

Measurement Fundamentals

The purpose of this exercise is to review an existing method and identify the measurement fundamentals that are present. The complete list of fundamentals covered in class is listed below, not all may be present in the lab method provided.

“Lab Exercise 5: Survival Assay-Sensitivity to UV Exposure” from Basics of Cell Culture, A student laboratory manual, by Golnar Afshar, PhD will be used.

Method:

- ✓ What will be measured?

- ✓ Will these measurements be taken directly, indirectly, by ratio or by some other method?

- ✓ What would be the order of operations?

System:

- ✓ What standards will this process comply with?

- ✓ What devices/ fixtures/ instrumentation will be needed to conduct the measurement?

Standards Usage:

- ✓ What Level of standards will be needed? Where will standards be needed? How will you ensure traceability?

Confidence (Uncertainty) Programs:

- ✓ What happens if your target for accuracy/ precision has not been met?

- ✓ How will you check for error?

- ✓ What might be sources of the following error types?

Systemic Error:

Environmental Error:

Observational Error:

Gross Human Error:

Lab Exercise 5: Survival Assay- Sensitivity to UV Exposure

After plating, cells attach to the surface of the culture vessel and start dividing. A single cell divides into two daughter cells which divide into four and then eight and so forth, thus forming a **colony**. A colony refers to a population of cells that are all descendents of a single parental cell. Cells in a colony sit close to each other and if there are enough cells in a colony, they can easily be seen without the help of the microscope.

When plating, many of the cells die in the process and never reattach to the flask. Among those who do attach, some may die later. Some of the cells may stay alive but will not divide enough times to make a colony. **Plating efficiency (or cloning efficiency)** is a measurement used to identify the percentage of cells that survive and are able to divide and form colonies after being plated. Plating efficiency is a value that measures cell survival when cells are plated at low densities (2-50 cells/cm²). When cells are plated at lower densities, they are far apart and are not able to assist each other for survival. These cells are on their own, and if they survive they will divide and form colonies. Each colony represents a single cell that has managed to survive. To measure plating efficiency, cells are plated at low densities and allowed to grow 7-10 days. During this time the cells that have survived will divide and form colonies. The colonies are then counted and plating efficiency (PE) is calculated:

$$PE = \frac{\text{Number of colonies}}{\text{Number of cells plated}} \times 100$$

Plating efficiency is used to study differences in cell survival and growth rate within cells of the same population or among cells of different populations. The

number of colonies represents cell survival and the size of colonies represents growth rate. The bigger colony size can be interpreted as faster growth rate.

Plating efficiency is one of the techniques often used for **cytotoxic studies**. Cytotoxic studies involve measurement of altered metabolism or loss of viability due to a toxic factor. *In vitro* cytotoxicity studies are often used by environmental scientists and pharmaceutical companies to screen for potentially toxic drugs and environmental factors. Plating efficiency is a form of a cytotoxicity assay that measures cell viability several divisions after exposure to the toxic reagent. To study the effects of a toxic reagent or drugs on cell survival, cells are plated at low densities in separate vessels and exposed to different dosages of the reagent. After several divisions, the number of colonies is counted. PE and **surviving fraction (SF)** values are compared among cells that were exposed to different dosages and control cells with no exposure.

$$SF = \frac{\text{Number of colonies for each dosage}}{\text{Number of colonies for control cells}}$$

The lower SF values represent less survival as a result of exposure. In addition, average colony size can reveal the effect of the toxic reagent on growth rate. The SF values can be plotted against dosages to generate a survival curve.

In the following exercise you will determine the PE and SF values for cells that have been exposed to different dosages of UV (ultra violet) light. The objective is to determine the level of sensitivity of your cells to UV light. You may do the experiment with two or more cell lines and compare their UV sensitivity levels.

Ultraviolet light exposure causes formation of covalent bonds between two adjacent **pyrimidines** (C and T) in cellular DNA forming **pyrimidine dimers** (Goodsell, 2001). Pyrimidine dimers block progression of replication forks and, if left unrepaired, may lead to base deletions or substitutions and cause mutations. The mutations are subsequently propagated as the cell goes through more divisions and can have disastrous effects for the organism (for example, skin cancer). Fortunately, our cells are equipped with a number of proteins that are specialized in recognition and repair of pyrimidine dimers. Mutations in the genes that code for these proteins can make the repair mechanism inefficient and cause devastating diseases. People who are affected by these mutations are very sensitive to light exposure and develop severe sunburns and tumors and die at young ages.

In the following exercise you will compare the sensitivity levels of two cell lines to UV radiation. The following exercise will be done over a week. On the first day, you will plate your cells at low density in multi-well plates. After one or two days, the cells are exposed to several dosages of UV light. The cells are allowed to divide and form colonies for one week. The colonies are then stained and counted. Finally, you will draw a survival curve and determine the sensitivity of your cells to UV light. Some of the steps may be performed by the instructor or the lab aids.

