

(2)

V79 COLONY FORMING ASSAY AND HPRT MUTAGENICITY

Experiment Name : ³HTdR toxicity (cluster, 100% labeling);

Exp. # ~~100~~ ML - HORT/A

Investigator: ~~A. Bishayee~~ MAREK

Date:

~~12/17/98~~ Oct 2, 2000

DAY

PBS
without
Ca⁺⁺, Mg⁺⁺

1. Set the rocker-roller at 37°C incubator with 5% CO₂, set the Coulter Counter, wash cells (from two 150 cm² flask, subcultured 1:2, 24h before) with PBS, trypsinize cells, each resuspend in ~~2~~² ml MEMB, pool, pass five times through ~~5~~¹⁰ cc syringe with 21 gauge needle, perform cell count by transferring 100 μl in Coulter cup containing 20 ml isotone (Coulter balanced electrolyte solution)
2. Dilute to ~4,000,000 cells/ml in MEMB [Actual count : 7.77×10^6 cells/ml]
3. Transfer 1 ml of cell suspension into ten 12 ml tubes (Falcon ~~plastic~~^{polypropylene} 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% CO₂ ~~10/02/00~~ Date/Time: 18:00 - 20:30 (2.5 hrs)
5. Prepare MEMB containing radioactivity in hood
10 μl ³HTdR (Stock : 1 μCi/μl on ~~7/27/00~~) + 2.4 ml MEMB
6. After 3-4 h, remove test tubes from roller and add MEMB with or without radioactivity according to Table below. ~~10/02/00 - 12/02/00~~ Date/Time: 21:30 → ~~10/02/00~~

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

10 μl + x = 2400 μl
2390 μl

1. I need 2.3 ml MEMB
2. Prepare 2.4 ml MEMB
3. Final concentration of STOCK
4. 4 μl - 1000 μl
5. x - 2500 μl

x = 10 μl e.g. 10 μl of stock (1 μCi/ml)

9	1	1.0	0.500	0.500
10	2	1.0	0	1

- DAY 7. Return test tubes to roller for 12 h ² Oct. 2, 2000 Date/Time: 13:30 (16.5 hrs)
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). Oct. 3, 2000 Date/Time: 13:40 (16 hrs 40min)

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 [Ⓢ] I used regular 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. Oct. 3, 00 Date/Time: 16:00

* ICE COLD is necessary for experiment with DMSO only because DMSO is toxic at very conc.

- Ⓢ 23. Transfer 30 ul supernatant in three sets of 20 ml scintillation vials containing 6 ml liquid scintillation cocktail (AquaSol) ^{F volume} from 150 ul supernatant removed earlier (Step 10) and count them for radioactivity Oct. 3, 00 Date/Time: 16:30

- DAY 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

Oct., 6, 00 Date/Time: 16:10

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

A- $8 \times 3 \times 3 = 72 + 6 = 78$ (P60) - survival

B- $10 \times 1 = 10$ (P100) - for HPGT-expression

24
31
30
2
27

- 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 μ l 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~~¹⁰⁰ μ l of cell suspension (in triplicate) to 20 ml scintillation vial containing 6 ml cocktail (Aquasol) - 30 vials
37. Incubate petridishes for 1 week
38. Count vials for radioactivity Oct, 6, 00 Date/Time: 19:15
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.

Reverted
~



HPRT/A Mutagenicity

41. Day 0 - Oct. 6 \rightarrow Plate 10×10^6 cells (P100 mm) + 7 ml MEMB
 42. Day 3 - Oct. 9 \rightarrow Replate 10×10^6 cells (P100 mm) + 7 ml MEMB
 43. Day 5 - Oct. 11 \rightarrow Replate 10×10^6 cells (P100 mm) + 7 ml MEMB
 44. Day 7 - Oct 13 \rightarrow Replate 10×10^6 cells
- Oct 15 - a) I Challenge + survival (\pm 6TG)
b) replate 10×10^6 cells (P100 mm) + 7 ml MEMB for 2nd Chall.
- Oct. 17 - II challenge ONLY
-
- Oct. 22-23 - Fix, stain - I st challenge
 - Oct. 24-25 - Fix, stain - II st challenge.

