. After 72 h, carefully remove the supernatant from the top, resuspend pellet in 200 ul wash MEMA and transfer the contents by using pasteur pipets to 10 corresponding14-ml tubes (Falcon plastic test tube, 17x100 mm, labeled 1-10 both on cap and wall) containing 10 ml wash MEMA in each **Date/Time:** 02/07/00; 10-00 a.m.
25. Again add 200 ul wash MEMA in microcentrifuge tubes, resuspend and transfer the cell suspensions in corresponding 14 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 60 mm tissue culture dishes with 4 ml MEMA
    - load 40 sterile tubes with 4.5 ml wash MEMA in each 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 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.
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 6 ml scintillation vial containing 6 ml cocktail.
37. Incubate petridishes for 1 week
38. Count vials for radioactivity **Date/Time :** 02/07/00; 5-30 pm.
39. After 1 week, wash colonies 3 times with normal (1X) saline, and 2 times with methanol. Stain colonies with 0.5% crystal violet
40. Count colonies. There must be between 25 and 250 colonies for the dish to be a valid data point.

point.

TABLE-1

Expt. # : I **Date/Time :** 02/07/00; 5-30 pm.

Tube #	Medium count for 30 ul (cpm)	Avg. cpm	dpm [cpm/0.58]	µ Ci/ml (A_t) on counting [dpm/66600]	µ Ci/ml (A_o) on addition [$A_t/e^{-\lambda t}$]
1					
2		3766	6493	0·0975	
3		7676	13235	0·1987	
4		15452	26641	0·400	
5		29406	50701	0·7613	
6					
7		3895	6716	0·1008	
8		7712	13296	0·1996	
9		14867	25633	0·3849	
10		30372	52365	0·7863	

30 µl medium

02/04/00

5-30pm

44	**- 8	1	1M	79.00	22.50	1.00	70.27	92.0
45	**- 9	1		83.00	21.95	1.00	72.29	92.0
46	**-10	1		3543.00	3.36	1.00	74.37	94.0
			2M					
				3856				
				5899				
				7783				
			3M	7164				
				8083				
52	**-16	1		14750.00	1.97	0.70	86.43	94.0
53	**-17	1	4M	15795.31	1.99	0.64	87.98	93.0
54	**-18	1		15812.50	1.99	0.64	89.74	92.0
55	**- 1	1		27544.58	1.87	0.41	91.48	92.0
56	**- 2	1	5M	29707.04	1.95	0.35	93.04	91.0
57	**- 3	1		30969.23	1.99	0.33	94.23	93.0
58	**- 4	1		83.00	21.95	1.00	96.16	92.0
59	**- 5	1	6M	77.00	22.79	1.00	98.09	92.0
60	**- 6	1		86.00	21.57	1.00	100.42	92.0
61	**- 7	1		3517.00	3.37	1.00	102.59	91.0
62	**- 8	1	7M	4018.00	3.16	1.00	104.58	92.0
63	**- 9	1		4151.00	3.10	1.00	106.46	91.0
64	**-10	1		7754.00	2.27	1.00	108.49	90.0
65	**-11	1	8M	8088.00	2.22	1.00	110.53	92.0
66	**-12	1		7294.00	2.34	1.00	112.55	90.0
67	**-13	1		15094.81	1.98	0.68	114.29	90.0
68	**-14	1	9M	13385.71	1.97	0.77	116.39	89.0
69	**-15	1		16124.80	1.99	0.62	118.09	93.0
70	**-16	1		29182.86	1.98	0.35	119.41	91.0
[redacted]	**-17	1	10M	30648.57	1.93	0.35	120.73	93.0
72	**-18	1		31286.49	1.86	0.37	122.42	92.0
73	**- 1	1		23.00	41.70	1.00	124.85	86.0
74	**- 2	1		9.00	66.67	1.00	126.73	85.0
75	**- 3	1		7.00	75.59	1.00	128.62	86.0
76	**- 4	1		10.00	63.25	1.00	130.64	85.0
77	**- 5	1		7.00	75.59	1.00	132.61	83.0

200 µl cells

USER: 6 ID:H3 HOWELL PRESET TIME: 1.00 MON 07 FEB 2000 17:34
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:0 1.00000
H[illegible] LIFE(DAYS):N

