15

V79 COLONY FORMING ASSAY

Experiment: 100 cluster conformation, Cs-137 rays); **Exp.**
Investigator: M. Lenarczyk **Date:** April 2, 2001

Procedure to get V79 cells with low background for HPRT- mutants.

Cells were propagated in normal culture medium upto 70-80 confluency.

Then they were split into 2 T175 flask and they were under HAT (1x) condition (from HAT 100x stock solution)

Next day they the cells were trypsinized @ split in same 2 T175 flasks and were cultured under HAT condition

After two days under HAT culture condition cells were split into 2 T175 flasks and were cultured for next two days in normal medium without HAT.

Day before experiment cells were re-plated (app. 10 x 10(6) into each of two T175 (from app. 80% confluency = 30.5 x 10(6) (flask A) and 31.5 x 10(6) (flask C).

Next day cells were washed with HBSS, trypsinized and counted **Date: April 02, 2001**

Flask A = count – 162 x 10(4) cells x 20 ml = 32.4 x 10(6) cells (app. 80-90% confluency)

Flask C = count – 168 x 10(4) cells x 20 ml = 33.6 x 10(6) cells (app 80-90 % confluency)

Experimental procedure

1. Set the rocker-roller at 37°C incubator with 5% CO_2,
   pool, pass 5x through 3 cc syringe with 21 gauge needle,
2. Dilute to ~4,000,000 cells/ml in MEMB [Actual count : 4 000 000 cells/ml)
   a) cells were counted using hematocytometer
   b) flask A + flask C = (32.4 + 33.6) x 10(6) cells = 66 x 10(6) cells
      1. re-suspend 66 x 10(6) cells in10 ml S-MEM
      2. take 9.09 ml (app. 60 x 10(6) cells) and add 5.91 ml S-MEM = 60 x 10(6) / 15 ml = 4 x 10(6)/ml
      3. count the cells : counts is (306 + 322)/ 2 x 10(4) cells = 3.14 x 10(6)
      4. take 1.27 ml (4 x 10(6) cells) and transfer them into Falcon polypropylene test tube, (7x100 mm) labeled 1-10 both on cap and wall 12 ml Falcon tube
      5. add 0. 73 ml S-MEM into each tube to make final volume = 2.00 ml

Sample	Cs-137 (Gy)	Temp. (10.5° C)
1	control	100% cluster
2	control	100% cluster
3	Gy	100% cluster
4	Gy	100% cluster
5	Gy	100% cluster
6	Gy	100% cluster
7	Gy	100% cluster
8	Gy	100% cluster
9	Gy	100% cluster
10	Gy	100% cluster
11	Gy	control after ROLL

* Before I put the cells in cluster I also plated them for survival to check "background" PE
74×10^4 cells $\xrightarrow{\text{dilute 10x}}$ 74×10^3 $\xrightarrow{\text{dilute 10x}}$ 74×10^2/ml
0.203 ml of 74×10^2/ml = 1500 cells
0.203 ml + 29.8 ml MEMA = 1500 cells/30 ml
a) 2 ml/P60' → 100 cells/P60' + 2 ml MEM/P60' ×3
b) 4 ml/P60' → 200 cells/P60 ×3
→ 37°C, 7 days

**

3. Keep the tubes in the roller overnight at 37°C, 5% CO_2 **Date/Time: April 02, 2001 / 18:00**
4. After overnight incubation period (hrs) remove tubes from incubator, and centrifuge at 2000 rpm at 4°C for 10 min (***precooled centrifuge***).
   Date/Time: April 3, 2001 : 10:30
5. Decant supernatant, click tubes, vortex, re-suspend in 10 ml wash MEMA
6. Centrifuge tubes for 10 min at 2000 rpm, 4°C
7. Decant supernatant, click tubes, vortex, re-suspend in 10 ml wash MEMA
8. Centrifuge tubes for 10 min at 2000 rpm, 4°C
9. Decant supernatant, click tubes, vortex, re-suspend in 10 ml wash MEMA
10. Centrifuge tubes for 10 min at 2000 rpm, 4°C
11. Decant supernatant, click tubes, vortex, re-suspend in 7 ml of MEMA (**culture medium**)
12. Centrifuge tubes for 10 min at 2000 rpm, 4°C
13. Decant supernatant, click tubes, vortex, transfer the cell suspension in polypropylene micro-centrifuge tubes Helena Plastics, (400 µl) using 200 µl pipet tips
14. Again add 200 µl ice cold MEMA, re-suspend and transfer the cell suspensions in the same polypropylene micro-centrifuge tubes (Total volume ~400 ul)
15. Centrifuge tubes for 5 min at 1000 rpm, 4°C
16. Transfer tubes at 10.5°C for 72 h. **Date/Time: April 3, 2001 /**

17. After 72 hrs remove cluster from 10.5 C and transfer them in plastic container contains ice. The cluster must be little bit above the ice.
18. Irradiate cluster with gamma rays, (Cs-137, acute exposure). Be sure that only one cluster is irradiated at time.

