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Cytotoxicity and Cell Growth Assays

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I. INTRODUCTION

Various assays are in use to determine the effect of a drug (broadly defined chemical or other inhibitory substance) on cells propagated *in vitro*. They range from simple assays that measure cell viability after drug exposure, i.e., dye exclusion that measures membrane integrity and effect of the drug on cell growth (simply enumerating cells), to other assays that measure cell viability, indirectly, by assessing the ability of the cell to reduce compounds such as XTT, MTS, SRB, and alamarBlue or to generate ATP. The advantages of these assays are that they are performed easily and, with the use of 96-well plates, many dilutions or many compounds can be tested rapidly.

Other assays that measure the ability of a cell to incorporate radiolabeled thymidine into DNA come closer to measuring the effect of drugs on the proliferative potential of the cell population. The gold standard, but a more difficult and time-consuming assay that measures the proliferative potential of cells, is the clonogenic assay. This assay measures the percentage of cells in the population capable of giving rise to clones, thus measuring the effect the compound has on the proliferating fraction of the population.

II. CYTOTOXICITY ASSAYS

This section describes dye exclusion assays and other commonly used assays that measure cell viability: XTT, MTS, SRB, AlamarBlue, [^{3}H]-thymidine incorporation, and ATP formation.

A. Dye Exclusion Test Using Trypan Blue

The trypan blue exclusion test is a rapid method to assess cell viability in response to environmental insults. It is simple and inexpensive.

The dye exclusion test is based on the ability of viable cells to be impermeable to trypan blue, naphtalene black, erythrosine, and other dyes. When membrane integrity of the cells is compromised, there is uptake of the dye into the cells so that viable cells, which are unstained, appear clear with a refractile ring around them and nonviable cells appear dark blue colored with no refractile ring around them. The following method is for trypan blue, the dye used most commonly.

An automatic system, the Vi-Cell cell viability analizer (Beckman Coulter Inc., Fullerton, CA), has been introduced. As compared to the manually performed assay, this technique allows rapid analysis and quantitation. It performs the test with video imaging of the flow-through cell.

Materials and Instrumentation

Trypan blue solution (0.4%) (Cat. No. T 8154) is from Sigma-Aldrich (St. Louis, MO); hemocytometer (improved Neubauer) (Cat. No. 02-671-5), lab counter (Cat. No. 02-670-12), and Eppendorf tubes (Cat. No. 05-402-24A) are from Fisher Scientific (Agawam, MA). Micropipettes (Cat. No. P-3950-200) and pipette tips (Cat. No. P3020-CPS) are from Denville Scientific (Metuchen, NJ).

Steps

1. Prepare cells by trypsinization or resuspension. Make sure that the cells are well resuspended as aggregates make the counting inaccurate. Avoid

allowing the cells to settle or adhere to the flask before transfering to the improved Neubauer chamber.
2. Mix thoroughly 50 µl of cell suspension with 50 µl of trypan blue in a 500-µl Eppendorf tube. Leave the mixture no more than 1–2 min because longer incubation with the dye may be toxic to viable cells and will result in overestimating the number of dead cells.
3. Place a coverslip over the hemocytometer so that it covers the central 1 mm^2 of the semisilvered counting area.
4. With a micropipette, collect the mixture of 1:1 cell suspension and trypan blue and transfer it to the edge of the hemocytometer chamber.
5. Let the mixture flow under the coverslip by capillary action, being careful not to overfill or underfill the chamber, as it will affect the counting.
6. If any surplus fluid is present over the edges, use absorbing paper to remove it.
7. Place the Neubauer chamber under the microscope and select the 10× objective. Focus on the center of the semisilvered counting area where the grid lines are evident by contrast.
8. Triple parallel grid lines surround a 1-mm^2 area divided in 25 smaller squares further subdivided in 16 smaller squares that are used for counting.
9. Unstained cells with a refractile ring around them are the viable cells, whereas dark blue colored cells that do not have refractile ring around them are nonviable cells.
10. Count the total amount of cells, stained and unstained. The percentage of unstained cells gives you the percentage of viable cells with this method. For routine culture, count 100 cells/mm^2. Counting more cells makes the test more accurate.

B. Cell Viability Assays

1. XTT/PMS Assay

This procedure exploits the fact that the internal environment of proliferating cells is more reduced than one of nonviable cells. Tetrazolium salts are used to measure this reduced state. Among them, XTT is preferred to MTT because it is more soluble. However, there are some disadvantages with this method. XTT is generally cytotoxic and destroys the cells under investigation, allowing only a single evaluation. It requires the presence of phenazine methosulfate for efficient reduction.

