7

V79 COLONY FORMING ASSAY

Experiment Name : ^{3}HTdR toxicity (cluster, 50% labeling) ± 100µM lindane **Exp. # :** 7
Investigator: R Howell **Date:** 10/22/01

Preparation of cells: confluent T25 flask split 1:5 into T225 on 10/19/01. Cells trypsined from T225 at 1:30 pm on 10/22/01. About 70% confluent.

Serum/Lot #s:

1. Set the rocker-roller at 37°C incubator with 5% CO_2, set the Coulter Counter, wash cells (from one 225 cm^2 flasks, seeded with 25 x 10^6 cells the day before) with PBS-PS, trypsinize cells with 2 ml trypsin 3 min at 37°C, resuspend in 10 ml MEMB, pool, pass five times through 10 cc syringe with 21 gauge needle, perform cell count by transferring 100 ul in Coulter cup containing 20 ml Isotone II (Coulter balanced electrolyte solution).
2. Dilute to ~2,000,000 cells/ml in MEMB [Actual count : cells/ml)

3. Transfer 1 ml of cell suspension into 20 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:** 2:00 pm
5. Prepare MEMB containing radioactivity in hood

1100 µl ^{3}HTdR (Stock : 1 µCi/µl on 10/15/01) + 4.4 ml MEMB

Manufacturer: PerkinElmer NET-0272 Lot #: 3106449 Calibration: 10/15/01

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. Also add 1 ml MEMB to each of second set of tubes.

Tube #	^{3}HTdR µCi/ml	Cells in MEMB (ml)	MEMB (ml)	MEMB+ ^{3}HTdR [200 µCi/ml] (ml)
1	0	1.0	1.0	0
2	25	1.0	0.75	0.25
3	50	1.0	0.50	0.50
4	75	1.0	0.25	0.75
5	100	1.0	0	1.0
6	0	1.0	1.0	0
7	25	1.0	0.75	0.25
8	50	1.0	0.50	0.50
9	75	1.0	0.25	0.75
10	100	1.0	0	1.0

with lindane

Need 5ml @ 200µCi/ml
200
5.5
1000
1000
1100.0 µCi
1.1 ml ^{3}HTdR + 8.4 ml MEMB

7. Return test tubes to roller for 12-14 h. **Date/Time:** 5:15 pm
8. Next day, while test tubes are in roller label 10 gamma-tubes (13 X 100 mm VWR glass test tube)
9. After ~12-14 h incubation period, remove all tubes and centrifuge at 2000 rpm at 4°C for 10 min (***precooled centrifuge***). **Date/Time:** 10/23 9:30am
10. Remove buckets from centrifuge and carefully remove 150 µl of supernatant from tubes containing radioactivity and place in pre-labeled gamma-tubes.
11. Decant supernatant, click tubes, vortex, resuspend in 10 ml wash MEMA
12. Centrifuge tubes for ~~10~~ 6 min at 2000 rpm, 4°C
13. Decant supernatant, click tubes, vortex, resuspend in 10 ml wash MEMA
14. Centrifuge tubes for ~~10~~ 6 min at 2000 rpm, 4°C
15. Decant supernatant, click tubes, vortex, resuspend in 10 ml of wash MEMA
16. Centrifuge tubes for ~~10~~ 6 min at 2000 rpm, 4°C
17. Decant supernatant, click tubes, vortex, resuspend unlabeled cells in ~~8~~ 7 ml MEMA.

For tubes 6-10 labeled & unlabeled resuspend in 5 ml MEMA w/ 100µM lindane
Also need lindane MEMA for wash & for transfer
Sonic's stock 500X for 100µM in DMSO. Prepare 55 ml MEMA w/ 100µM lindane
Resuspended tubes 6-10 with lindane MEM along with unlabeled tubes 0.11 ml 500X lindane + 55 ml MEMA

See pg. 2a

18. ~~Transfer unlabeled cells to the corresponding tubes containing 2,000,000 labeled cells.~~
19. ~~Wash unlabeled cell tube with 5 ml MEMA and transfer to corresponding labeled cell~~ tube.
20. ~~Syringe the pooled cells 5 times with 5 ml syringe with 21 G~~ needle.
21. Centrifuge tubes for 10 min at 2000 rpm, 4°C.
22. Decant supernatant completely, click tubes, vortex.

