

8k

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1 Aim

Establish the optimal number of B16-F1-Luc2-BR2 cells with which to seed a growth curve.

The goal is to demonstrate steady growth of the cells during the 5 day period of the experiment.

This will be used to inform future studies of agents which may inhibit growth.

We begin with 8×10^3 cells/ 12-well plate.

2 Materials

- **B16-F1-Luc2-BR2** cells.

These were generated as follows:

- B16 cells were obtained from American Type Culture Collection (ATCC).
- To facilitate quantitative measurement of tumor growth, they were modified as described previously [1].

The cells were stably transfected with the gene encoding luc2 (luciferase) using the pGL4.51 [luc2/CMV/Neo] vector (Promega Corp, Madison, WI) and FuGENE 6 Transfection Reagent (Roche Applied Science, Indianapolis, IN) following conditions specified by the manufacturer.

- They were then injected into the right ventricle of a mouse.
These animals were sacrificed when bioluminescence was detected in the brain.
- Cells metastatic to the brain were recovered put into culture.

- 12-well plastic culture plate (Falcon®).
- Media: DMEM (Gibco ®) +10% FCS + 600 µg/mL G418 + 1x glutamine. pH 7.4.
FCS = Fetal Calf Serum.
- Trypsin/ EDTA.
EDTA = EthyleneDiamineTetraAcetic acid.
- Hemocytometer (manual and automated (Countess™ by Invitrogen™)).
- 15 mL plastic centrifuge tube (VWR™).
- Other supplies:
 - Trypan blue stain 10%.
 - Eppendorf tubes (plastic, sterile).
 - PCR tubes (for mixing cells and Trypan blue).
 - Disposable slides for hemocytometer.

3 Methods

3.1 Preparatory work

On day -3, pass the cells at a ratio of 1 : 4.

I.e. add 0.4 mL from the original flask to 1.6 mL of new media.

The T-25 flask is shown (prior to cell passage) in figure 1.

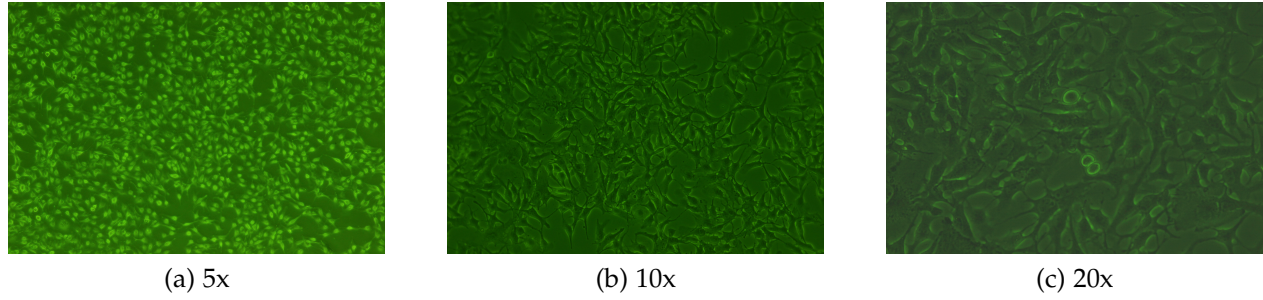


Figure 1: Visual confluency of cells in T-25 flask at various magnifications

Count the cells. The results are shown in table 1.

Based on this the automated counter was thought to be reliable for this purpose.

For a seeding density of 1.2×10^4 per well, approximately $12 \times 1.2 \times 10^4 = 1.44 \times 10^5$ cells are required.

This is $\frac{1}{8}$ to $\frac{1}{10}$ of one T-25 flask.

Method	Trial	Cells/ mL	Cells in flask (2 mL)
Manual	1	4.04×10^5	8.08×10^5
	2	4.72×10^5	9.44×10^5
Automated	1	4.3×10^5	8.6×10^5
	2	3.2×10^5	6.4×10^5

Table 1: Counts of viable cells by method

3.2 Day 0

Monday 3rd February, 2014

1. Cell counts from the T-25 flask are shown in table 2.

The mean from the 3 counts is thus 4.93×10^5 mL.

Trial	Cells per mL
1	3.92×10^5
2	3.06×10^5
3	7.82×10^5

Table 2: Cells counts on day 0

Seed a 12-well plate with 8×10^3 cells in 1 mL of media.