Date/Time:

19. After irradiation, carefully remove the supernatant from the top, re-re-suspend pellet in 200 µl 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
20. Again add 200 µl wash MEMA in micro-centrifuge tubes, re-suspend and transfer the cell suspensions in 12 ml tubes
21. Centrifuge the tubes for 10 min at 2000 rpm, 4°C ***(precooled centrifuge)***
22. 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.
23. Decant supernatant, click tubes, vortex, re-suspend in 10 ml wash MEMA
24. Centrifuge tubes for 10 min at 2000 rpm, 4°C
25. Decant supernatant, click tubes, vortex, re-suspend in 10 ml wash MEMA
26. Centrifuge tubes for 10 min at 2000 rpm, 4°C
27. Decant supernatant, click tubes, vortex, re-suspend in 2 ml wash MEMA, pass five times through 3 cc syringe with 21 gauge needle
28. Determine cell concentration : transfere100 µl of cell suspension in vial with 20 ml Isotone II in Coulter vial
29. Vortex tube, transfer 0.5 ml into tube X.5, vortex tube X.5, (10x dilution - 10(5) cells)
    transfer 0.5 ml into dilution tube X.4, vortex tube X.4 (100x dilution) – 10(4) cells)
    transfer 0.5 ml to tube X.3, vortex tube X.3 (1000x dilution) – 10(3) cells)
    transfer 0.5 ml to tube X.2 and vortex. (10000x dilution) – 10(2) cells)
    Keep tubes on ice.
30. 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.
31. Transfer 200 µl of cell suspension (in triplicate) to 20 ml scintillation vial containing 6 ml

cocktail (Eco-Lume)

32. Incubate dishes for 1 week
33. After 1 week, wash colonies 3 times with normal (1X) saline, and 2 times with methanol. Stain colonies with 0.05% crystal violet
34. Count colonies. Should be between 25 and 250 colonies per dish to be a valid data point.

↓

~

↓

For survival → 1 - x2
2 - x2
3 - x2
4 - x2
5 - x2
6 - x2
7 - x2
8 - x3, x2
9 - x3, x2
10 - x3, x2

# of cells / P60's x3

# of cells / P100 — For HPRT expression

For MN

1 -
2 -
3 -
4 -
5 -
6 -
7 -
8 -
9 -
10 -

0.5×10^5 for 24h with Cyt & 48h with Cyt ~3 µg/ml Cyt.

Counts of cells recovered from cluster 4
(clusters were per 3 day at 10.5°C)

TABLE-3

Expt. # : V79/Cs-137 acute **Date/Time :** 4-6-2001

Plate for M per P (ml)

Tube #	Coulter count for 100 µl cell suspension	Avg. count − 13 (Background)	Cells/ml [Avg. count x 400]		
1	5609 5468 5562	5533	2 213 333		0.0
2	6538 6596 6634	6576	2 630 533		0.0
3	6586 6628 6538	6571	2 628 400	5 256 800	0.0
4	6639 6918 6735	6751	2 700 400		0.0
5	6245, 6282, 6161	6216	2 486 533	4 973 067	0.08
6	6956 6709 6923	6863	2 739 867		0.07
7	6367 6431 6532	6430	2 572 133		0.08
8 →	4597 4525 4148	4410	1 764 133	5 292 400	0.11
9	6452 6373 6309	6365	2 546 000		0.08
10	6655 6810 6805	6744	2 697 467		0.07

IN 3 ml ←

Bkgr. - 13
Pellet - 500 µl

Sample #	Count	Total (in 2ml) x10^6	5x10^5/P100 (volume)
1	360	7.2	~~0.069~~ 0.139
2	339	6.7	0.150
3	80	1.6	0.690
4	330	6.6	0.152
5	94	1.9	0.53
6	240	4.8	0.210
7	260	5.2	0.192
8	337	6.6	0.148
9	290	5.8	0.172
10	306	6.1	0.167