- Because mistakenly resuspended unlabeled in 7ml, had to resuspend labeled in 3 ml
- Syringed labeled cells tubes 1-5 21 G 5CC 5X

Coulter count 100Ml w/ 500 Ml manometer

1)	1543 1499	1521	608,400/ml
2)	1551 1470 1545	1522	608,400
3)	1058 1008 989	1018	407333
4)	856 892 867	872	348666
5)	872 852 881	868	347333

Examined cells, looked very clumpy. Decided not to make corrections to volumes based on cell counts.

~~S~~yringed all remaining tubes (labeled, unlabeled)
- Transfered 7ml of unlabeled to labeled
- Centrif. 6 min, 2000 rpm, 4°C
- Decant thoroughly, vortex, click
- Transfer 200Ml to 400Ml microfuge tube
- Wash 1X with 200Ml MEMA or MEMA 100Ml lindane (tubes 6-10)
  & transfer to 400Ml tube
- Spin 5 min 1000 rpm 4°C
- Transfer tubes to 10.5°C

23. Transfer the cell suspension in polypropylene microcentrifuge tubes with attached caps (Helena Plastics, 400 µl) using 200 µl pipette tip.
24. Again add 200 µl ice cold MEMA, resuspend and transfer the cell suspensions in the same polypropylene microcentrifuge tubes (Total volume ~400 µl)

25. Centrifuge tubes for 5 min at 1000 rpm, 4°C
26. Transfer tubes at 10.5°C for 72 h. **Date/Time:** 10/23/01 1:00pm
27. Transfer 30 µl supernatant in three sets of 7 ml scintillation vials and add 5 ml liquid scintillation cocktail (Ecoscint) from 150 ul supernatant removed earlier and count them for radioactivity **Date/Time:**
28. 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 pipette **Date/Time:**
29. Again add 200 µl wash MEMA in microcentrifuge tubes, resuspend and transfer the cell suspensions in 12 ml tubes
30. Centrifuge the tubes for 10 min at 2000 rpm, ~~4°C~~ RT (~~*precooled centrifuge*~~)
31. 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 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.
32. Decant supernatant, click tubes, vortex, resuspend in 10 ml wash MEMA
33. Centrifuge tubes for 10 min at 2000 rpm, ~~4°C~~ RT
34. ~~Decant supernatant, click tubes, vortex, resuspend in 10 ml wash MEMA~~
35. ~~Centrifuge tubes for 10 min at 2000 rpm, 4°C~~
36. Decant supernatant, click tubes, vortex, resuspend in 2 ml wash MEMA, pass five times through 5 cc syringe with 21 gauge needle
37. Determine cell concentration by transferring 100 µl to Coulter cup
38. 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.
39. 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.
40. Transfer 100 200 µl of cell suspension (in triplicate) to 20 ml scintillation vial containing 6 ml cocktail (Ecolume)
41. Incubate tissue culture dishes for 1 week
42. Count vials for radioactivity **Date/Time:**
43. After 1 week, wash colonies 3 times with normal (1X) saline, and 2 times with methanol.