For 8×10^3 cells, this is $\frac{8}{493} = 0.0163$ mL = 16.3 μ L.

Add $13 \times 16.3 \mu\text{L} = 0.212$ mL to a 15 mL centrifuge tube and add media to make a total volume of 13 mL.

Mix well. Add 1 mL to each of the 12 wells.

2. Shake the plates gently to evenly distribute the cells.
Return to the incubator at 17:30.
3. Incubate at 37 °C, in 5% CO₂ for 24 h.
4. Pass the remaining cells at 1 : 3.
I.e. take 0.5 mL of media with cells from the T-25 flask and add 1.5 mL of new media.

3.3 Days 1-4

1. For plates which are not being counted:
Aspirate the old media aspirated replace with 1 mL of new media.
2. For plates to be counted:
Add 1 mL of trypsin/ EDTA to each in the row to be counted.
Rock the plates.
Remove the trypsin/ EDTA after <30 s.
3. Add 250 µL of trypsin/ EDTA.
4. Incubate at 37 °C for 3 min.
5. Shake the plate to ensure all cells are dislodged from the base.
Check with the microscope.
6. 'Quench' the trypsin/ EDTA quenched with 250 µL of media.
Pipette up and down ×5 to ensure the cells are thoroughly mixed.
7. Remove 10 µL of cell suspension and add 10 µL Trypan Blue in a sterile PCR tube.
Mix using a pipette.
8. Place 10 µL of the mixture into a sample well of the hemocytometer.
9. Count the cells using the hemocytometer.
These counts are shown in table 3.
10. Return 12-well plates to the incubator.

4 Results

Day	Well	Trial	Cells/ mL	Mean of 2 readings
1	A	1	3.28×10^5	193×10^3
		2	0.66×10^5	
	B	1	1.72×10^5	148×10^3
		2	1.24×10^5	
	C	1	1.20×10^5	159×10^3
		2	1.98×10^5	
2	A	1	0.7×10^5	78×10^3
		2	0.86×10^5	
	B	1	0.52×10^5	63×10^3
		2	0.78×10^5	
	C	1	0.46×10^5	79×10^3
		2	1.12×10^5	
3	A	1	0.26×10^5	33×10^3
		2	0.4×10^5	
	B	1	0.46×10^5	56×10^3
		2	0.66×10^5	
	C	1	0.066×10^5	33×10^3
		2	0.6×10^5	
4	A	1	0.22×10^5	13×10^3
		2	0.04×10^5	
	B	1	0.22×10^5	15×10^3
		2	0.08×10^5	
	C	1	0.04×10^5	10×10^3
		2	0.16×10^5	

Table 3: Cells counts day 1 - 4

5 Code

5.1 data

```
(df1 <- data.frame(Day = rep(0:4, each=3),
  Well = rep(LETTERS[1:3], 5),
  Count = c(rep(8e3, 3),
    193e3, 148e3, 159e3,
    78e3, 63e3, 79e3,
    33e3, 56e3, 33e3,
    13e3, 15e3, 10e3)))
```

```
##      Day Well Count
## 1      0    A  8000
```

```
## 2      0      B      8000
## 3      0      C      8000
## 4      1      A     193000
## 5      1      B     148000
## 6      1      C     159000
## 7      2      A      78000
## 8      2      B      63000
## 9      2      C      79000
## 10     3      A      33000
## 11     3      B      56000
## 12     3      C      33000
## 13     4      A      13000
## 14     4      B      15000
## 15     4      C      10000
```