Replating 1st on Apr. 9, 2007

cell. 9~~73~~ 4954977

B019655

Experiment:
Date/Time: Apr. 2, 2001

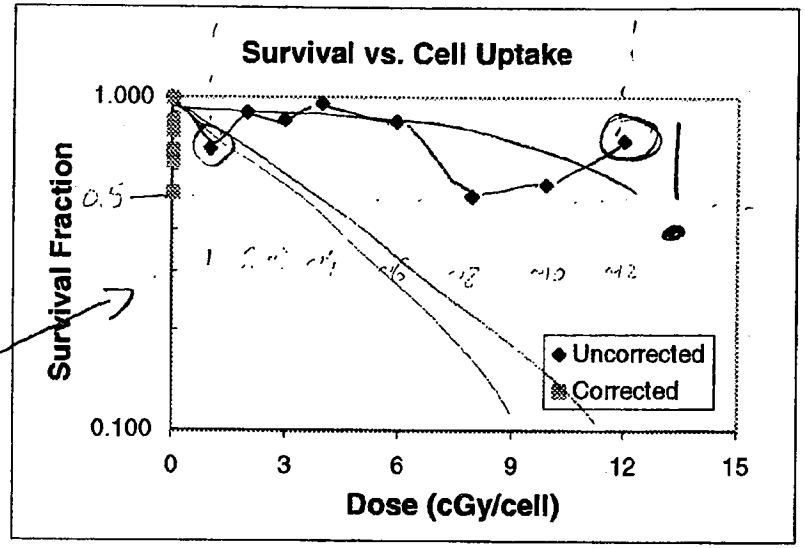
Tube #	Activity Conc. (kBq/ml)	Activity/Cell (mBq/cell)	Survival Uncorrected	Survival Corrected
1	*0.000*	*0.000*	*1.0000*	*1.0000*
2	*0.000*	*0.000*		
3	#DIV/0!	#VALUE!	0.6990	0.6441
4	#DIV/0!	#VALUE!	0.9029	0.8098
5	#DIV/0!	#VALUE!	0.8544	0.8322
6	#DIV/0!	#VALUE!	0.9612	0.8496
7	#DIV/0!	#VALUE!	0.8447	0.7953
8	#DIV/0!	#VALUE!	0.5049	0.6931
9	#DIV/0!	#VALUE!	0.5437	0.5172
10	#DIV/0!	#VALUE!	0.7379	0.6625

	Dose (Gy)	SE
1(contr	0	1
2 (contr	0	1
3	0.99	0.6990291
4	1.98	0.9029126
5	2.97	0.8543689
6	3.97	0.961165
7	5.95	0.8446602
8	7.93	0.5048544
9	9.91	0.5436893
10	11.99	0.7378641

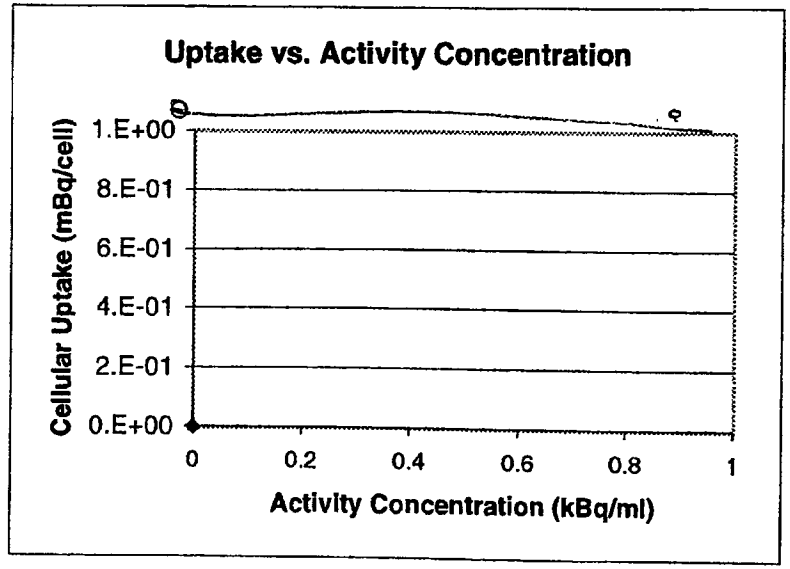
IRRADIATING TIME FROM

+ 48 min 0

FT 10.5 °C



Is that hypoxic effect involved in this experiment ?