20

* may have mixed up tubes 1-5

5 tubes w/ lindane
Should wash in presence of lindane. Dilutions then remove.
Need 110 ml wash MEMA w/ lindane
0.22 ml lindane 500X + 110 ml

Exp. 7 V79

USER: 6 ID:HOWELL H3 PRESET TIME: 1.00 TUE 30 OCT 2001 09:34
SAMPLE REPEAT: 1 CYCLE REPEAT: 1 SCR:N RS232:N
[redacted] 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:@ 1.00000
HALF LIFE(DAYS):N

SAM	POS	CH	CPM	2SIG%	TIME	EL TIME	AVG H#	ERR	
1	**- 1	1	49.00	28.57	1.00	1.42	79.0		1M
2	**- 2	1	65.00	24.81	1.00	3.06	79.0		
3	**- 3	1	54.00	27.22	1.00	4.63	78.0		
4	**- 4	1	335760.00	0.89	0.15	5.35	80.0		2M
5	**- 5	1	340640.00	0.88	0.15	6.08	80.0		
6	**- 6	1	335030.00	1.09	0.10	6.80	80.0		
7	**- 7	1	665200.00	0.78	0.10	7.55	79.0		3M
8	**- 8	1	679266.62	0.63	0.15	8.31	79.0		
9	**- 9	1	678900.00	0.77	0.10	9.06	80.0		
10	**-10	1	1015620.00	0.63	0.10	9.84	80.0		4M
11	**-11	1	1006173.31	0.51	0.15	10.62	79.0		
12	**-12	1	1013119.94	0.51	0.15	11.41	80.0		
13	**-13	1	1355813.25	0.44	0.15	12.22	80.0		5M
14	**-14	1	1372006.62	0.44	0.15	13.03	80.0		
15	**-15	1	1346480.00	0.45	0.15	13.85	80.0		
16	**-16	1	8.00	70.71	1.00	15.42	75.0		6M
17	**-17	1	4.00	100.0	1.00	16.98	74.0		
18	**-18	1	8.00	70.71	1.00	18.60	75.0		
[redacted]	**- 1	1	336073.31	0.89	0.15	19.37	80.0		7M
[redacted]	**- 2	1	340150.00	1.08	0.10	20.10	79.0		
21	**- 3	1	337946.66	0.89	0.15	20.82	80.0		
22	**- 4	1	675446.62	0.63	0.15	21.58	80.0		8M
23	**- 5	1	682666.62	0.62	0.15	22.33	79.0		
24	**- 6	1	670130.00	0.77	0.10	23.10	79.0		
25	**- 7	1	990613.31	0.52	0.15	23.88	78.0		9M
26	**- 8	1	983046.62	0.52	0.15	24.66	79.0		
27	**- 9	1	1035779.94	0.51	0.15	25.45	80.0		
28	**-10	1	1349900.00	0.44	0.15	26.26	80.0		10M
29	**-11	1	1314573.25	0.45	0.15	27.07	79.0		
30	**-12	1	1352226.62	0.44	0.15	27.89	80.0		
31	**-13	1	11.00	60.30	1.00	29.44	93.0		1C
32	**-14	1	11.00	60.30	1.00	31.01	93.0		
33	**-15	1	4.00	100.0	1.00	32.64	92.0		
34	**-16	1	98446.66	1.65	0.15	33.34	93.0		2C
35	**-17	1	91846.66	1.70	0.15	34.04	93.0		
36	**-18	1	97120.00	1.66	0.15	34.74	93.0		
37	**- 1	1	197240.00	1.42	0.10	35.51	92.0		3C
38	**- 2	1	175070.00	1.51	0.10	36.22	94.0		
39	**- 3	1	199286.66	1.16	0.15	36.94	93.0		
40	**- 4	1	301113.31	0.94	0.15	37.66	94.0		4C
41	**- 5	1	276480.00	1.20	0.10	38.38	92.0		
42	**- 6	1	287820.00	1.18	0.10	39.11	93.0		
43	**- 7	1	407926.66	0.91	0.15	39.84	93.0		5C
[redacted]	**- 8	1	414960.00	0.98	0.10	40.57	93.0		
[redacted]	**- 9	1	424200.00	0.97	0.10	41.31	94.0		
46	**-10	1	4.00	100.0	1.00	42.93	92.0		