SAM	POS	CH	CPM	2SIG%	TIME	EL TIME	AVG H#	ERR
1	**- 1	1	27.00	38.49	1.00	2.15	112.0	
2	**- 2	1	30.00	36.51	1.00	4.17	110.0	
3	**- 3	1	23.00	41.70	1.00	6.19	111.0	
4	**- 4	1	13271.25	1.94	0.80	7.97	112.0	
5	**- 5	1	12626.25	1.99	0.80	9.74	110.0	
6	**- 6	1	12590.30	1.96	0.82	11.64	111.0	
7	**- 7	1	31440.00	1.91	0.35	13.17	111.0	
8	**- 8	1	30264.71	1.97	0.34	14.43	112.0	
9	**- 9	1	28545.00	1.87	0.40	15.99	110.0	
10	**-10	1	58753.49	1.78	0.22	17.23	109.0	
11	**-11	1	59021.05	1.89	0.19	18.33	111.0	
12	**-12	1	61205.00	1.81	0.20	19.50	112.0	
13	**-13	1	106540.00	1.94	0.10	20.78	110.0	
14	**-14	1	104428.57	1.48	0.17	22.03	111.0	
15	**-15	1	106613.49	1.52	0.16	23.23	111.0	
16	**-16	1	23.00	41.70	1.00	25.16	111.0	
17	**-17	1	29.00	37.14	1.00	27.29	110.0	
18	**-18	1	26.00	39.22	1.00	29.62	110.0	
19	**- 1	1	14966.67	1.97	0.69	31.48	111.0	
20	**- 2	1	14533.79	1.95	0.72	33.07	111.0	
21	**- 3	1	14631.43	1.98	0.70	34.75	111.0	
[illegible]	**- 4	1	30148.57	1.95	0.35	36.07	111.0	
23	**- 5	1	29010.26	1.88	0.39	37.57	112.0	
24	**- 6	1	28357.89	1.93	0.38	39.37	110.0	
25	**- 7	1	57936.59	1.84	0.20	40.81	109.0	
26	**- 8	1	58310.00	1.85	0.20	41.97	110.0	
27	**- 9	1	54775.00	1.91	0.20	43.15	109.0	
28	**-10	1	~~94251.53~~	~~1.67~~	~~0.16~~	~~44.33~~	~~108.0~~	
29	**-11	1	101293.33	1.62	0.15	45.46	110.0	
30	**-12	1	102072.00	1.77	0.12	46.97	110.0	

TABLE-2

Expt. # : (

Date/Time : 02/07/00

Tube #	Radioactivity for 200 ul cell suspension (cpm)	Avg. cpm	dpm [cpm/0.58]	μ Ci/ml (A_t) on counting [dpm/444000]	μ Ci/ml (A_o) after 12 h incubation [$A_t/e^{-\lambda t}$]
1		~~12829~~	~~22118~~		
2		~~12829~~ 30083	~~22118~~ 51867	0·0498	
3		30083	51867	0·1168	
4		59659	102861	0·2317	
5		105860	182517	0·4111	
6					
7		14710	25362	0·0571	
8		29171	50295	0·1132	
9		57007	98287	0·2214	
10		101682	175314	0·3948	

	Coulter count	cell conc. (#/ml)	Vol containing 10^6 cells (ml)	02/07/00
1.	839, 822, 847	3,344,000	0.299	
2	727, 710, 734	2,894,666	0.345	
3	729, 724, 751	2,938,666	0.340	
4	785, 793, 768	3,128,000	0.319	
5	737, 733, 703	2,897,333	0.345	
6	824, 832, 843	3,332,000	0.300	
7	847, 866, 833	3,394,666	0.294	
8	746, 732, 719	2,929,333	0.341	
9.	759, 729, 732	2,960,000	0.337	
10	703, 695, 697	2,793,333	0.358	

TABLE-3

Expt. # : (**Date/Time :** 02/07/00

Tube #	Coulter count for 100 ul cell suspension	Avg. count	Cells/ml [Avg. count x 4000]	pCi/cell [uCi/ml x 10^6 / Cells/ml]	KBq/cluster [pCi/cell x 148]
1			3,344,000		
2			2,894,666	0.0148	2.20
3			2,938,666	0.0397	5.88
4			3,128,000	0.0740	10.96
5			2,897,333	0.1419	20.99
6			3,332,000		
7			3,394,666	0.0168	2.49
8			2,929,333	0.0386	5.72
9			2,960,000	0.0748	11.07
10			2,793,333	0.1413	20.91

without 0% DMSO

with 0% DMSO

TABLE-4

Expt # : 1 **Date :** 02/14/00

Tube.dilution	Colony 1	Colony 2	Colony 3	Avg Colony	SF
1·2	157	139	142	146	
2·2	88	96	105	96·33	0·6598
3·2	23	25	27	25	0·1712
4·3	95	103	112	10·33	0·0708
5·4	83	92	87	0·8733	0·0059
6·2	132	141	129	134	
7·2	99	108	118	108·33	0·8084
8·2	71	78	96	81·66	0·6094
9·2	27	33	40	33·33	0·2487
10·3	130	120	109	11·96	0·0893