Molecular and Clinical Radiobiology Workshop
Clonogenic Cell Survival Assay

Goals

1) Become comfortable with cell irradiation
2) Measure clonogenic survival
3) Become familiar with the clonogenic assay
4) Generate cell-survival curves
5) Analyze effects of radiation on cell survival

Experimental Design

Non-small cell lung cancer cells were plated in 10cm dishes 48 hours prior to irradiation to provide 70% confluence (see dish with stained cells).

After 48h, cells were counted using an automated cell counter (TC-10).

Serial dilutions were made and cells were plated according to table 1 in 6-well plates. For each condition, 3 wells were seeded with cells.

The irradiator (Figure 1) was pre-warmed and set to 160kV and 6.3mA.

Table 1 – Cell seeding density for different doses and corresponding irradiation time

Cells Seeded	Dose (Gy)	Beam On Time (min)
100	0	0
200	2	3.1
400	4	6.2
1000	6	9.4
3000	8	12.6
8000	10	15.7

Figure 1 – Faxitron X-ray irradiation machine. The machine is self-shielded and provides x-ray beams upto a maximum of 160 kV and 6.3 mA.

Preparation

1. Complete growth medium as required for the cell line typically containing 10% fetal bovine serum plus antibiotics (penicillin–streptomycin) and glutamine.
2. For every 500 mL of medium, add 55 mL of serum, 5 mL of 200 mM L -glutamine, and 5 mL of 10,000 U/mL penicillin–streptomycin solution.
3. Medium should be stored at 4°C but warmed to 37°C prior to use.
4. Trypsin-EDTA, to make single-cell suspensions from monolayer cultures. Stored at 4°C but warmed to room temperature prior to use.
5. Phosphate buffered saline.
6. Plasticware, for carrying out tissue culture including 6-well dishes; 100-mm dishes; 5-, 10-, and 25-mL pipets.
7. Micropipets and corresponding tips (10uL, 200uL, 1000uL).
8. Plastic tubes including 15mL and 1mL tubes.

Protocol

1. Aspirate media and wash cells 3x with PBS.
2. Aspirate PBS from third wash completely.
3. Add 1mL of pre-warmed Trypsin-EDTA and incubate for 2 min at 37^0C.
4. Add media and harvest detached cells by placing them in 15 mL falcon tube.
5. Spin tube containing cell suspension at 2000 rpm for 5 min.
6. Aspirate media carefully without disturbing pellet.
7. Resuspend pellet in 200uL of media. Pipette rigorously.
8. Add 800uL to bring the volume to 1mL. Pipette rigorously.
9. In a 1mL tube (Eppendorf tube) add 10uL from the cell suspension and mix with 10uL of Trypan blue. Mix well.
10. Add 10uL of the cell/dye solution to each side of the hemacytometer (or use automated cell counter).
11. Count cells and note the cell concentration and cell viability.
12. Perform serial dilutions for each radiation dose so that the desired number of cells will be obtained by adding between 100 and 1000 μ L of volume to the dishes. If the number of cells needed requires a volume exceeding 1000 μ L, use a more concentrated dilution. Plate a number of cells consistent with obtaining a colony count of 50–100.
13. Seed cells in triplicates onto 6-well plates.
14. Incubate plates for 24h.
15. Irradiate each set of plates at the appropriate dose
    a. Tip: Start with the 10 Gy and include the 8 Gy and 6 Gy. Irradiate upto 6 Gy, then remove the 6 Gy flasks while leaving the 8 Gy and 10 Gy. Then Irradiate for an additional 2 Gy and remove the 8 Gy flasks and so on.
16. Once irradiation is completed, return plates to the incubator (maintaining a temperature of 37.5^0C and a CO_2 concentration of 5%).
17. Incubate for 9-14 days.
18. After 9-14 days, aspirate media and wash plates with PBS 3x.
19. Add fixative (4% paraformaldehyde or 10% formalin) for 2 hours at room temperature.
20. Stain wells with 0.5% Methylene blue over night.
21. Count and analyze colonies.
    a. Ensure that your counting is consistent: If you counted a very small and lightly stained colony in one flask, you must do so for all other flasks*.

*There are colony counters that can be used for automated counting.

Analysis

1. Calculate the plating efficiency
2. Plot cell survival against dose
3. Plot a typical cell-survival curve
4. Calculate the SF2
5. Perform curve fitting using the Linear Quadratic Model
6. Calculate the alpha/beta ratios

Suggested Readings

Anupama Munshi, Marvette Hobbs, and Raymond E. Meyn. Clonogenic Cell Survival Assay. Methods in Molecular Medicine, vol. 110: Chemosensitivity: Vol. 1: In Vitro Assays Edited by: R. D. Blumenthal © Humana Press Inc., Totowa, NJ.

Buch K, Peters T, Nawroth T, et al. Determination of cell survival after irradiation via clonogenic assay versus multiple MTT Assay-A comparative study. Radiation Oncology; 2012. 7:1.

Rafehi H, Orlowski C, Georgiadis GT, et al. Clonogenic Assay: Adherent Cells. Journal of Visualized Experiments. 2011.