B007496

SAM	POS	CH	CPM	2SIG%	TIME	EL TIME	AVG H#	ERR	
47	**-11	1	7.00	75.59	1.00	44.51	94.0		6C
48	**-12	1	5.00	89.44	1.00	46.14	93.0		
49	**-13	1	105500.00	1.95	0.10	46.84	92.0		7C
50	**-14	1	98766.66	1.64	0.15	47.55	92.0		
51	**-15	1	96853.33	1.66	0.15	48.25	94.0		
52	**-16	1	219980.00	1.35	0.10	48.97	93.0		8C
53	**-17	1	209893.33	1.13	0.15	49.67	92.0		
54	**-18	1	191873.33	1.18	0.15	50.39	92.0		
55	**- 1	1	308220.00	1.14	0.10	51.17	91.0		9C
56	**- 2	1	309030.00	1.14	0.10	51.90	93.0		
57	**- 3	1	307220.00	1.14	0.10	52.62	92.0		
58	**- 4	1	374213.31	0.84	0.15	53.35	94.0		10C
59	**- 5	1	352166.66	0.87	0.15	54.08	92.0		
60	**- 6	1	373870.00	1.03	0.10	54.82	93.0		
61	**- 7	1	7.00	75.59	1.00	56.43	-2.0		Bkgd.
62	**- 8	1	28782.86	1.99	0.35	57.40	1.0		Std.

Exp. 7

Break up Cluster

Seed for Survival, Expression

10/26/01

Coulter Counts

Bkgd. 4 for 500µl setting

100µl cells + 20ml Isotone II + 100µl manometer

					2×10^6 cells
1)	1259	1259	1318	1278.7	2.56×10^6/ml	0.78 ml
2)	1212	1179	1241	1210.7	2.42×10^6/ml	0.83 ml
3)	981	970	939	963.3	1.93×10^6/ml	1.04 ml
4)	1022	1088	1066	1058.7	2.12×10^6/ml	0.94 ml
5)	1093	1038	1056	1062.3	2.12×10^6/ml	0.94 ml
6)	1413	1471	1471	1451.7	2.90×10^6/ml	0.69 ml
7)	1111	1041	1077	1076.3	2.15×10^6/ml	0.93 ml
8)	1057	1078	1010	1048.3	2.00×10^6/ml	0.95 ml
9)	1041	957	1016	1004.7	2.00×10^6/ml	1.0 ml
10)	891	884	937	904.0	1.81×10^6/ml	1.11 ml

Exp. 7 10/29/01

Replating for Expression

- Remove five T75 flasks, remove medium by aspiration.
- Wash 1X 10 ml PBS, add 1 ml trypsin, 37°C, 3min
- Add 7ml MEMA, transfer to 14 ml tube
- Syringe 5X, 21G, 5cc
- Count 100µl cells, 20 ml Isotone, 100µl manometer

1)	1155 1144 1191	1163.3	2.32×10^6	0.86 ml
2)	1404 1533 1438	1458.3	2.92×10^6	0.68 ml
3)	993 971 942	968.6	1.94×10^6	1.03
4)	917 895 899	903.6	1.81×10^6	1.10
5)	746 777 764	762.3	1.52×10^6	1.31
6)	1180 1121 1285	1195.3	2.39×10^6	0.84ml
7)	1026 932 997	985	1.97×10^6	1.02ml
8)	939 925 905	923	1.85×10^6	1.08
9)	760 732 720	737.3	1.47×10^6	1.36
10)	698 702 706	702	1.40×10^6	1.42

Exp. 7 11/2/01

Replating for Expression

- Aspirate media from T150, wash 1X PBS, 2 ml trypsin, 3min 37°C, resuspend in 7 ml MEMA, transfer to 14 ml tube
- Syringe 5X, 21G, 5cc
- Coulter Count 500mA 5.0 Lower thresh
  100 Ml cells, 20 ml Isotone II, 100 Ml manometer

