Video Article

Clonogenic Assay: Adherent Cells

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URL: https://www.jove.com/video/2573
DOI: doi:10.3791/2573

Keywords: Cellular Biology, Issue 49, clonogenic assay, clonogenic survival, colony staining, colony counting, radiation sensitivity, radiation modification

Date Published: 3/13/2011

Citation: Rafehi, H., Orlowski, C., Georgiadis, G.T., Ververis, K., El-Osta, A., Karagiannis, T.C. Clonogenic Assay: Adherent Cells. *J. Vis. Exp.* (49), e2573, doi:10.3791/2573 (2011).

Abstract

The clonogenic (or colony forming) assay has been established for more than 50 years; the original paper describing the technique was published in 1956[1]. Apart from documenting the method, the initial landmark study generated the first radiation-dose response curve for X-ray irradiated mammalian (HeLa) cells in culture[1]. Basically, the clonogenic assay enables an assessment of the differences in reproductive viability (capacity of cells to produce progeny; i.e. a single cell to form a colony of 50 or more cells) between control untreated cells and cells that have undergone various treatments such as exposure to ionising radiation, various chemical compounds (e.g. cytotoxic agents) or in other cases genetic manipulation. The assay has become the most widely accepted technique in radiation biology and has been widely used for evaluating the radiation sensitivity of different cell lines. Further, the clonogenic assay is commonly used for monitoring the efficacy of radiation modifying compounds and for determining the effects of cytotoxic agents and other anti-cancer therapeutics on colony forming ability, in different cell lines. A typical clonogenic survival experiment using adherent cells lines involves three distinct components, 1) treatment of the cell monolayer in tissue culture flasks, 2) preparation of single cell suspensions and plating an appropriate number of cells in petri dishes and 3) fixing and staining colonies following a relevant incubation period, which could range from 1-3 weeks, depending on the cell line. Here we demonstrate the general procedure for performing the clonogenic assay with adherent cell lines with the use of an immortalized human keratinocyte cell line (FEP-1811)[2]. Also, our aims are to describe common features of clonogenic assays including calculation of the plating efficiency and survival fractions after exposure of cells to radiation, and to exemplify modification of radiation-response with the use of a natural antioxidant formulation.

Video Link

The video component of this article can be found at https://www.jove.com/video/2573/

Protocol

1. Cell Culture and Experimental Set-up

1. Human keratinocytes are maintained as monolayers in 75 cm^2 tissue culture flasks containing 15 mL of keratinocyte-SFM (K-SFM) medium (GIBCO, serum-free medium) supplemented with L-glutamine (2 mM), epidermal growth factor (5 ng/ mL), bovine pituitary extract (40 µg/ mL) and 20 mg/ mL gentamicin. Cells are grown in a humidified 5% CO_2 environment at 37°C.
2. Cells are seeded into 12 x 25 cm^2 tissue culture flasks containing 5 mL of K-SFM medium.

Single cell suspensions are prepared by trypsinization. Cells are washed with phosphate buffered saline and incubated with a 0.05% trypsin / EDTA solution for 5-10 minutes. When the cells start to become rounded and ~30% are detached, 3 volumes of Dulbecco's modified eagle medium containing 10% fetal bovine serum is added to neutralize the trypsin. The cells are detached by pipetting up and down (20 times). Cells are counted using a hemocytometer.

Appropriate cell numbers are seeded according to the doubling time of the cell line (approximately 20 hours for human FEP-1811 keratinocytes). The aim is to achieve ~90% confluency (~10^6 cells per flask) on the day of the experiment.

An experiment consisting of 12 flasks is optimal for a single clonogenic assay (six unirradiated control and six irradiated flasks) which can be completed in approximately four hours.

2. Treatment and Irradiation

1. Treat cells for an appropriate time with a relevant radiation-modifying compound and expose cells to ionising radiation either γ-radiation or X-rays.

Typically six flasks serve as plating efficiency (untreated) and drug only controls. The other six flasks are irradiated.

In this example human keratinocytes are treated with various concentration of Cinnulin PF (CPF; Integrity Nutraceuticals International, Spring Hill, TN, US; representative data for 20 µg/ mL is shown below), a water-soluble natural antioxidant formulation, for 1 hour at 37°C. Cells are irradiated with 4 Gy using a ^{137}Cs source (Gammacell 1000 Elite irradiator; Nordion International, ON, Canada; 1.6 Gy/min).

3. Plating

1. Following treatment, single cell suspensions are obtained as described earlier.
2. The number of cells in each sample are counted carefully using a hemocytometer and diluted such that appropriate cell numbers are seeded into petri dishes (five replicates of each in 15 mm dishes).
   The plating efficiency and / or surviving fraction should be anticipated when deciding the number of cells to seed per plate. The aim is to achieve a range of between 20 - 150 colonies.

Petri dishes are arranged in a humidified plastic cloning box and incubated in a 5% CO_2 environment at 37°C for colony formation.

The incubation time for colony formation varies from 1-3 weeks for different cell lines; it is accepted that the time must be equivalent to at least six cell divisions. In this example, the control dishes for human keratinocytes require eight days to form sufficiently large clones consisting of 50 or more cells.

