

COC Biotechnology Program



Sterile Techniques, Tissue Culture and Cell Counting

Version 12-7-03

- **Tissue culture is a technique used by scientists to grow cells from various tissues in a laboratory setting.**
- **In this experiment you will propagate your own cell line for three weeks, conducting bi-weekly propagation as well as quantifying and characterizing your cell culture.**
- **The type of cells you will be working with are insect cells—they are easy to obtain and can grow under a variety of conditions (unlike their mammalian counterparts).**
- **Cell cultivation requires stringent protocols and sterile technique. Therefore, in addition to the propagation of your cell line you will become familiar with these techniques and protocols.**
- **You will also record and graph the health of your culture by using an instrument called a hemocytometer to count both living and dead cells in your cell line.**
- **Finally, in conjunction with the additional labs you will determine the average protein concentration of the cells in your culture.**

For more information on the College of the Canyons Biotechnology program contact Jim Wolf, Associate Professor of Biology/Biotechnology at (661)362-3092 or email: jim.wolf@canyons.edu

I. Objectives:

1. To become proficient at sterile technique.
2. To understand the importance of tissue culture in the field of molecular biology.
3. To successfully propagate your own insect cell line for three weeks.
4. To become familiar with a hemocytometer and be able to use it to quantify living and dead cells in your cell line.

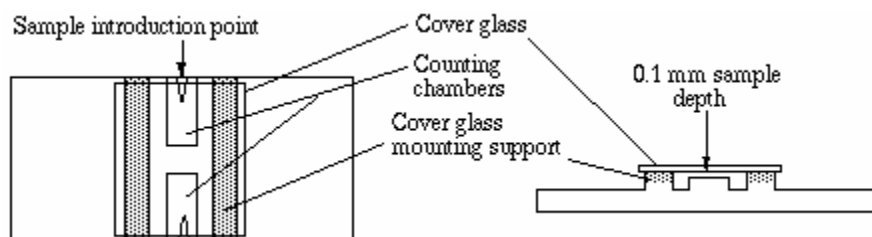
II. Background:

Over the next few weeks we will be learning the art (oops, I meant science) of tissue culture. Many of the most significant advances in the field of molecular biology have been made via tissue culture techniques. I say art of tissue culture because a lot of what has been learned is through trial and error and serendipity. Each organism has unique tissues, and so the tissue culture techniques are both organism and tissue specific.

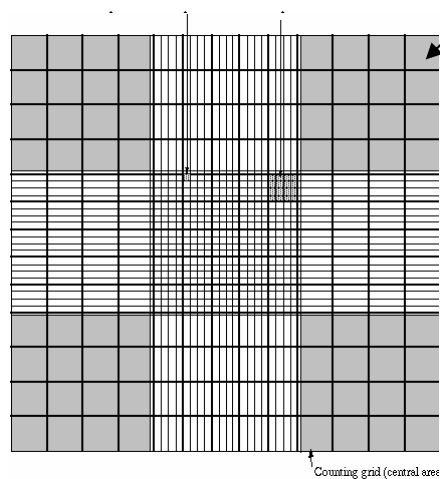
Once a tissue culture protocol has been worked out it is rarely changed. Some tissue culture techniques and actual cell lines date back over fifty years! There are three broad categories of tissue culture: plant, mammalian and other. Mammalian tissues are arguably the most difficult, for these cells require a constant stream of CO₂ gas in addition to the normal cell requirements.

The group of cells we will be working with are insect cells. They are easy to obtain and can grow under a variety of conditions. The first few steps in becoming competent in working with cell cultures is to practice sterile technique, propagate a cell line and inspect the line for health and growth. To accomplish this, each of you will propagate your own cell line for three weeks, conducting bi-weekly propagation as well as quantifying and characterizing your cell culture. In addition to this you will record and graph the health of your cell culture.

To count cells, a hemocytometer is used. The hemocytometer is a slide with grids inscribed on it. These grids are a set distance apart and create many small squares. If we count the number of cells in a particular square, and know the volume of the square, it will give us the number of cells per unit volume.



Ultimately you want to know #cells/ml. You will be counting cells in any of the larger corner grids, labeled gray in the illustration.



