

Clonogenic Cell Survival Assay

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Summary

The clonogenic cell survival assay determines the ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large colony or a clone. This cell is then said to be clonogenic. A cell survival curve is therefore defined as a relationship between the dose of the agent used to produce an insult and the fraction of cells retaining their ability to reproduce. Although clonogenic cell survival assays were initially described for studying the effects of radiation on cells and have played an essential role in radiobiology, they are now widely used to examine the effects of agents with potential applications in the clinic. These include, in addition to ionizing radiation, chemotherapy agents such as etoposide and cisplatin, antiangiogenic agents such as endostatin and angiostatin, and cytokines and their receptors, either alone or in combination therapy. Survival curves have been generated for many established cell lines growing in culture. One can use cell lines from various origins including humans and rodents; these cells can be neoplastic or normal. Because survival curves have wide application in evaluating the reproductive integrity of different cells, we provide here the steps involved in setting up a typical experiment using an established cell line in culture.

Key Words

Survival curve; cell survival; plating efficiency; radiation.

1. Introduction

Clonogenic cell survival is a basic tool that was described in the 1950s for the study of radiation effects. Much of the information that has been generated on the effect of radiation on mammalian cells has been obtained from clonogenic cell survival assays.

Various mechanisms have been described for cell death; however, loss of reproductive integrity and the inability to proliferate indefinitely are the most common features. Therefore, a cell that retains its ability to synthesize proteins and DNA and go through one or two mitoses, but is unable to divide and

produce a large number of progeny is considered dead. This is very commonly referred to as loss of reproductive integrity or reproductive death and is the end point measured with cells in culture. On the other hand, a cell that is not reproductively dead and has retained the capacity to divide and proliferate indefinitely can produce a large clone or a large colony of cells and is then referred to as “clonogenic.” A cell survival curve describes a relationship between the insult-producing agent and the proportion of cells that survive.

The ability of a single cell to grow into a large colony that can be visualized with the naked eye is proof that it has retained its capacity to reproduce. The loss of this ability as a function of dose of radiation or chemotherapy agent is described by the dose-survival curve. Most laboratories now extensively use established cell lines for studying the effects of various agents either alone or in combination. Therefore, the aim of this chapter is to go through the steps involved in setting up a typical clonogenic cell survival experiment using established cells lines growing as monolayer cultures.

In brief, cells from an actively growing stock culture in monolayer are prepared in a suspension by the use of trypsin, which causes the cells to detach from the substratum. The number of cells per milliliter in this suspension is then counted using a hemocytometer or a Coulter counter. From this stock culture, if 50 cells are seeded into a dish, e.g., and the dish is incubated for approx 2 wk, each single cell divides many times and forms a colony that is easily visible with the naked eye, especially if it is fixed and stained (the steps involved in this process are briefly outlined in **Fig. 1**). All the cells that make up the colony are the progeny of a single cell. For the 50 cells seeded into the dish, the number of colonies counted may be anywhere from 0 to 50. One would ideally expect the number to be 50, but that is rarely the case for several possible reasons, including suboptimal growth medium, errors in counting the number of cells initially plated, and the loss of cells by trypsinization and general handling. The term plating efficiency (PE) indicates the percentage of cells seeded into a dish that finally grow to form a colony. Therefore, in the previous example, if there are 25 colonies in the dish, then the PE becomes 50%. If a parallel dish is seeded with cells exposed to a dose of 6 Gy of gamma rays and incubated for approx 2 wk before being fixed and stained, then the following may be observed:

1. Some cells may remain single, not divide, and, in some cases, may show evidence of nuclear deterioration as they die by apoptosis. These cells would be scored as dead.
2. Some cells may go through one or two divisions and form small colonies of just a few cells. These cells would be scored as dead.
3. Some cells may form large colonies, indicating that the cells have survived the treatment and have retained the ability to reproduce indefinitely.