Materials and Instrumentation

Falcon Microtest tissue culture plates (96 wells) (Cat. No. 35-3072) and Falcon polystirene pipette (Cat. No. 35-7551) are from Becton-Dickinson (Franklin Lakes, NJ); RPMI 1640 medium with L-glutamine (Cat. No. 11875-093), fetal bovine serum (FBS) (Cat. No. 10437-028), phosphate-buffered saline (PBS), 7.4 (Cat. No. 10010-023), and trypsin–EDTA (Cat. No. 25200-056) are from GIBCO BRL (Rockville, MD); multichannel pipette (50–300 µl) (Cat. No. P3970-18), micropipettes (200 µl) (Cat. No. P-3950-200), and pipette tips (Cat. No. P3020-CPS) are from Deville Scientific (Metuchen, NJ); XTT sodium salt [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt] (Cat. No. X 4626) and phenazine methosulfate (*N*-methylphenazonium methyl sulfate salt) (Cat. No. P 9625) are from Sigma-Aldrich (St. Louis, MO); and microplate reader SpectraMax Plus is from Molecular Devices (Sunnyvale, CA).

Two 96-wells plates are required: one for cells and one for drug dilutions.

a. Preparation of Cells in Plate A

Steps

1. The method may be used on cells that are adherent or growing in suspension.
2. Culture cell lines in RPMI 1640 media with 10% FBS, 1% glutamine, and 1% pen/strep or other appropriate media.
3. For harvesting, the cells should be in log-phase growth (300–500 × 10^3 cells/ml) or, if dealing with adherent cells, trypsinization must be done before cells reach 80% confluence.
4. Harvest 100,000 cells per each 96-well plate and resuspend in a total volume of 10 ml/medium with 20% FBS, 1% glutamine, and 1% pen/strep.
5. From 100,000 cells in 10 ml medium, pipette 100 µl of medium+cells in each well to have 1000 cells/well.
6. Leave the first row for the blank. The second row is a control (cells without drug).
7. For adherent cells, allow 1 h for cells to reattach before adding the drug under study.

The amount of medium per well in each experiment may change depending on the amount of drug that is added after the cells are plated. The following example uses 100 µl of medium containing 1000 cells and 100 µl of drug, resulting in 200 µl of medium in each well.

b. Drug Preparation in Plate B

Steps

1. Pipette 125 µl of RPMI 1640 medium in each well of a 96-well plate.
2. Add 125 µl of the drug in each well in the first row. Then, after mixing, transfer 125 µl of the mix to

the following row and repeat the procedure up to the last row. In this way X concentration of the drug will be present in the first row, a $X/2$ concentration in the second row, and so on.

3. Pipette 100 µl from the 10th row of the plate (plate B) with the drug dilutions to the last row of the plate with the cells (plate A) so that the lower concentrations do not affect subsequent transfers.

4. When all the transfers are completed, add 100 µl of plain RPMI 1640 medium to the control row. You will have 200 µl of medium with 10% FBS in each well.

5. Add 200 µl of medium to the blank row. Start the incubation.

c. Assay Procedure

Steps

1. Warm 5 ml of plain RPMI 1640 medium at 50°C for each plate tested. This temperature allows the XTT salt to dissolve better.
2. Add 5 mg of XTT powder to the 5 ml of RPMI 1640 (it is important that no more than 1 mg of XTT/ml of medium is used).
3. Prepare a stock solution of 5 m*M* PMS. Add 326 µl of warm PBS to the vial containing the 0.5 mg of PMS (FW 306.3).
4. Add 25 µl of the stock 5 m*M* PMS to the solution containing 5 ml of medium + 5 mg of XTT.
5. Pour the solution in a reservoir and, with a multichannel pipette, transfer 50 µl of it per each well. The ratio of 0.25 ml of the XTT/PMS solution/ml of cell culture must be maintained. In the procedure described earlier there is 200 µl of medium/well × 96 wells, or a total of 1820 µl, and 50 µl of the XTT/PMS solution should be added to each well.
6. Incubate at 37°C for 2–4 h.
7. Measure absorbance with a microplate reader at the wavelength of 450 and 630 nm as a reference wavelength.

Pitfalls

Warming up the RPMI 1640 media is critical for total XTT solubilization. When dissolved incompletely, XTT salt will affect the results. The incubation time after XTT is added may vary and could be longer than the 2–4 h as suggested earlier.