Each small square is 0.25mm wide with a depth of 0.1mm. Thus, the volume of each square is:

$$0.25\text{mm} \times 0.25 \text{ mm} \times 0.1 \text{ mm} = 0.00625 \text{ mm}^3$$

At least ten squares should be counted. If you count 700 cells in ten squares, then the average is 70 cells per square:

$$\frac{700\text{cells}}{10\text{squares}} = 70 \frac{\text{cells}}{\text{square}}$$

Now simply use dimensional analysis to determine the number of cells per mL:

$$\frac{70 \text{ cells}}{1 \text{ square}} \times \frac{1 \text{ square}}{0.00625 \text{ mm}^3} \times \frac{1000 \text{ mm}^3}{1 \text{ cm}^3} \times \frac{1 \text{ cm}^3}{1 \text{ ml}} = 1.12 \times 10^7 \frac{\text{cells}}{\text{ml}}$$

To check for cell viability the dye Trypan Blue is used. A healthy cell will have a vital cell membrane capable of keeping out many organic macromolecules. Healthy cells **exclude** Trypan Blue, but once the cell dies, the membrane is no longer capable of keeping the dye out, making it appear blue or dark (although the cell may otherwise appear fine).

Note: Light intensity is very critical when assessing cells with Trypan Blue. Every effort should be made to optimize the lighting in the microscope to maximize contrast. It is very rare that a sample will not have any dead cells in it, so double check your light environment to ensure that the blue dyed cells are indeed visible.

Practice Exercise: Working from provided stock, count cells as described in steps 8.31 - 8.34. For comparison, look at provided dead cells to ensure you can see blue coloration under the microscope.

III. SOP/Lab Activities:

Important: Always note any addenda that are posted by the instructor.

1. **Scope:** Maintain continuous culture for tissue culture experiments.
2. **Definitions:** Inoculum: Portion of old culture that is added to new media to encourage growth.
3. **References:** SOP Manual, Biotechnology 230, College of the Canyons
4. **Reagents/ Supplies:**
 - 4.1 Stock tissues culture (*Drosophila sp.* Cell suspension.)
 - 4.2 50 ml suspension culture flask
 - 4.3 1 ml disposable transfer pipette
 - 4.4 10 ml transfer pipet
 - 4.5 pipette pump
 - 4.6 isopropyl alcohol in spray bottle
 - 4.7 tissue culture hood
 - 4.8 Stock Hyband tissue culture broth
 - 4.9 2 - 1.5 mL labeled microfuge tubes

5. Responsibility:

- 5.1 Transfer and propagation of cell line is the responsibility of students in Biotechnology 230.

6. Hazard Communication

- 6.1 **Danger:** Isopropyl Alcohol: Flammable and irritant if inhaled.
- 6.2 **Danger:** UV Light (avoid prolonged exposure to skin and eyes).

7. Attachments: Verification ledger: Form 101-TC (posted on fume hood)

8. Procedure:

8.1 Sterile field preparation: *Note: The first person to propagate should set up enough equipment for the entire class. The last person should clean out the hood and replace any unused equipment.*

Note that you will be propagating two lines. *One will be transferred every day that you do transfers, and the other will be transferred every other day (so effectively once a week). For the samples that are transferred every day, the inoculum will be increased to 1.0 ml (opposed to 0.5 ml for the weekly propagated cell line).*

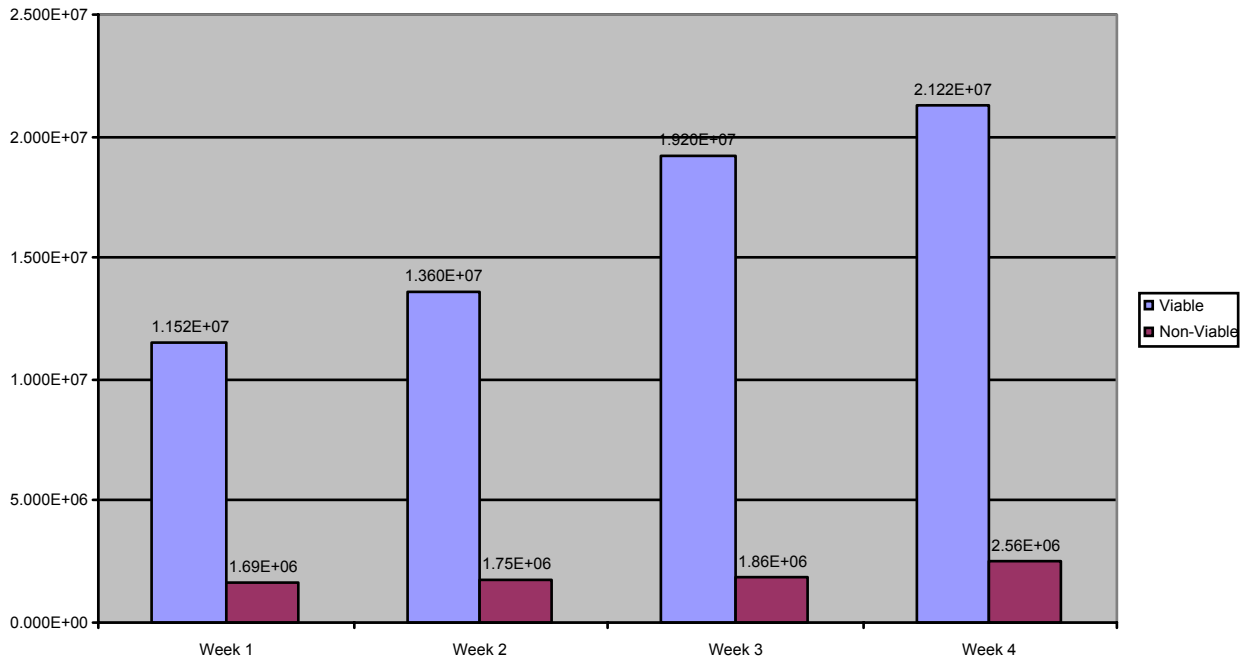
- 8.11 Place tissue culture flasks, a disposable transfer pipettes, 10 ml transfer pipettes and pipette pump into the culture hood. Spray down with isopropyl alcohol and illuminate hood with UV light source. Let dry.
- 8.12 You will be assigned a stock culture and media solution for your use only. Label them clearly and keep track of them.