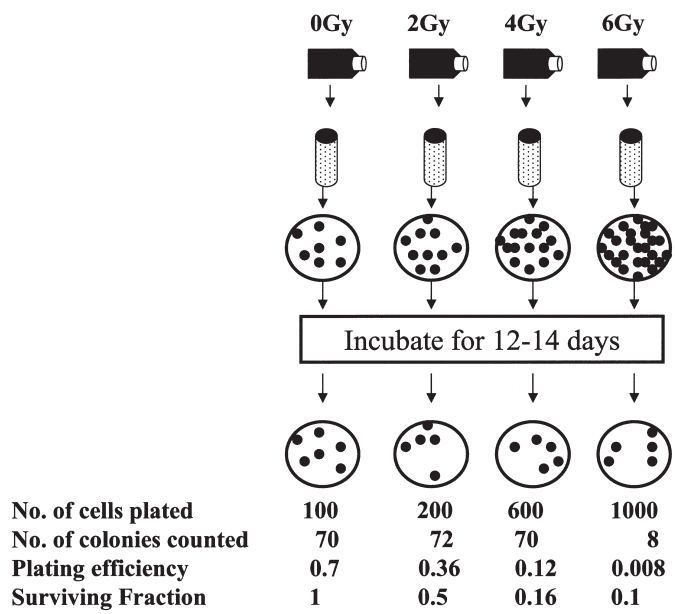


Fig. 1. Schematic representation of steps involved in setting up a clonogenic cell survival assay.

2. Materials

2.1. Preparation of Cell Lines Prior to Setting Up Clonogenic Assays

1. Cell lines that need to be tested for their ability to form colonies.
2. Complete growth medium as recommended by the manufacturer typically containing 10% fetal bovine serum plus antibiotics (penicillin–streptomycin) and glutamine. 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. Medium should be stored at 4°C but warmed to 37°C prior to use.
3. Trypsin-EDTA, to make single-cell suspensions from monolayer cultures. Store at 4°C.
4. Plasticware, for carrying out tissue culture including flasks (T-25 and T-75); 100-mm dishes; and 5-, 10-, and 25-mL pipets.
5. Micropipets and corresponding tips.
6. 70% ethanol, for wiping the surface of the hood as well as the surface of all medium bottles prior to bringing them into the hood.
7. Cidecon (detergent disinfectant with bactericidal and virucidal properties), to wipe the surface of the shelf on which the dishes will be incubated.
8. Phosphate-buffered saline (PBS) (calcium magnesium free). Store at 4°C.
9. Isoton II (diluent for counting cells using a Coulter counter). Store at room temperature.

2.2 Staining of Plates

1. 0.5% Gentian Violet (made up in methanol). Store at room temperature in a dark bottle. Do not pour it down the sink.

3. Methods

3.1. Cells Growing in Monolayers or Attached Cells

The procedure that we outline in this chapter is a basic protocol for setting up radiation clonogenic assays. However, this protocol can be modified to test the effect of different agents on the cell line of interest, either alone or in combination. These could include gene therapy vectors, chemotherapy agents, tyrosine kinase inhibitors, antiangiogenic agents—basically any agent that has to be tested for its ability to affect reproductive cell death.

3.2. Preparation of Cell Lines Prior to Setting Up Clonogenic Cell Survival

1. Label six T-25 flasks in preparation for setting each flask with a known number of cells as 0, 2, 4, and 6 Gy (depending on the experiment) for the various doses of radiation to be given. Add 5 mL of growth medium to the flasks and keep them aside in a hood.
2. Trypsinize the stock flask of cells containing the cells that have to be tested for their radiosensitivity. Make sure that the cells are in single-cell suspension and obtain an accurate cell count. We use a Coulter counter to obtain a cell count. If a Coulter counter is not available, cells can be counted using a hemocytometer. Using a Pipetman, add 250,000 cells (the cell number can vary depending on the cell type) to the 5 mL of medium in each T-25 flask. Shake gently to distribute the cells evenly.
3. Place the flasks in a 37°C incubator set at 5% CO₂ and be sure to leave the cap one thread loose so as to allow CO₂ exchange (*see Note 1*).
4. Allow the cells to settle and attach as a monolayer.

3.3. Irradiation of Flasks and Performance of Plating Experiment for Clonogenic Assay

1. Prepare the hood and clean an incubator shelf. Because these cells are going to be left untouched in an incubator for up to 2 wk, and the possibility of contamination is high, clean the shelf thoroughly with Cidecon and 70% ethanol. Keep the cleaned shelf in the hood. Make sure that more than one bottle of complete medium is available; this experiment may require >500 mL of medium.
2. Prepare the 100-mm dishes and 15-mL tubes in advance. One will need 100-mm dishes in triplicate, and two cell numbers will be plated for each dose of radiation. Because cells will be exposed to six doses of radiation, 36 dishes will be needed. Label the bottom of each dish as the lid, for the dishes will be loose during staining, and the bottom is where the colonies will form, which is what will actually

be counted. Label the first set of triplicate dishes as 0 Gy A and 0 Gy B (A and B for the two cell numbers to be used). Repeat this for all dose levels: 2, 4, 6, 8, and 10 Gy. Place 10 mL of complete medium in each dish. Place all dishes, stacked in threes, on the incubator shelf and put back in the incubator until ready for plating.