Medium activity

USER: 6 ID:H3 HOWELL PRESET TIME: 1.00 TUE 03 OCT 2000 16:40
 SAMPLE REPEAT: 1 CYCLE REPEAT: 1 SCR:N RS232:N
 H#: 1 AGC:N QCF:N RCM:Y (23)
 RCM-TIME: 0.10 INT:999.95
 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	$\frac{-}{X}$	CPM	2SIGZ	TIME	EL TIME	AVG H#	RCMZ	ERR
1	**	1		21.00	43.64	1.00	1.42	82.0	2.40	
2	**	2	45.00	12.00	57.74	1.00	3.00	79.0	4.02	
3	**	3		12.00	57.74	1.00	4.57	86.0	1.76	
4	**	4		12.00	57.74	1.00	6.14	82.0	5.67	
5	**	5	13.67	21.00	43.64	1.00	7.72	80.0	4.86	
6	**	6		8.00	70.71	1.00	9.28	81.0	7.18	
7	**	7		178.00	14.99	1.00	10.85	79.0	0.39	
8	**	8	112	173.00	15.21	1.00	12.42	79.0	0.40	
9	**	9		165.00	15.57	1.00	13.99	81.0	0.46	
10	**	10		703.00	7.54	1.00	15.57	83.0	0.10	
11	**	11	687	677.00	7.69	1.00	17.13	80.0	0.10	
12	**	12		681.00	7.66	1.00	18.70	80.0	0.09	
13	**	13		1428.00	5.29	1.00	20.26	78.0	0.05	
14	**	14	1477.3	1410.00	5.33	1.00	21.83	80.0	0.05	
15	**	15		1504.00	5.16	1.00	23.41	79.0	0.04	
16	**	16		2851.00	3.75	1.00	24.98	80.0	0.03	
17	**	17	1285.2	3039.00	3.63	1.00	26.56	79.0	0.02	
18	**	18		2797.00	3.78	1.00	28.13	78.0	0.02	
19	**	1		8024.00	2.23	1.00	29.75	80.0	0.01	
20	**	2	1794.6	7942.00	2.24	1.00	31.32	82.0	0.01	
21	**	3		7859.00	2.26	1.00	32.89	79.0	0.01	
22	**	4		11384.44	1.98	0.90	34.36	81.0	0.01	
23	**	5	11112	10936.84	1.96	0.95	35.88	79.0	0.01	
24	**	6		11016.84	1.95	0.95	37.41	83.0	0.01	
25	**	7		14500.00	1.99	0.70	38.67	80.0	0.01	
26	**	8	15877.3	15835.38	1.97	0.65	39.88	80.0	0.01	
27	**	9		15407.69	2.00	0.65	41.10	81.0	0.01	
28	**	10		31174.29	1.91	0.35	42.00	79.0	0.01	
29	**	11	31022.8	30794.29	1.93	0.35	42.91	81.0	0.01	
30	**	12		31034.29	1.92	0.35	43.82	79.0	0.01	

MEDIUM ACTIVITY

TABLE-1

Expt. #: ML-HPRT/1

Date/Time: Oct, 3, 2000 / 16:30

Tube #	Medium count for 10 ul (cpm)	Avg. cpm	dpm [cpm/0.52]	$\mu\text{Ci/ml (A)}$ on counting [dpm/444]	$\mu\text{Ci/ml (A}_0)$ on addition [A/e ^{-λt}]
1	21, 12, 12				
2	12, 21, 8				
3	178, 173, 165				
4	703, 677, 681				
5	1428, 1410, 1504				
6	2851, 3039, 2797				
7	8024, 7942, 7859				
8	11384, 10937, 11017				
9	14500, 15835, 15408				
10	31174, 30794, 31034				

of cells recovered from clusters
after 72 hrs at 10:5°C

TABLE-3

Expt. #: HPRT/1

Date/Time :

Total # of recovered cells
% of recovery from cluster

Tube #	Coulter count for 100 ul cell suspension	Avg. count Background ↓	Cells/ml [Avg. count x 4000] x 10 ⁶	pCi/cell [uCi/ml x 10⁶ Cells/ml]
1	304, 266, 288	858/3 - 1	1.14	2.28 0.57
2	395, 341, 307	1043/3 - 1	1.37	2.76 69%
3	414, 346, 336	1096/3 - 1	1.46	2.96 73%
4	379, 408, 331	1118/3 - 1	1.49	75%
5	360, 367, 316	1043/3 - 1	1.38	2.76 69%
6	354, 290, 269	913/3 - 1	1.21	2.42 61%
7	246, 233, 198	677/3 - 1	0.89	1.78 45%
8	319, 322, 333	974/3 - 1	1.29	2.58 65%
9	298, 294, 314	906/3 - 1	1.20	2.40 60%
10	373, 328, 335	1036/3 - 1	1.38	2.76 69%