B019656

~~74×10⁴~~ → 10× (1)

10× (1)

SF- → 203 µl

+

69 } /2 = 74×10⁴/ml
79 }

(1500 - x → 203
dilute 10× → 20.3 µl

3×100 = 300 } 900
3×200 = 600 }

~~2000~~ = P60' = 4ml

~~900~~

900 ± 12+6 = 18

900 = 18

4 ml ⇒ 200/P 600'

900-18

~~2000~~ -

1500 → in 30ml

200 = 4ml/P60' 100 = 2ml/P60

74×10⁴ → 10× → 10× →

take 203 µl + 29.8ml MED

2ml = 100cell 4ml = 200

↓

+2ml medium

30ml S-MEM

① 30ml - SEM
② 3ml FCS
0.3ml Lipids
0.3ml P/S

~~A~~ —
1, 2] CONTROLS
3, 4, 5, 6, 7, 8, 9, 10] Cs-137 (doses)] 10.5°C
11, 12] CONTROLS — ROLL THEM OVERNIGHT

13 - Control - ~~w/o DO~~ AFTER MICROWAVESTING THREE CELLS

A – 162×10^4 × 20ml – [illegible]

B – 236×10^4 × 20ml – 47.2

C – 168×10^4 × 20ml – 33.6

32.4
33.6
66.0 ×10^6 — 10 — ~~6.6ml~~ — 60 → 9.09

60. ×10^6 – 15 ml

5.91 (SMEP)

13×4×10^6

15×4=

60 – 15ml.

306
322
628/2 = 3.14/ml

3.14 – 1ml
4.00 – x = 1.27

1.27 (4×10^6 cells) ~~[illegible]~~ SMEP

ROLL

18.00 2.1V

14.4

9:00 3.V

V79 / Cs-137

Propagate

28 - 29 III → UNDER HIT

30 - III - split → NO HIT

31 - III - split → NO HIT

4-01-2001 — 30.5 × 10^6 / flask (100% confluency)] → plate 10 × 10^6 into A, B, C
30.5 × 10^6 / flask (100% confluency)

04.2001 — A - 162 × 10^4/ml × 20 ml = 32.4 × 10^6 cells

B - 236 × 10^4/ml × 20 ml = 47.2 × 10^6 cells

a) C - 168 × 10^4/ml × 20 ml = 33.6 × 10^6 cells.

A+C = 32.4 + 33.6 = 66 × 10^6 cells.

66 × 10^6 (pellet) → + 10 ml medium (mix) → take 9.09 ml = 60 × 10^6 cells / 9.09 ml

+ 5.91 S-MEM → 60 × 10^6 / 15 ml ≈ 4 × 10^6/ml

COUNT ← 306 / 322 ← 2/628 ← 3.14 × 10^6 / ml

3.14 × 10^6 - 1 ml

4 × 10^6 → x ml → 1.27 ml → into tube + 0.73 ml S-MEM

Roll tubes in 37°C - 18:00 ~~/4/02/01~~ → 14/03/01 DAY

b) plated 3 × 100 cells / P 60] for PE.
3 × 200 cells / P 60

c) plated A1 - A3, B1 - B3, C1 - C3 } app. 5 × 10^4 cells for ~~dose~~ exp. with different conc/time cytochalasin B

Results of survival

(A) Control 1 → cells were harvested and plated for SF, s
same cells were used for rolling & the irradiation with Cs-137

Day	Plated	Colonies			
4-2-2001	100	119	108	119	(A)
4-2-2001	200	221	217	237	(B)

Procedure: 7.4×10^4 cells/ml
↓ 10x dilute
↓ 10x dilute
7.4×10^2/ml
↓
0.203 ml (~1500 cells) + 29.8 ml MEM

2 ml/P60' x3 + 2 ml MEM → 100 cells/P60' (A)

4 ml/P60 x 3 → 200 cells/P60' (B)

(B) Control for sample 11 → this is control for SF after overnight (~10) rolling at 37°C

Day	Sample #	Plated cells	Colonies		
4-3-2001	11	100	166	100	141

500 – Bch-47 | 50 | 50 | 50 – Bch – 4
5.0 – 99.9 | 99.9 | 5.0 – 59.9 | 29.9 | 19.9 | 9.9

2600	2633	254	132	114	112	37
	2644	288	156	125	90	55
	2591	232	185	116	101	40

1.030286

1.543406

1.03×10^6

~~0.5×10^6~~

0.2×10^6

0,19 /ml + HPRT.