3. Place 18, 15-mL tubes on a clean rack three deep. Label the first tube 0Gy, 1:1; the next one 0 Gy 1:10; and the next 0 Gy 1:100. Repeat this for all dose levels. Place 4.5 mL of complete medium in the back two tubes but not in the 1:1 dilution tubes.
4. Put the flasks on ice. Clean a rectangular tub and fill it halfway with ice. Remove the flasks from the incubator and close the caps tightly. Place the flasks on ice, and insert half depth into the ice. Tilt the flasks to the bottom so that the medium does not rest against the cap. Start a timer for 20 min.
5. While waiting, prepare the Coulter counting vials. Label each as 0, 2, 4, and 6 Gy. Place 9.9 mL of Isoton (to be used for counting cells if using a Coulter counter) into each vial. Place Isoton in a control vial and run through the counter to get a background measurement; repeat until a satisfactory low background is obtained. Make sure that the Coulter is set to the appropriate size parameter for the cell line.
6. When the 20-min time is up, irradiate the flasks using an appropriate irradiator according to the desired dose.
7. Return to the hood, keeping the flasks on ice outside the hood. Begin the trypsinization procedure for each flask. Start with the 0-Gy flask. Aspirate the medium, rinse the cells gently with PBS, and then trypsinize. Place the harvested cells in the 15-mL tube labeled 0 Gy, 1:1. Make sure that you have a good single-cell suspension. From this cell suspension, take 100 μ L and place in the appropriate counting vial for 0 Gy. Now trypsinize the next flask. While the flask is on the warming tray, count the previous counting vial and record the counts on a dilution sheet (*see Fig. 2* for a setup of a dilution sheet that we commonly use in the laboratory). This expedites the experiments and lessens the chance of cell divisions taking place unequally during the time of trypsinization and counting.
8. Continue this procedure until all the flasks have been trypsinized and counted. There should now be cells in each of the 1:1 dilution tubes, with a known number of cells/milliliter, all documented on the dilution sheet.
9. 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. This may require only 100 cells for the control plate whereas at 6 Gy this may require 4000 cells or more. Remember that the larger the insult to the flask (i.e., increasing radiation dose or increasing drug concentration if using chemotherapy agents), the lower the plating efficiency and the more cells are needed to obtain the desired colony count (*see Note 2*).
10. It is important to resuspend the cell pellet, which has probably settled to the bottom of the tube by the time all the flasks have been counted. Place 0.5 mL of the 1:1

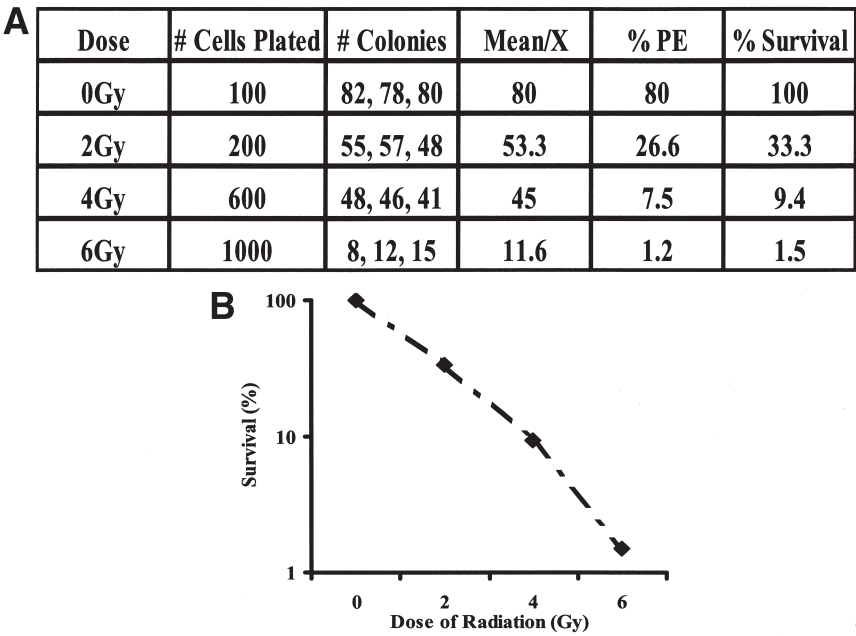


Fig. 2. (A) Setup of dilution sheet used during clonogenic cell survival assays; (B) survival curve plotted using hypothetical numbers derived from dilution sheet