2. MTS/PMS Assay

The tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt MTS, in the presence of the electron coupling reagent phenazine methosulfate (PMS)] is bioreduced by viable cells into a formazan product that is soluble in culture media. The advantage of MTS over XTT is that it is more soluble and nontoxic, allowing the cells to be returned to culture for further evaluation. The disadvantage is that like XTT it requires the presence of PMS for efficient reduction.

Materials and Instrumentation

Falcon Microtest tissue culture plates (96 wells) (Cat. No. 35-3072) and Falcon polystirene pipette (Cat. No. 35-7551) are from Becton-Dickinson (Franklin Lakes, NJ); RPMI 1640 medium with L-glutamine (Cat. No. 11875-093), fetal bovine serum (Cat. No. 10437-028), Dulbecco's PBS (Cat. No. 14190-136), and trypsin–EDTA (Cat. No. 25200-056) are from Gibco BRL (Rockville, MD); multichannel pipette (50–300 µl) (Cat. No. P3970-18), micropipettes (200 µl) (Cat. No. P-3950-200), and pipette tips (Cat. No. P3020-CPS) are from Deville Scientific (Metuchen, NJ); phenazine methosulfate (*N*-methylphenazonium methyl sulfate salt) (Cat. No. P 9625) is from Sigma-Aldrich (St. Louis, MO); CellTiter 96 AQ_{ueous} MTS reagent powder (Cat. No. G1111) is from Promega Co. (Madison, WI); and microplate reader Spectra®Max Plus[384] is from Molecular Devices (Sunnyvale, CA).

The **preparation of cells and drug preparation** are similar to the XTT/PMS assay.

Assay Procedure

Steps

1. Add 2 mg of MTS powder to each 1 ml of Dulbecco's PBS. Per each 96-well plate add 4 mg of MTS to 2 ml of DPBS.
2. Prepare a stock solution of PMS at a concentration of 0.92 mg/ml.
3. Add 100 µl of PMS to the MTS solution immediately before addition to the cultured cells.
4. Pour the solution in a reservoir and, with a multichannel pipette, transfer 20 µl of it per each well.
5. Incubate the plate for 1–4 h at 37°C in a humidified, 5% CO_2 atmosphere.
6. Measure absorbance with a microplate reader at a wavelength of 490 and 630 nm as a reference wavelength.

Comments

The incubation time after MTS/PMS is added may vary and could be longer than the 1–4 h suggested earlier.

3. Sulforhodamine B Assay (SRB)

The SRB assay is based on binding of the dye to basic amino acids of cellular proteins, and colorimetric evaluation provides an estimate of total protein

mass, which is related to cell number. This assay has been widely used for the *in vitro* measurement of cellular protein content of both adherent and suspension cultures. The advantages of this test as compared to other tests include better linearity, higher sensitivity, a stable end point that does not require time-sensitive measurement, and lower cost. The disadvantage lies in the need for the addition of TCA for cell fixation. This step is critical because, if not added gently, TCA could dislodge cells before they become fixed, generating possible artifacts that will affect the results.

Materials and Instrumentation

Falcon microtest tissue culture plates (96 wells) (Cat. No. 35-3072) and Falcon polystirene pipette (Cat. No. 35-7551) are from Becton-Dickinson (Franklin Lakes, NJ); RPMI 1640 medium with L-glutamine (Cat. No. 11875-093), FBS (Cat. No. 10437-028), PBS, 7.4 (Cat. No. 10010-023), and trypsin–EDTA (Cat. No. 25200-056) are from GIBCO BRL (Rockville, MD); multichannel pipette (50–300 µl) (Cat. No. P3970-18), micropipettes (200 µl) (Cat. No. P-3950-200), and pipette tips (Cat. No. P3020-CPS) are from Deville Scientific (Metuchen, NJ); trichloroacetic acid (TCA) (Cat. No. T9159), Trizma base [tris (hydroximethyl)aminomethane] (Cat. No. 25-285-9), acetic acid (Cat. No. A6283), and sulforhodamine B sodium salt (Cat. No. S 9012) are from Sigma-Aldrich (St. Louis, MO). Microplate reader SpectraMax®Plus is from Molecular Devices (Sunnyvale, CA).

The **preparation of cells and drug preparation** are similar to the XTT/PMS assay.

Assay Procedure

Steps

1. Prepare a stock solution of 50% TCA and add 50 µl of this cold solution (4°C) to each well containing 200 µl of medium + cells so that a final concentration of 10% TCA is reached in each well.
2. Place the 96-well plate for 1 h at 4°C to allow cell fixation.
3. Prepare a 0.4% SRB (w/v) solution in 1% acetic acid and add 70 µl of this solution to each well and leave at room temperature for 30 min.
4. Wash the plate with 1% acetic acid five times in order to remove unbound SRB.
5. Prepare a stock solution of 10 m*M* Trizma base and add 200 µl of this solution to each well in order to solubilize bound SRB. Place the 96-well plate on a plate shaker for at least 10 min.
6. Read abosrbance with a microplate reader at 492 nm, subtracting the background measurement at 620 nm.