500	50	50	50	50	50
5.0	50	5.0	50	5.0	5.0
99.9	999	9.9	59.9	89.9	29.9
479	424	64	312	330	204
[illegible]	480	83	313	350	
484	367	82	281	340	

V79 – Bch – 10

7.4×10^6/ml
Haemocyt.

1.932667 / ml | 1614667 / ml

x 2 | x 2

3865 333 | 3229333

① $1. \times 10^6$/ml →
10x ④
0,1 ml – 10^5
↳ 10x ⑦ → 10^4
10x ⑧ → 10^3 → 1000/ml (200) → 0,2 ml = 200
10x ⑨ → 10^2 → 100/ml
↳ 3x100

CoulterSurvival

riment: 0
e/Time: Apr. 2, 2001

Tube #	Coulter count 1st	2nd	3rd	Average	Cells/ml	Hemocytometer Count in Grid 1st	2nd	3rd	4th
						total # of cells/tube (in 2ml)			
1	5609	5468	5562	5546	2213333	4.42×10^6			
2	6538	6596	6634	6589	2630533	5.26×10^6			
3	6586	6628	6538	6584	2628400	5.24×10^6			
4	6639	6918	6735	6764	2700400	$5,40 \times 10^6$			
5	6245	6282	6161	6229	2486533	$\sim 5.0 \times 10^6$			
6	6956	6709	6923	6863	2739867	5.48×10^6			
7	6367	6431	6532	6443	2572133	$5,14 \times 10^6$			
IN 3ml ← 8	4597	4525	4148	4423	1764133 ×3ml	$5,28 \times 10^6$			
9	6452	6373	6309	6378	2546000	$5,08 \times 10^6$			
10	6655	6810	6805	6757	2697467	5.38×10^6			

NOTE! It seems that cells are propagated after 16 hrs

4×10^6 seeded → (4.42) $5.0 \div 5.4 \times 10^6$ – cells are propagated

Tube #	Predicted # Cells Seeded	Actual # Cells Seeded	Colony count 1st	2nd	3rd	Average	PE (%)	SF Uncorrected	SF Corrected
Control 1	200	221	29	42	31	34	14.176	1.00	1.0000
2	200	263	43	32	29				
3	200	263	21	31	20	24	9.131	0.6990	0.6441
4	200	270	27	37	29	31	11.480	0.9029	0.8098
5	200	249	35	25	28	29	11.797	0.8544	0.8322
6	200	274	43	34	22	33	12.044	0.9612	0.8496
7	200	257	17	29	41	29	11.275	0.8447	0.7953
8	200	176	19	23	10	17	9.825	0.5049	0.6931
9	200	255	19	24	13	19	7.332	0.5437	0.5172
10	200	270	31	19	26	25	9.392	0.7379	0.6625

1. Colonies were fixed & stained on Friday, April 13, 2001. e.g. one week from the day the cells were plated.
2. Very low PE for tube 1 & 2 → question – is that possible that V79 cells can die when they are in cluster for 72h at 10.5°C?

B019663

TABLE-4

START.

Expt # : V79, Cs-137 acute, 100% clus. **Date :** April, 2, 2001

PE

Tube.dilution	Colony 1	Colony 2	Colony 3	Avg Colony	SF NON CORRECTED
Controls 1.2/10000	29	42	31	34	1
2.2/10000	43	32	29		
3.2/10000	21	31	20	24	0,70
4.2/10000	27	37	29	31	0,90
5.2/10000	35	25	28	29	0,85
6.2/10000	45	34	22	33	0,96
7.2/10000	17	29	41	29	0,85
8.2/10000	19	23	10	17	0,51
9.2/10000	19	24	13	19	0,54
10.2/10000	31	19	26	25	0,74

For tub 8, 9, 10 also 1000 x diuled cells were plated for survival.

					PE
8.3/1000	142	131	140	138	14%
9.3/1000	151	172	150	158	16%
10.3/1000	154	152	164	157	16%

Note:
For all samples (e.g 8.3, 9.3, & 10.3) number of colonies is not very acurate. Some of colonies were attached each other. Therfore, counts are rather conservative.

Sample #	Count $\times 10^4$ in 2.5ml	Total # of cells 10^6 in 2.5ml	Volume for 5×10^5 cells / P100's
1	51×5	6.4	0.195
2	60×5	7.5	0.167
3	45×5	9.4	0.133
4	69×5	8.6	0.145
5	73×5	9.1	0.137
6	73×5	9.1	0.137
7	98×5	12.3	0.102
8	52×5	6.5	0.192
9	80×5	10.0	0.125
10	80×5	10.0	0.125

Replating Tmrw
April 12, 2007

57_155.

