

16k

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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.

In previous work, we have shown that 8×10^3 cells/ 12-well plate is insufficient to show sustained growth over 5 days. We now use 16×10^3 cells.

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.
- 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 Day 0

Monday 10th February, 2014

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

Cell counts on Day 0

```
df1 <- data.frame(Cells_per_ml = c(392e3, 332e3, 300e3))
library(xtable)
print(xtable(df1,
              caption="Cell counts on day 0",
              align=c("l", "c"),
              display=c("d", "e"),
              label="tab:ccd0"),
      booktabs=TRUE)
```

	Cells_per_ml
1	3.92e+05
2	3.32e+05
3	3.00e+05

Table 1: Cell counts on day 0

Mean

```
(m1 <- mean(df1$Cells_per_ml))
```

```
[1] 341333
```

The mean from the 3 counts was thus 340×10^3 mL.

Seed a 12-well plate with was 16×10^3 cells in 1 mL of media.

For 16×10^3 cells, this is $\frac{16}{340} = 0.047$ mL.

Add 13×0.047 mL = 0.61 mL to a 15 mL graduated cylinder and top up to 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.

3.2 Days 1-4

1. For plates which are not being counted:
Aspirate the old media and replace with 1 mL of new media.

2. For plates to be counted:
Add 1 mL of trypsin to each in the row to be counted.
Rock the plates.
Remove the trypsin after <30 s.
3. Add 250 μ L of trypsin.
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 quenched with 250 μ L of media.
Pipette up and down $\times 5$ to ensure the cells are thoroughly mixed.
7. Remove 10 μ L of cell suspension and add 10 μ L Trypan Blue in a sterile PCR tube.
Mix using a pipette.
8. Return plates to incubator.
9. Place 10 μ L of the mixture into a sample well of the hemocytometer.
10. Count the cells using the hemocytometer.
These counts are shown in table 2.

4 Results

```
df1 <- data.frame(Day = rep(0:4, each=3),
                  Well = rep(LETTERS[1:3], 5),
                  Count = c(rep(16e3, 3),
                             38e3, 27e3, 13e3,
                             43e3, 32e3, 33e3,
                             134e3, 32e3, 17e3,
                             194e3, 79e3, 83e3))
print(xtable(df1,
              caption="Cells per ml, day 0 - 4",
              align=c("l", rep("c", 3)),
              display=c("d", "d", "s", "fg"),
              label="tab:data"),
      booktabs=TRUE)
```

4.1 Standard Error

	Day	Well	Count
1	0	A	16000
2	0	B	16000
3	0	C	16000
4	1	A	38000
5	1	B	27000
6	1	C	13000
7	2	A	43000
8	2	B	32000
9	2	C	33000
10	3	A	134000
11	3	B	32000
12	3	C	17000
13	4	A	194000
14	4	B	79000
15	4	C	83000

Table 2: Cells per ml, day 0 - 4

```
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)))
print(xtable(df2,
  caption="Mean counts, with standard errors",
  align=c("l", rep("c", ncol(df2))),
  display=c("d", rep("fg", ncol(df2))),
  label="tab:means"),
  booktabs=TRUE,
  include.rownames=FALSE)
```

4.2 Plot

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

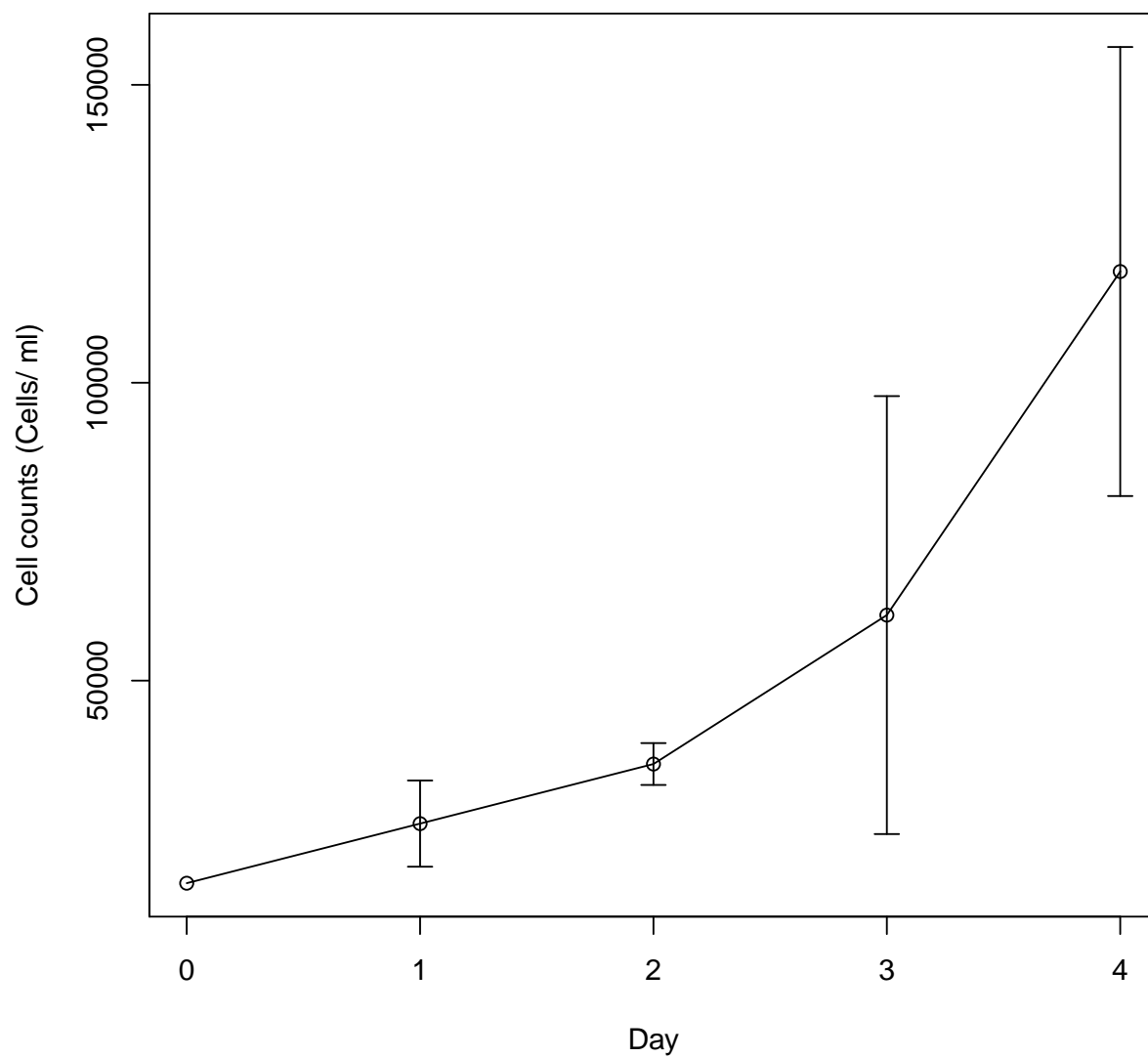
Day	mean	SE	mean+SE	mean-SE
0	16000	0	16000	16000
1	26000	7234	33234	18766
2	36000	3512	39512	32488
3	61000	36756	97756	24244
4	118667	37684	156351	80982

Table 3: Mean counts, with standard errors

```

        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))

```



5 Conclusion

A starting number of 16×10^3 cells appears more promising than 8×10^3 cells in terms of demonstrating sustained growth over the period of observation. See `8k.pdf` for comparison.

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.