Bkg. 20

1)	2383 2564 2471	2472.7	4.95×10^6/ml	0.40 ml
2)	2899 2843 2909	2883.7	5.77×10^6/ml	0.35
3)	1808 1840 1922	1856.7	3.71×10^6/ml	0.54
4)	1761 1852 1789	1800.7	3.60×10^6/ml	0.55
5)	1777 1721 1768	1755.3	3.51×10^6/ml	0.57 ml
6)	3059 3060 3081	3100	6.20×10^6/ml	0.32
7)	2791 2736 2752	2759.7	5.52×10^6/ml	0.36
8)	2193 2202 2202	2199	4.39×10^6/ml	0.45
9)	1754 1799 1716	1756	3.51×10^6/ml	0.57
10)	1489 1471 1509	1489.7	2.98×10^6/ml	0.67

- Transfer appropriate volume of cells to T150 containing 25 ml MEMA

Stain colonies with 0.05% crystal violet

44. Count colonies. There must be between 25 and 250 colonies for the dish to be a valid data point.

Tube	Dilution	Colony Counts

V79 Exp. 7 11/5/01

Processing for Mutant Selection

- Load 100 P100 w/ MEMA 10ml * Careful
  Load 30 P60 w/ 4 ml MEMA
  Load 40 dilution tubes with 4.5 ml wash MEMA

- Aspirate medium from T75
- Wash 1X 10 ml PBS
- Trypsinize 2 ml, 3 min, 37°C, hit flasks
- Resusp in 8 ml MEMA (wash) & transfer to 14 ml tube
- Spin 5 min 2000 rpm, RT
- Decant, resuspend in 5 ml wash MEMA
- Syringe 5X 21G 5cc

Coulter Counts (100 Aperture cells, 20 ml Isotone, LT 5.0, 100 Aperture manometer)

						vol. to get for 2×10^6 cells
1)	3438	3571	3566	3525	7.05×10^6/ml	0.142 cells + 0.358 MEM
2)	3715	3784	3761	3753.3	7.50×10^6/ml	0.133 cells + 0.367 MEM
3)						
4)						
5)						
6)						
7)						
8)						
9)						
10)						

V79 Exp 7.

Coulter Counts

			Cells/ml	5ml @ 2×10^6/ml for ml cells	ml MEM
1)	3438 3571 3566	3525	7.05×10^6/ml	1.41	3.59
2)	3715 3784 3761	3753.3	7.51×10^6/ml	1.33	3.67
3)	2164 1991 ~~1935~~				
4)	~~1749~~ 1714	Didn't spin down & resusp in 5ml			
3)	3733 3756 3801	3763.3	7.52×10^6	1.33	3.67
4)	3391 3243 3336	3323.3	6.64×10^6	1.50	3.50
5)	3322 3294 3405	3340.3	6.68×10^6	1.50	3.50
6)	3770 3693 3863	3775.3	7.55×10^6	1.32	3.68
7)	3675 3410 3525	3536.7	7.07×10^6	1.41	3.59
8)	3440 3353 3605	3466	6.93×10^6	1.44	3.56
9)	3503 3468 3397	3456	6.91×10^6	1.45	3.55
10)	3538 3371 3465	3458	6.92×10^6	1.45 wash	3.55

- Transfer designated ml cells + ml MEMA to fresh 14 ml tube to get 2×10^6 cells/ml
- Serially dilute 0.5ml of above to 4.5 ml wash MEMA & repeat 3x
- Seed P60's with last dilution (1ml)
- Seed P100's with 100Ml of 2×10^6 cells/ml dilution

- Normally add 6TG in 3 ml - need to concentrate it 10X.

- Need 30ml of 6TG @ $\frac{500\ Mg/ml}{3}$ = 5000 Mg

Stock = $\frac{5mg}{2ml}$ = 2.5 $\frac{mg}{ml}$ = 2500 Mg/ml

Need 2 ml stock + 28 ml MEM yields

30 ml @ 167 $\frac{Mg}{ml}$

So add 1ml to each of 3 2.5 mg 5ifma vials

- Add 0.3 ml of 6TG - medium to each P100