Clonogenic Assays Protocols

Clonogenic assays serve as a useful tool to test whether a given cancer therapy can reduce the clonogenic survival of tumor cells. A colony is defined as a cluster of at least 50 cells that can often only be determined microscopically. A clonogenic assay is the method of choice to determine cell reproductive death after treatment with ionizing radiation, but can also be used to determine the effectiveness of other cytotoxic agents.

<u>Crystal Violet Assay</u>

Materials and Reagents

- Cell culture medium (DMEM 10% FCS)
- Phosphate buffered saline (PBS)
- Trypsin/ EDTA
- Crystal violet
- Fixation solution
- Cell culture petri dishes or six-well plates
- Hemocytometer
- Stereomicroscope (e.g., Nikon Eclipse, model: TS100)
- CO_2 Incubator

Cell preparation:

1. Culture CF41 and MeLn cells according to their requirements.
2. Remove medium, and then rinse cells with 10 ml PBS.
3. Add 4 ml 0.25% trypsin to the cells and incubate for 1-5 min until the cells appear round and detach from the substrate.
4. Add 10 ml medium with 10% FBS, and detach the cells by pipetting.
5. Count the cells using a hemocytometer.
6. Note: It is critical to get a relatively accurate number for the cells.
7. Prepare desired seeding concentration: $1x10^3$ to $4x10^4$ cells per milliliter of complete culture medium, and then seed cell into 6mm dishes.

Assay setup:

1. Incubate cells for a few hours to overnight in a CO_2 incubator at 37 °C and allow them to attach to the plate/dish.
2. Treat the cells as necessary with chemicals, radiation or a combination of both.
3. Incubate the cells in a CO_2 incubator at 37 °C for 1-2 weeks until cells in control plates have formed colonies that are of a substantially good size (50 cells per colony is the minimum for scoring).

Fixation and staining:

1. Remove medium, and then rinse cells with 10 ml PBS.
2. Remove PBS and add 2-3 ml of fixation solution and leave the dishes/plates at room temperature (RT) for 5 min.
3. Remove fixation solution.
4. Add 0.5% crystal violet solution and incubate at RT for 2 h.
5. Remove crystal violet carefully and wash the dishes/plates in distilled water to rinse off crystal violet.
6. Air-dry the dishes/plates on a table cloth at RT for up to a few days.

Data analysis:

1. Count number of colonies with a stereomicroscope.
2. Calculate plating efficiency (PE) and surviving fraction (SF).
3. PE = no. of colonies formed/ no. of cells seeded x 100%
4. SF = no. of colonies formed after treatment/ no. of cells seeded x PE

Soft agar assay (Clonogenic anchorage-independent assay)

1. Prepare single-cell suspensions according to the Cell Preparation item up to step 6;
2. Mix $1x10^3$ to $4x10^4$ cells in 1mL of 0.6% agarose prepared in complete medium;
3. Plate cell-agarose mixes in culture dishes coated with 5mm of 1% agarose (already prepared by your instructors);
4. Incubate plates at 37 °C and 5% CO_2 for 2 weeks to allow colony formation;
5. The number of colonies formed and the colony area (mm2) are determined under the light microscope;
6. Analyze using ImageJ software (NIH, Bethesda, MD, USA).