Background = 1

Volume counted = 50 µl

Cell suspension activity

USER: 6 ID:H3 HOWELL PRESET TIME: 1.00
 SAMPLE REPEAT: 1 CYCLE REPEAT: 1 SCR:N RS232:N
 H#: 1 ACC:N GCF:N RCM:Y

FRI 06 OCT 2000 19:35

RCM-TIME: 0.10 INT:999.95
 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: 1.00000

SAM	POS	CH	CPM	2SIG%	TIME	EL TIME	AVG H#	RCM%	ERR
1	**	1	10.00	63.25	1.00	1.42	81.0	1.56	
2	**	2	10.00	63.25	1.00	3.00	80.0	2.03	
3	**	3	12.00	57.74	1.00	4.57	80.0	0.94	
4	**	4	9.00	66.67	1.00	6.13	81.0	1.57	
5	**	5	6.00	81.65	1.00	7.71	82.0	0.60	
6	**	6	30.00	36.51	1.00	9.28	87.0	0.46	
7	**	7	31.00	35.92	1.00	10.85	80.0	0.38	
8	**	8	24.00	40.82	1.00	12.42	79.0	0.50	
9	**	9	7.00	75.59	1.00	13.99	77.0	1.86	
10	**	10	130.00	17.54	1.00	15.56	82.0	0.14	
11	**	11	108.00	19.25	1.00	17.13	80.0	0.22	
12	**	12	112.00	18.90	1.00	18.69	80.0	0.16	
13	**	13	240.00	12.91	1.00	20.26	80.0	0.09	
14	**	14	221.00	13.45	1.00	21.82	82.0	0.09	
15	**	15	235.00	13.05	1.00	23.39	81.0	0.10	
16	**	16	370.00	10.40	1.00	24.97	80.0	0.06	
17	**	17	586.00	8.26	1.00	26.54	84.0	0.03	
18	**	18	506.00	8.89	1.00	28.11	83.0	0.04	
19	**	1	726.00	7.42	1.00	29.78	79.0	0.03	
20	**	2	813.00	7.01	1.00	31.34	80.0	0.03	
21	**	3	715.00	7.48	1.00	32.92	80.0	0.03	
22	**	4	1844.00	4.66	1.00	34.49	80.0	0.01	
23	**	5	1866.00	4.63	1.00	36.12	80.0	0.01	
24	**	6	1617.00	4.97	1.00	37.69	80.0	0.01	
25	**	7	2067.00	4.40	1.00	39.26	79.0	0.01	
26	**	8	2133.00	4.33	1.00	40.82	79.0	0.01	
27	**	9	2138.00	4.33	1.00	42.39	79.0	0.01	
28	**	10	4549.00	2.97	1.00	43.96	81.0	0.01	
29	**	11	4600.00	2.95	1.00	45.53	79.0	0.01	
30	**	12	3991.00	3.17	1.00	47.11	80.0	0.01	

NOTE: 100ul cells suspension into 6ml scintillation liquid.

CELLS SUSPENSION ACTIVITY

TABLE-2

Expt. #: ML-HPRT/1

Oct. 6, 00 Date/Time: 19:35

Tube #	Radioactivity for 100 ul cell suspension (cpm)	Avg. cpm	dpm [cpm/0.65]	$\mu\text{Ci/ml } (A_t)$ on counting [dpm/44000] 222000	$\mu\text{Ci/ml } (A_0)$ after 12 h incubation [$A_t/e^{-\lambda t}$]
1	10, 10, 12	11	17		
2	9, 6, 30	15	23		
3	31, 24, 7	21	32	0.00003	
4	130, 108, 112	117	180	0.004	
5	240, 221, 235	232	357	0.007	
6	370, 586, 506	487	749		
7	726, 813, 715	751	1155		
8	1844, 1866, 1617	1776	2732		
9	2067, 2133, 2138	2113	3251		
10	4549, 4600, 3991	4380	6738		

TABLE-4

Expt # :

Date :

	Tube.dilution	Colony 1	Colony 2	Colony 3	Avg Colony PE	SF
0	1,3	37	48	35	$\frac{37+5}{2}$	0.34 -1
0	2,3	38	36	31	$\frac{(14+125)}{2}$	
0.01	3,3	52	57	47	$\frac{52+146}{2}$ 0.36	-1.16
0.05	4,3	57	65	72	$\frac{65+149}{2}$ 0.44	-1.42
0.1	5,3	29	22	22	$\frac{24+139}{2}$ 0.17	-0.55 ?
0.2	6,3	50	51	47	$\frac{49+121}{2}$ 0.40	-1.29
0.5	7,3	38	50	43	$\frac{44+90}{2}$ 0.49	-1.58
0.75	8,3	39	60	48	$\frac{49+129}{2}$ 0.38	-1.23
1	9,3	41	34	38	$\frac{38+120}{2}$ 0.32	-1.00
2	10,3	19	19	20	$\frac{19+138}{2}$ 0.14	-0.45