4. Fixing and Staining Colonies

Complete the following steps in a fume hood.

1. Gently remove the media from each of the plates by aspiration.
2. Wash each plate with 5 mL 0.9% saline.
3. Fix the colonies with 5 mL 10% neutral buffered formalin solution for 15-30 minutes.
4. Stain with 5 mL 0.01% (w/v) crystal violet in dH_2O for 30-60 minutes.
5. Wash excess crystal violet with dH_2O and allow dishes to dry.

5. Colony Counting

Stereomicroscope

1. Colonies containing more than 50 individual cells are counted using a stereomicroscope.

Digital imaging and counting using imaging software

1. Digital images of the colonies are obtained using a camera or scanning device
2. Colonies are counted using imaging analysis software packages as described below.

Cell counting using ImageJ (Fiji Version 1.44a)

1. Open the image file in Fiji, go to File -> Open.
2. If required convert the image to 8-bit format, go to Image -> Adjust...-> Threshold.
3. Adjust threshold to reduce levels of non-specific background so that only the colonies are detected.
4. Count colonies using the following: go to Process -> Binary -> Find maxima.

For this image format, noise tolerance can be set to 0. Ensure that light background option is ticked and preview the detected maxima to check that all cell colonies have been correctly registered.

Discussion

In this example human FEP-1811 keratinocytes were treated with various concentrations up to 100 µg/ mL CPF; data is shown for 20 µg/ mL CPF for 1 hour at 37°C. Following treatment cells were irradiated with 4 Gy using a ^{137}Cs source (Gammacell 1000 Elite irradiator; Nordion International, ON, Canada; 1.6 Gy/min). For the control untreated and drug only treatments 100 cells were plated in each petri dish and 1000 cells per dish were plated for the irradiated samples. As indicated in the table above, colonies were counted using a stereomicroscope or digital images (Fig 1) were taken for counting using ImageJ. The average colony count for the five dishes was used to calculate plating efficiency and surviving fraction. The data indicated a radiation protective effect (protection factor ~3) with the natural antioxidant formulation.

Representative Results

Treatment	Counting Method	Cell plated	1	2	3	4	5	Plating Efficiency1	Surviving Fraction2
Untreated Cells	Manual (stereomicroscope)	100	19	22	38	14	20	0.23	1
	Manual (digital image)	100	22	23	34	15	22	0.23	1
	Find Maxima (Fiji)	100	23	21	33	14	21	0.22	1
20 mM CPF	Manual (stereomicroscope)	100	18	11	21	15	11	0.15	0.66
	Manual (digital image)	100	19	11	23	16	8	0.15	0.67
	Find Maxima (Fiji)	100	17	11	22	17	8	0.15	0.65
4 Gy	Manual (stereomicroscope)	1000	39	27	28	44	28	0.03	0.14
	Manual (digital image)	1000	42	33	31	42	31	0.04	0.16
	Find Maxima (Fiji)	1000	42	35	30	43	31	0.04	0.16
20 mM CPF + 4 Gy	Manual (stereomicroscope)	1000	115	105	98	108	101	0.11	0.47
	Manual (digital image)	1000	117	102	104	103	99	0.11	0.45
	Find Maxima (Fiji)	1000	121	102	104	102	97	0.11	0.47

[1] Plating efficiency = number of colonies counted / number of cells plated
[2] Surviving fraction = (number of colonies counted /number of cells plated) / plating efficiency

Table 1. Calculation of plating efficiency and survival and comparison of colony counts using various methods

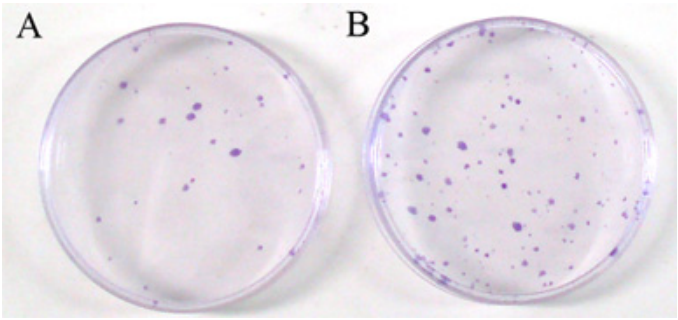

Figure 1. Digital image showing colonies produced by human, FEP-1811, keratinocytes following plating of 1000 cells and eight days incubation. (A) Cells were irradiated with 4 Gy and (B) cells were treated with 20 µg/ mL CPF for 1 hour at 37°C prior to irradiation with 4 Gy. A radiation protective effect with the natural antioxidant formulation is evident.

Disclosures

No conflicts of interest declared.

Acknowledgements

The support of the Australian Institute of Nuclear Science and Engineering is acknowledged. TCK was the recipient of AINSE awards. Epigenomic Medicine Lab is supported by the National Health and Medical Research Council of Australia (566559). HR is supported by an Australian post-graduate and BakerIDI bright spark awards. This work is funded by the CRC for Biomedical Imaging Development Ltd (CRC-BID), established and supported under the Australian Government's Cooperative Research Centres program. CO is the recipient an Australian post-graduate award and a CRC-BID supplementary scholarship.

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