5.2 stdError

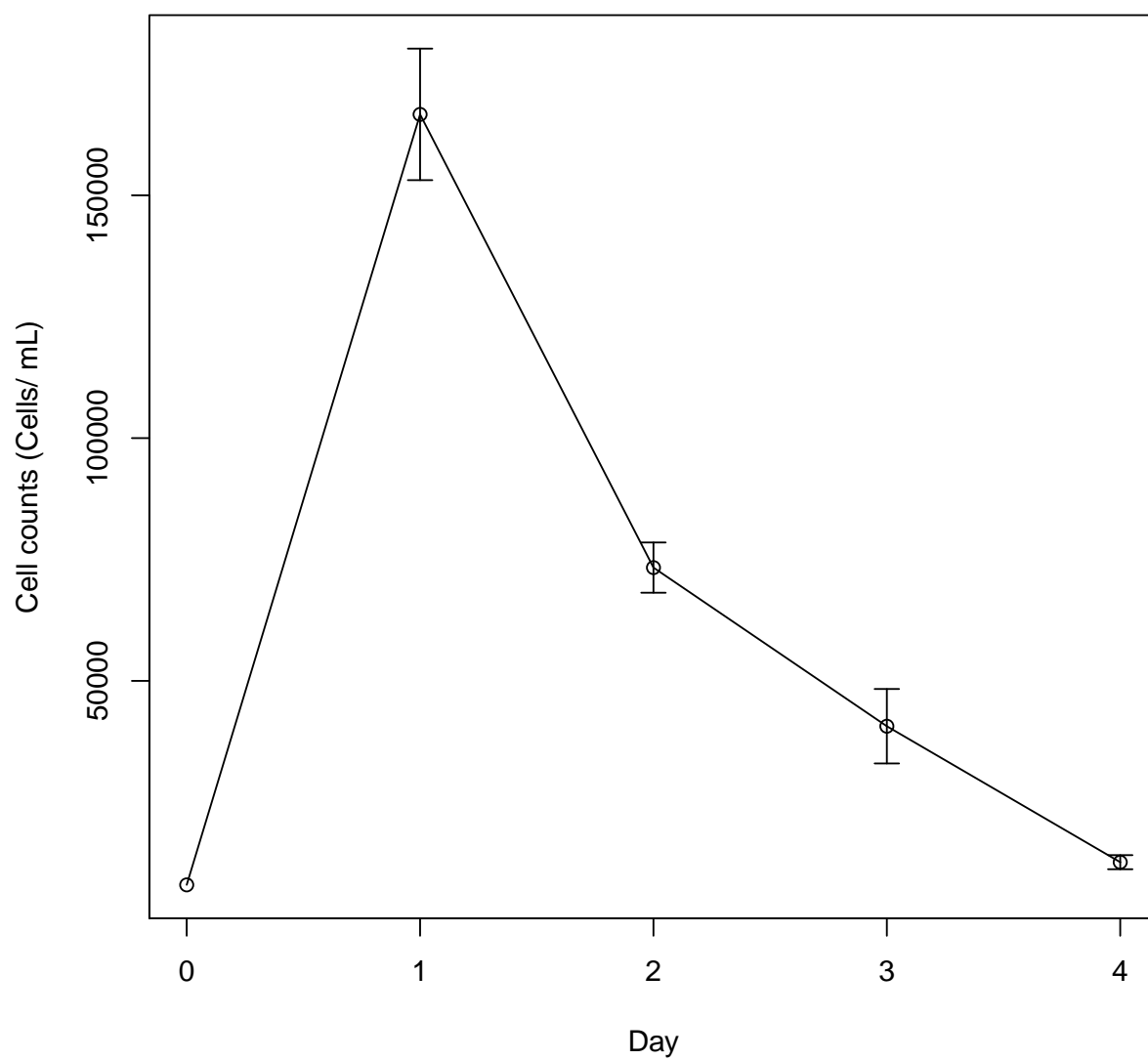
```
stdErr <- function(x) sqrt(var(x)) / sqrt(length(x))
library(plyr)
(df2 <- ddply(df1, .(Day),
              function(df)
                c(mean = mean(df$Count),
                  SE = stdErr(df$Count),
                  'mean+SE' = mean(df$Count) + stdErr(df$Count),
                  'mean-SE' = mean(df$Count) - stdErr(df$Count))))
```

##	Day	mean	SE	mean+SE	mean-SE
## 1	0	8000	0	8000	8000
## 2	1	166667	13544	180211	153123
## 3	2	73333	5175	78508	68159
## 4	3	40667	7667	48333	33000
## 5	4	12667	1453	14120	11214

5.3 plot

```
library(plotrix)
# confidence interval plot
with(df2, plotCI(x=Day, y=mean, ui=mean+SE, li=mean-SE,
                 xlab="Day",
```

```
ylab="Cell counts (Cells/ mL)",  
axes=FALSE))  
axis(side=1, at=c(0:4))  
axis(side=2, at=seq(0, 150e3, by=50e3))  
box()  
with(df2, lines(Day, mean))
```



6 Conclusion

Although the cells show vigorous growth initially, this is not sustained over the 5 day period of observation.

References

- [1] Abdelwahab, Mohammed G., et al. The ketogenic diet is an effective adjuvant to radiation therapy for the treatment of malignant glioma. PloS one 7.5 (2012): e36197.