SURVIVAL

010

Colony counting for 1027/1 16-Oct, 2000

1	1				
	2				
	3	48	(37)	(35)	- 120:3 = 40
2	1				110
	2	178			
	3	(36)	(38)		114
3	1	↓ too			
	2	186	(185)		
	3	37	(57)	(52)	
4	1	↓ too			
	2	(205)	159	197	
	3	65	72	(57)	
5	1	↓ too			
	2	(38)	(33)	(43)	
	3	22	(29)	22	
6	1	↓ too			
	2	(145)	183	(171)	
	3	(57)	(50)	57	
7	1	↓ too			
	2	123	143	140	
	3	43	(38)	(50)	
8	1	↓ too			
	2	(95)	(174)	196	
	3	48	(39)	60	
9	1	↓ too			
	2	186	160		
	3	(47)	(34)	(38)	
10	1	↓ too			
	2	(153)	(133)	154	
	3	20	(19)	19	

MediumActivity

Experiment: ML - Oct. 2, 2000
 Date: 10/2/2000

Tube #	Medium count (CPM)			CPM Average	CPM corrected for control	DPM CPM(y e)	At $\mu\text{Ci/ml on counting}$	Ao $\mu\text{Ci/ml at addition}$ [A/(e-0.693t/T)]	Ao kBq/ml at addition
	1st	2nd	3rd						
1	21	12	12	14	0	0	0	0	0
2	12	21	8	0	0	0	0	0	0
3	178	173	165	172	158	243	0.0036	0.0036	0.1348
4	703	677	681	687	673	1035	0.0155	0.0155	0.5750
5	1428	1410	1504	1447	1433	2205	0.0331	0.0331	1.2249
6	2851	3039	2797	2896	2881	4433	0.0666	0.0666	2.4629
7	8024	7942	7859	7942	7927	12196	0.1831	0.1831	6.7762
8	11384	10937	11017	11113	11098	17074	0.2564	0.2564	9.4867
9	14500	15835	15408	15248	15233	23436	0.3519	0.3519	13.0213
10	31174	30794	31034	31001	30986	47671	0.7158	0.7158	26.4868

Experiment: ML - Oct. 2, 2000
 Date: 10/02/00

Tube #	Suspension count (CPM)			CPM Average	CPM corrected for control	DPM CPM/(y e)	A _t μCi/ml on counting	A ₀ μCi/ml after uptake	A ₀ kBq/ml after uptake
	1st	2nd	3rd						
1	10	10	12	13	0	0	0.00000	0	0.0000
2	9	6	30	0	0	0	0.00000	0	0.0000
3	31	24	7	21	8	12	0.00005	0.00005	0.0020
4	130	108	112	117	104	160	0.00072	0.00072	0.0266
5	240	221	235	232	219	337	0.00152	0.00152	0.0562
6	370	587	506	488	475	731	0.00329	0.00329	0.1218
7	726	813	715	751	739	1136	0.00512	0.00512	0.1895
8	1844	1866	1617	1776	1763	2712	0.01222	0.01222	0.4522
9	2067	2133	2138	2100	2087	3211	0.01446	0.01447	0.5354
10	4549	4600	3991	4380	4367	6719	0.03026	0.03028	1.1203