Pitfalls

The addition of TCA for fixation is critical and if it is not done with caution can cause dislodgement of the cells before fixation and subsequent alteration of the results.

4. Alamar BlueAssay

AlamarBlue is used to monitor the reducing enviroment of proliferating cells. Because it is not toxic, cells exposed to it can be returned to culture or used for other purposes. AlamarBlue takes advantage of mitochondrial reductase to convert nonfluorescent resazurin to fluorescent resorufin.

Proliferation measurements with alamarBlue may be monitored using a standard spectrophotometer, a standard spectrofluorometer, or a spectrophotometric microtiter well plate reader.

Materials and Instrumentation

AlamarBlue (Cat. No. DAL1100) is from Biosource (Camarillo, CA); Falcon microtest tissue culture plates (96 wells) (Cat. No. 35-3072) and Falcon polystirene pipettes (Cat. No. 35-7551) are from Becton-Dickinson (Franklin Lakes, NJ); RPMI 1640 medium with L-glutamine (Cat. No. 11875-093), FBS (Cat. No. 10437-028), PBS, 7.4 (Cat. No. 10010-023), and trypsin–EDTA (Cat. No. 25200-056) are from GIBCO BRL (Rockville, MD); multichannel pipettes (50–300 µl) (Cat. No. P3970-18), micropipettes (200 µl) (Cat. No. P-3950-200), and pipette tips (Cat. No. P3020-CPS) are from Deville Scientific (Metuchen, NJ); and microplate reader SpectraMax Plus^{384} is from Molecular Devices (Sunnyvale, CA).

The **preparation of cells** is similar to XTT/PMS assay, except for the following.

Steps

1. The assay can be performed on adherent cells or suspension culture.
2. Culture cells in RPMI 1640 medium with 10% FBS, 1% glutamine, and 1% pen/strep and amphotericin to avoid microbial contaminants that may reduce AlamarBlue.
3. For harvesting, the cells in suspension must be in log-phase growth (300–500 $\times$ 10^3 cells/ml) or, if dealing with adherent cells, trypsinization must be done before the cells reach 80% confluence.
4. Harvest 100,000 cells for each 96-well plate resuspended in a total volume of 10 ml/medium with 20% FBS, 1% glutamine, 1% pen/strep, and amphotericin.
5. Pipette 100 µl of medium+cells in each well to have 1000 cells/well.

Assay Procedure

Steps

1. Leave the first row for the blank. The second row is used as a control (cells without drug)
2. For adherent cells, allow 1 h for cells to reattach before adding the drug in study.
3. Add 25 µl of alamarBlue *is then added* to a resulting final volume of 250 µl of media+cells.
4. Measure viability after a 1-h incubation at 37°C in humidified 5% CO_2 when the medium in the control row turns from blue to pink. If the reduction observed is insufficient, you may allow the incubation to proceed for a longer period of time.
5. Place the 96-well plate in a automated plate-reading spectrofluorophotometer, with excitation at 530 nm and emission at 590 nm. Fluorescence is expressed as a percentage of control (cells with no drug) after reading the substraction of background fluorescence (blank without cells). AlamarBlue reduction can also be measured spectrophotometrically at two wavelengths, 570 and 600 nm, which are the wavelengths where the reduced and oxidized forms of AlamarBlue absorb maximally.

Pitfalls

The whole procedure has to be performed under aseptic conditions because proliferating bacterial and fungal cells are able to reduce AlamarBlue and may affect the results.

5. *ATP Cell Viability Assay*

ATP is the most important source of energy for the living cells and can be quantitated in a luminometer by measuring the light generated using the luciferase-luciferin reagent. Typically, apoptotic cells exhibit a significant decrease in ATP levels due to loss of cell integrity.

The ATP cell viability assay is based on two steps. In the first step, ADP is added as a substrate for adenylate kinase and, in the presence of this enzyme, ADP is converted to ATP. In the second step, the enzyme luciferase catalizes the formation of light from ATP and luciferin. The intensity of the light emitted is measured using a luminometer or a β counter. When the measurement is done on cells in culture using microtiter plates, it is necessary to perform this procedure using white-walled microtiter plates suitable for measuring luminescence.