Plating of the cells

1. Turn on the hood and wipe down with alcohol.
2. Gather the material you need and wipe them down with alcohol before placing them in the hood:

- Serological pipettes
- Micropipette tips
- Sterile centrifuge tubes
- $\text{Ca}^{+2}/\text{Mg}^{+2}$ PBS-warmed
- Trypsin- warmed
- Hemocytometer
- Five 6-well plates

3. Observe the cells under the microscope and note the confluency of your cultures. Work with one cell line at a time.

4. Remove the media, wash with PBS and trypsinize cells with 1 ml trypsin. (Refer to the page 34 for detailed protocol of trypsinizing.)

5. After cells are detached and floating, add 4mls of media and mix by pipetting.

6. Using a hemocytometer count the number of cells and calculate cell concentration. (Refer to page 26, counting cells using a hemocytometer, for a detailed protocol).

7. Plate 200 cells into one row or 3 wells of the six-well plate (density of about 20 cells/cm²) according to the diagram in figure 12. Repeat for the other four plates. (The number of cells that you need to plate may be different depending on the plating efficiency of your cells). When plating cells in multi-well plates, it is best to prepare a master-mix first and then aliquot into the wells rather than plating each well separately. This is to reduce the chances of mistakes in plating each well and keeping the wells consistent. For this experiment we will plate three wells per plate for each cell line, which is a total of 15 wells. You can

use 2 ml of media for each well. You must always prepare a master-mix with slightly larger volume than you need to account for pipetting errors. Therefore, make 32 ml of the master-mix with 2 ml extra. Calculate the volume of cells you need to take out in order to make your master-mix (V_1). Use the $(C_1)(V_1) = (C_2)(V_2)$ formula.

	Cell line 1	Cell line 2
# of cells counted in 10 squares of the hemocytometer		
Dilution factor for counting		
Cell concentration, C_1		
Final cell concentration of the master-mix (cells/ml), C_2		
Final volume of the master mix (ml), V_2	32 ml	32 ml
Initial volume of the culture, V_1		
Volume of complete media		

8. Prepare the master-mix by mixing the appropriate volume (V_1) of cells from your culture to a large tube with enough complete media to bring up the volume to 32 ml.

9. Mix well by pipetting.

10. Aliquot 2 ml into each well.

11. Label the plates as shown in (Fig. 11) and put them in the incubator.

12. If you are using more than one cell line, repeat the above procedure for the other cell line, using the second set of three wells on the six-well plates.

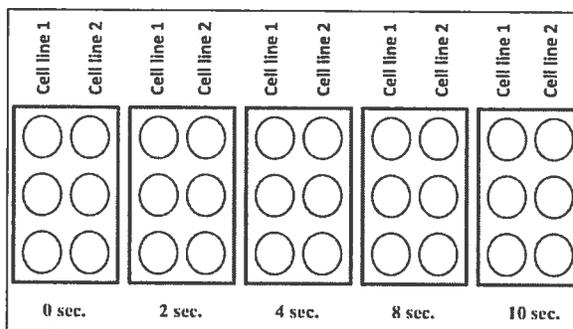


Figure 11- Diagram of the six-well plates. Seed 3 wells (or a row) of each plate with each cell line.

UV exposure

One day after plating:

13. Turn on the hood and wipe down with alcohol. Gather your material and wipe down with alcohol:

- Serological pipettes
- PBS (with Ca^{+2}/Mg^{+2})
- Complete media, warmed
- UV protection goggles

14. Remove the media from your wells.

15. Wash the wells with 0.5 ml of PBS per well. Remove the PBS such that the wells are dry.

16. Expose each of the plates to one of the following doses: 0, 2, 4, 8 or 10 seconds of UV light. To expose the plates:

- Wear protective goggles.

- Remove all other cells that you are NOT treating with UV from under the hood.
- Place the plate to be treated under the UV light.
- Remove the lid from the cells that you are going to treat with UV.
- Turn on the UV lamp for 0, 2, 4, 8 and 10 seconds.

17. After UV treatment, quickly add 2 ml of fresh complete media per well.

18. Put the plates back in the incubator and do not move for 7-10 days.

Counting colonies

In order to see the colonies clearly, you will stain the colonies with a blue/violet stain called **crystal violet**. Crystal violet stain is mixed with methanol, which is a fixative. Methanol will kill the cells but keeps the cell structures intact and prevents disintegration of the cells.

19. Aspirate out the media.

20. Wash the cells once with about 0.5-1 ml per well of PBS (with $\text{Ca}^{+2}/\text{Mg}^{+2}$). Remove the PBS.

21. Add 0.5 ml of Crystal violet/Methanol to each well.

22. Allow the cells to stain at room temperature for about 30-60 min.

23. Transfer the stain to the waste bottle (the stain can be filtered and re-used). Do not use the aspirator: crystal violet will stain the tubing of the aspirator, which is difficult to clean.

24. Gently pour some tap water into each well and wash the excess stain 2-3 times. Dump the water waste in the sink with running water.

25. Invert the plates and let them dry.

26. Count number of colonies for each well. Colonies that are too tiny (less than 50 cells) are not counted. Different sizes of colonies represent variations in growth rates among cells in the same culture.

27. Calculate the average number of colonies for each set of three wells with the same treatment.

28. Calculate PE and surviving fraction values for each dosage and each cell line.

29. Graph a surviving curve and analyze your data.

Analysis questions:

1. Count the average number of colonies for each set of three wells and calculate the PE value for each dosage? Repeat for each cell line. How do the PE values change with different dosages?