CoulterSurvival

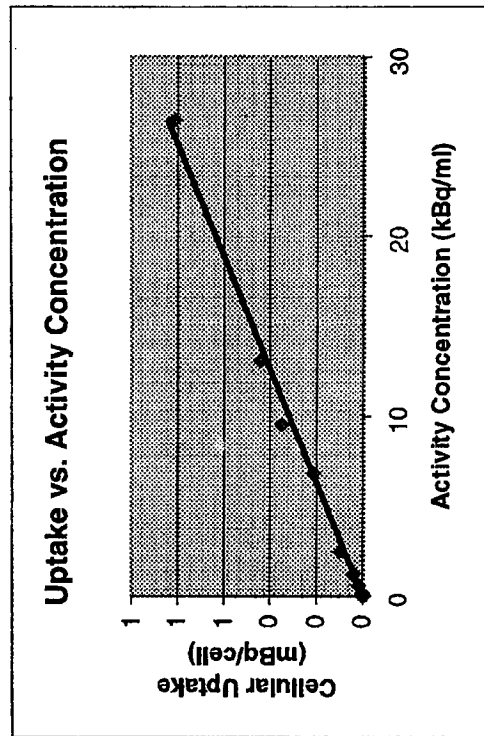
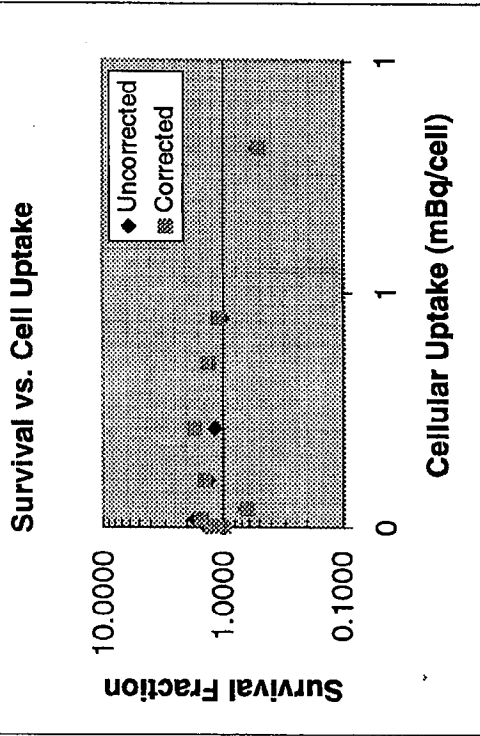
Experiment: ML - Oct. 2, 2000
 Date/Time: 10/2/00

Tube #	Coulter count			Average Cells/ml	Hemocytometer Count in Grid				
	1st	2nd	3rd		1st	2nd	3rd	4th	
		304							
1	394	266	288	316	1260000				
2	395	341	307	348	1386667				
3	414	346	336	365	1457333				
4	379	408	331	373	1486667				
5	360	367	316	348	1386667				
6	354	290	269	304	1213333				
7	246	233	198	226	898667				
8	319	322	333	325	1294667				
9	298	294	314	302	1204000				
10	373	328	335	345	1377333				

Tube #	Predicted # Cells Seeded	Actual # Cells Seeded	Colony count			Average	PE (%)	SF Uncorrected	SF Corrected
			1st	2nd	3rd				
1	200	126	37	48	35	38	29.762	1.00	1.0000
2	200	139	38	36	31				
3	200	146	52	57	47	52	35.682	1.3867	1.2592
4	200	149	57	65	72	65	43.498	1.7244	1.5350
5	200	139	29	22	22	24	17.548	0.6489	0.6193
6	200	121	50	51	47	49	40.659	1.3156	1.4348
7	200	90	38	50	43	44	48.591	1.1644	1.7147
8	200	129	39	60	48	49	37.848	1.3067	1.3356
9	200	120	41	34	38	38	31.285	1.0044	1.1040
10	200	138	19	19	20	19	14.037	0.5156	0.4953

Experiment: 10/2/00
 Date/Time:

Tube #	Activity Conc. (kBq/ml)	Activity/Cell (mBq/cell)	Survival Uncorrected	Survival Corrected
1	0.000	0.000	1.0000	1.0000
2	0.000	0.000		
3	0.135	0.001	1.3867	1.2592
4	0.575	0.018	1.7244	1.5350
5	1.225	0.041	0.6489	0.6193
6	2.463	0.100	1.3156	1.4348
7	6.776	0.211	1.1644	1.7147
8	9.487	0.349	1.3067	1.3356
9	13.021	0.445	1.0044	1.1040
10	26.487	0.813	0.5156	0.4953



Experiment: 10/2/00
 Date/Time:

Tube #	Activity Conc. (kBq/ml)	Activity/Cell (mBq/cell)	Survival Uncorrected	Survival Corrected
1	0.000	0.000	1.0000	1.0000
2	0.000	0.000		
3	0.135	0.001	1.3867	1.2592
4	0.575	0.018	1.7244	1.5350
5	1.225	0.041	0.6489	0.6193
6	2.463	0.100	1.3156	1.4348
7	6.776	0.211	1.1644	1.7147
8	9.487	0.349	1.3067	1.3356
9	13.021	0.445	1.0044	1.1040
10	26.487	0.813	0.5156	0.4953