- dilution into the 1 : 10 dilution tube (containing 4.5 mL of medium). Similarly, mix each dilution well before aliquoting 0.5 mL to the next-higher dilution tube.
11. When the final dilution tube for each dose has been made, mark the top of each tube with an X and place the other tubes that are not needed outside the hood. This will minimize the chance of grabbing the wrong tube at the time of plating.
 12. Place the calculated volume of cell solution slowly into each appropriate 100-mm dish. Start with the bottom dish and work up. Place the solution on the medium drop by drop, spreading the drops evenly over the entire surface area to prevent clumping and overlap of colonies.
 13. After placing the solution in the three 100-mm dishes, rock the plates north–south and east–west to distribute the cells evenly. Avoid swirling the solution, which allows cells to group at the sides, making counting difficult.
 14. When all of the dishes have been plated, return the shelf to the incubator. Label the inside door of the incubator with the name of the experiment, initials, date of the project in and date expected to come out (*see Note 3*).
 15. As a rule of thumb, incubate for 10–12 d for cells with 13 h or less of generation time, approx 14 d for 14 h or more. We routinely set up clonogenic assays for these periods of time without any contamination.

3.4. Staining of Plates

Staining plates with Gentian Violet is easy, but care must be taken not to get it on one's clothes, because it is difficult to remove. It is suggested that a laboratory coat and double glove be used for the staining procedure.

1. Take the shelf from the incubator and place it by the sink. Empty the medium from six plates into a flask with bleach in the sink. Place 0.5% Gentian Violet onto each of the six plates. Gentian Violet is diluted from a stock with methanol.
2. Run a gentle stream of lukewarm water into a pan in the sink. Take the stained plates and transfer the stain from them into an additional six plates. Rinse the stained plates upside down in the pan to prevent the colonies from loosening and washing off.
3. Continue **steps 1 and 2** until all the plates have been stained. Let the plates air-dry overnight; they will be ready for counting the next day (*see Note 4*).

3.5. Counting of Colonies

1. Take the air-dried colonies and count the colonies in each dish to obtain the plating efficiency. We use a dissecting scope to view the colonies under a magnified field. A cluster of blue-staining cells is considered a colony if it comprises at least 25–50 cells. However, it is important to keep the cutoff constant so that there are no variations introduced between experiments. Count the colonies and note the numbers for both the A and B dilutions in a chart (prepare one similar to the one shown in **Fig. 2**).
2. Average the three colony counts for each dilution A and B and divide the mean by the number of cells plated. This will give the PE:

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

The PE of some cells may be close to 80–90%. This is especially true for human tumor cells of various origins. However, the PE of normal human fibroblasts is usually very low (ranging from as low as 1 to 12–15%).

3. Following determination of PE, calculate the fraction of cells surviving a given treatment. First, normalize all the plating efficiencies of the treated samples to that of the control unirradiated plates, considering that to be 100%. The surviving fraction (SF) is determined by dividing the PE of the treated cells by the PE of the controls, and then multiplying by 100:

$$SF = \frac{\text{PE of treated sample}}{\text{PE of control}} \times 100$$

Plot the data on an Excel spreadsheet with the dose of radiation on the x-axis and survival on the y-axis.

4. Notes

1. The CO₂ requirement may vary depending on the cell type. If the flasks have lids with a membrane that allows gas exchange, there is no need to loosen the cap.
2. Note that you can use prior cell survival data, if available, to extrapolate exactly the number of cells to plate for a desired colony count.
3. If possible, it is a good idea to dedicate an incubator to clonogenic cell survival. This avoids unnecessary bumping of colonies as people open and close the door to the incubator. As the colonies grow, bumping the incubator or shelf can cause the cells to shed and settle as new colonies, thereby leading to an increase in the colony count and erroneous results.
4. We usually do not pour the used stain down the sink but, instead, collect it in a bottle dedicated to spent stain.

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