

Protocol

The tissue culture facility in the lab can accommodate 6 students at a time. Half the class will begin by performing the tissue culture portion of today's experiment while the other half will begin in the main lab loading their samples from last lab on an agarose gel.

Part 1: Agarose Gel Electrophoresis

Load your samples on a 1% agarose gel in the following order:

Lane	Sample	Volume to load
1	Molecular Weight Marker-see Day3	5 ul
2	pCX-NNX diagnostic digest 1	25 ul
3	Candidate 1 diagnostic digest 1	25 ul
4	Candidate 2 diagnostic digest 1	25 ul
5	Candidate 3 diagnostic digest 1	25 ul
6	empty	xxx
7	pCX-NNX diagnostic digest 2	25 ul
8	Candidate 1 diagnostic digest 2	25 ul
9	Candidate 2 diagnostic digest 2	25 ul
10	Candidate 3 diagnostic digest 2	25 ul

Once all the samples are loaded, the power will be applied (100V for 45 minutes) and the gel will be photographed. While you are waiting, you can review and discuss the assigned article.

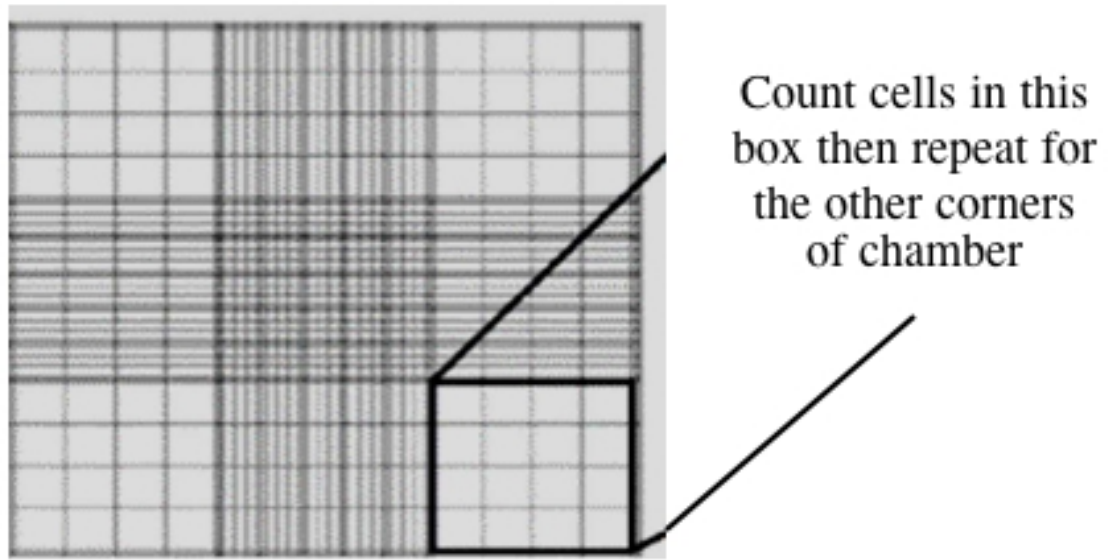
Part 2: Tissue Culture

Each of you will have a 60 mm dish of mouse embryonic stem (MES) cells that you will use to seed a six-well dish. You and your partner will seed the dishes at different concentrations so you should decide who will seed at 1:100 and who will seed at 1:400.

1. Prewarm all the required reagents in the water bath.
2. Look at your cells as you remove them from the incubator. Look first at the color and clarity of the media. Fresh media is reddish-orange in color and if the media on your cells is yellow or cloudy, it could mean that the cells are overgrown, contaminated or starved for CO₂. Next look at the cells on the inverted microscope. Note their shape and arrangement in the dish and how densely the cells cover the surface.
3. Move the cells into the sterile hood, as well as the PBS, trypsin, and media that you will need. One of the greatest sources for TC contamination is moving materials in and out of the hood since this disturbs the air flow that maintains the sterile environment inside the hood. Anticipate what you will need during your

experiment to avoid moving your arms in and out of the hood while your cells are inside.

4. Aspirate the media from the cells.
5. Wash the cells by adding 3 ml PBS with a 5 ml pipet. Tip the dish in all directions to rinse all the cells, and then aspirate the liquid out of the dish.
6. Repeat the PBS wash, leaving the cells dry for the next step.
7. To dislodge the cells from the dish, you will add trypsin, a proteolytic enzyme. Using a 2 ml pipet, add 0.5 ml of trypsin to the dish. For one minute precisely (use your timer), tip the dish in each direction to distribute the trypsin over the cells then aspirate the trypsin off the cells. Incubate the cells ("dry") at 37° for 10 minutes, again using your timer to precisely time this incubation.
8. While you are waiting, you can add 1 ml of gelatin to each well of a six-well dish. This should be done in the sterile hood with sterile technique. The gelatin will be removed before you seed the dish with your MES cells but it is important to pre-treat the dish this way. The gelatin must remain in the wells for at least 10 minutes.
9. With a 5 ml pipet, add 4 ml of media to the trypsinized MES cells and pipet the liquid up and down ("triterate") to remove the cells from the plastic and suspend them in the liquid. Remove a small amount of the liquid to an eppendorf tube and take it to the inverted microscopes.
10. Fill one chamber of a hemocytometer with 10 ul of the cell suspension. This slide has an etched grid of nine large squares. The square in the center is further etched into 25 squares each with a volume of 0.1 ul and 16 tiny chambers (4x4 pattern). The concentration of cells in a sample can be determined by counting the cells that fall within the 4x4 pattern and then multiplying by 10,000 to determine the number of cells/ml. You should count the cells in the four corner squares of the 25 square grid, then average the numbers to determine the concentration of cells in your suspension.



Counting cells using a hemocytometer

11. You and your partner will seed at different concentrations. Decide if you will try the 1:100 or 1:400 dilution and add the appropriate amount of cell suspension to 10 ml of media in a 15 ml conical tube.
12. Remove the gelatin from the six-well dish you have prepared and add 3 ml of your cell dilution to each well. Be sure to label your dish with your name, today's date, the cell line (called "J1") and the type of media you have used. Return your cells to the incubator.
13. Aspirate any remaining cell suspensions to destroy them and clean up the hood. Dispose any vessels that held cells in the Biohazard waste and any sharps in the grey bins. The next group who uses your hood should find the surfaces wiped down, no equipment left inside, the sash closed and the germicidal lamp on.