8.2 Cell line propagation:

- 8.21 Remove tissue culture media from refrigerator. Get stock culture and **inspect stock culture under tissue culture microscope to ensure no contamination.** While covering cap so no alcohol gets in, spray both down with isopropyl alcohol. Put into culture hood, turn **off** UV light and let dry.
- 8.22 Wait 15 minutes to allow culture media to warm up to room temperature.
- 8.23 Transfer 5 ml aliquot of stock culture broth into each new tissue culture flask using 10 ml transfer pipette.
- 8.24 Inoculate tissue flasks (using only uncontaminated samples) with 0.5 or 1.0 ml of cell culture (depending on frequency of transfer (see above)). Use new transfer pipette for each inoculation.
- 8.25 Agitate the old cell flask and then aliquot a 100 μ L portion of the cell culture you just removed the sample for propagation (**not the new sample**). *Note: again, ensure that your sample is taken from your old cultures, not the new one you just made!*
- 8.26 Label flasks appropriately and clean out your debris from hood.
- 8.27 Place new cultures into incubation chamber @ 25° C and return tissue culture media to refrigerator. Save the original cultures until told to dispose of them.

Remember to identify your samples propagated weekly and biweekly.

Once you have obtained all of the data, construct a histogram like the one below for each cell line (The example is weekly – the biweekly transfer histogram will have twice the data). Collecting all of the data will take three weeks.



IV. Post-Lab Questions/Activities: The following post lab questions are for your benefit. The questions will help you to address a range of topics relating to the lab activity. Along with the post lab handouts, these questions will help to ensure that you have both correct information regarding the lab data and crucial lab processes. Complete the post lab questions at the end of the lab and post lab handouts (keys for both of these are available from your instructor) before making any lab-notebook entries.

- 1) Why is Trypan Blue used and how does it help with quantifying your cell culture?
- 2) Is there any correlation between the number of viable and non-viable cells and time since transfer?
- 3) Was there any significant change in your cell count from week to week? If so, what may have caused it?
- 4) Is there any correlation between total cell counts and time since transfer?

Hint: for questions 2-4 compare the cells transferred weekly versus the cells transferred every lab period. Also consider comparing percents as opposed to numbers (i.e. 25 % more cells are seen in 125,000 cells when compared to 100,000 cells.)

- V. **Notebook Entries:** Data from the lab should be the focus of this section and if there are any incorrect results, you should discuss this as well as expected results. Section V will contain both your results and discussion. Your data should drive the discussion. An informed discussion is dependent on understanding the post lab questions/activities.

Your intro should:

- Mention role of cell culture in modern biotech.
- Type of cell being transferred.
- Frequency and assaying using Trypan blue (3 weeks).

Results should be:

- Two histograms (one weekly, one biweekly) both showing viable and non-viable cell numbers.
- Daily count tally and final numbers should also be in this section.

Discussion should consider the following:

- Look closely at the data and see if you can note any trends.
- What are total cell numbers? Do they change in response to transfer frequency?
- Express the viable and non-viable as a percentage of total cells. Are there relationships here? Are biweekly more healthy than weekly?
- Recall that all data is suspect as you are just refining techniques.

The previous lab protocol can be reproduced for educational purposes only. It has been developed by Jim Wolf, and/or those individuals or agencies mentioned in the references.