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[illegible] clone production with HeLa cells in tissue culture. The use of X-irradiated cells to s
S.A. **41** (1955), pp. 432_437.

[illegible]

3μg/ml - CHO

5μg/ml - PL61 ⇒ CHO clone + single copy of plasmid cr1A neo + gpt

Set / Dish	24h A	48h B	72h C	
1	←	1μg/ml →		2 μl/dish
2	←	3μg/ml →		6 μl/dish
3	←	6μg/ml →		12 μl/dish

−1 day − plated ~ $\frac{5\times10^4}{P60}$

0 day = Mgn. 3

STOCK cr1 B = 2μg/μl

V79 COLONY FORMING ASSAY

Experiment Name : 100cluster, no labeling); **Exp.**
Investigator: M.Lenarczyk **Date:**
4/2/2001

1. Set the rocker-roller at 37°C incubator with 5% CO_2, set the Coulter Counter, wash cells (from two 150 cm^2 flask, sub-cultured 1:2, 24h before) with PBS, trypsinize cells, each resuspend in 9 ml MEMB, pool, pass 5x through 3 cc syringe with 21 gauge needle, count the cells 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 : 4×10^6 cells/ml)
   Flask A+C = $(32.4+33.6)\times10^6$ cells = 66×10^6 cells
   66×10^6 cells + 10ml S-MEM → take 9.09 ml (~ 60×10^6) + 5.91 ml S-MEM = 60×10^6/15ml
   After checking → $\frac{306}{322}$ → 628/2 → 314×10^4/ml, → take 1.27ml + 0.73ml S-MEM (~ 4×10^6/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 overnight at 37°C, 5% CO_2 **Date/Time: April 02, 2001 / 18:00**
   Make a table with # of samples.
5. After overnight incubation period (~~16.5~~ hrs), remove tubes and centrifuge at 2000 rpm at 4°C for 10 min (***precooled centrifuge***). **Date/Time: April 3, 2001 : 10:30**
   → should be 3hrs + 12 hrs = 15 hrs.
~~6. Remove buckets from centrifuge and carefully remove 150 µl of supernatant and place in prelabeled gamma-tube.~~

V 0
7. Decant supernatant, click tubes, vortex, resuspend in 10 ml wash MEMA
8. Centrifuge tubes for 10 min at 2000 rpm, 4°C

V 1
9. Decant supernatant, click tubes, vortex, resuspend in 10 ml wash MEMA
10. Centrifuge tubes for 10 min at 2000 rpm, 4°C

2
11. Decant supernatant, click tubes, vortex, resuspend in 10 ml wash MEMA
12. Centrifuge tubes for 10 min at 2000 rpm, 4°C

3
13. Decant supernatant, click tubes, vortex, resuspend in 7 ml of MEMA
14. Centrifuge tubes for 10 min at 2000 rpm, 4°C
15. 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
16. Again add 200 ul ice cold MEMA, resuspend and transfer the cell suspensions in the same polypropylene microcentrifuge tubes (Total volume ~400 ul)
17. Centrifuge tubes for 5 min at 1000 rpm, 4°C
18. Transfer tubes at 10.5°C for 72 h. **Date/Time: April 3, 2001 /**

→ sample 11 – control for DE + HPRT background + MN(?) →
AFTER ROLL OVERNIGHT –
# of cells after roll is →

100% cluster

1 - control
2 - control
3
4
5
6
7
8
9
10

2 ml → cell suspension
↓ -0,5 ml
x5 0,5 ml + 4,5 ml medium (10×)
↓ -0,5 ml
x4 0,5 ml + 4,5 ml medium (100×)
↓ -0,5 ml
x3 0,5 ml + 4,5 ml medium (1000×
↓ -0,5 ml
x2 0,5 ml + 4,5 ml medium (10000×)

9,3

10,2 (= 10,3)

151
172
150
473 : 3 = 157,
3
17
15
23
21
20
≈ 158

154
152
164
470 : 3 = 156
3
17
15
20
18
20

256 - 100
158 -

$\bar{x} - \frac{158}{255} \times 100$

$\bar{x} - \frac{157}{270} \times 100 =$

158

158 158

255 - 100
158 - x

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