Materials and Instrumentation

White-walled tissue culture plates (96 wells) (Cat. No. LT07-102) and ToxiLight nondestructive cytotoxicity assay (Cat. No. LT07-117) are from BioWhittaker-Cambrex (Rutherford, NJ); Falcon polystyrene pipette (Cat. No. 35-7551) is from Becton-Dickinson (Franklin Lakes, NJ); RPMI 1640 medium with L-glutamine (Cat. No. 11875-093), FBS (Cat. No. 10437-028); PBS, 7.4 (Cat. No. 10010-023), and trypsin–EDTA (Cat. No. 25200-056) were from GIBCO BRL (Rockville, MD); multichannel pipette (50–300 µl) (Cat. No. P3970-18), micropipettes (200 µl) (Cat. No. P-3950-200), and pipette tips (Cat. No. P3020-CPS) are from Deville Scientific (Metuchen, NJ); and the Reporter microplate luminometer (Cat. No. 9600-001) is from Turner BioSystem (Sunnyvale, CA).

The **preparation of cells** is similar to the XTT/PMS assay except that white-walled microtiter plates are used.

Drug Preparation

Steps

1. Pipette 75 µl of RPMI 1640 medium in each well of a 96-well plate.
2. Add 75 µl of the drug in each well in the first row. Then, after mixing, transfer 75 µl of the mix to the following row and repeat the procedure up to the last row. In this way you will have 1*X* concentration of the drug in the first row, a *X*/2 concentration in the second row, and so on.
3. Pipette 50 µl from the 10th row of the plate with the drug dilutions to the last row of the plate with the cells so that the lower concentrations will not affect the subsequent transfers.
4. When all the transfers are completed, add 50 µl of plain RPMI 1640 medium to the control row. Each well will contain 100 µl of medium with 10% FBS.
5. Add 100 µl of medium to the blank row. Start the incubation.

Assay Procedure

Steps

1. Reconstitute the AK detection reagent by adding 10 ml of Tris–AC buffer and, after mixing it, gently allow the reagent to equilibrate at room temperature for 15 min.
2. After the planned period of incubation for cells and drug in the white-walled microplates, remove the plate from the incubator and allow the plate to equilibrate to room temperature prior to measurement.
3. Add 100 µl of the AK detection reagent to all the wells with a multichannel pipette.
4. Wait 5 min before reading to allow for detectable ADP conversion to ATP. Measurement of the light emission should be performed within 30 min from the addition of the AK detection reagent.

5. Place the plate into a luminometer or a β counter. Measure the light emission. Results are expressed as relative light units (luminometer) or counts per second (β counter).

6. *[^{3}H]-Thymidine Incorporation Assay*

This assay is based on the ability of proliferating cells to incorporate [^{3}H]-thymidine into replicating DNA. Despite its precision to produce accurate data on DNA synthesis, this assay has some disadvantages. It uses radioactivity, requires extensive sample preparation, and the method is sample destructive as compared to a clonogenic assay. The assay described is for human cells grown on agar; the assay may also be used for cells grown in suspension or attached to glass or plastic.

Materials and Instrumentation

Falcon Microtest tissue culture plates (24 wells) (Cat. No. 35-3047), Falcon polystirene pipette (Cat. No. 35-7551), and BlueMax Falcon 15-ml tubes (Cat. No. 35-2097) are from Becton-Dickinson (Franklin Lakes, NJ); RPMI 1640 medium with L-glutamine (Cat. No. 11875-093), FBS (Cat. No. 10437-028), and PBS, 7.4 (Cat. No. 10010-023) are from GIBCO BRL (Rockville, MD); micropipettes (200 µl) (Cat. No. P-3950-200) and pipette tips (Cat. No. P3020-CPS) are from Deville Scientific (Metuchen, NJ); agar (Cat. No. A 7002), sodium azide (Cat. No. S 2002), and TCA (Cat. No. T 9159) are from Sigma-Aldrich (St. Louis, MO); KHO (Cat. No. SP208-500), 20-ml Wheaton glass liquid scintillation vials (Cat. No. 03-341-25G), and ScintiVerse scintillation liquid (Cat. No. SX18-4) are from Fisher Scientific Co. (Suwanee, GA); thymidine [6-^{3}H] specific activity 10 Ci (370 GBq/mmol) at a concentration of 1 m Ci/ml (Cat. No. 355001MC) is from Perkin Elmer Life Science (Boston, MA); and LS 6500 liquid scintillation counter is from Beckman Coulter Inc. (Fullerton, CA).

a. Preparation of Cells

Steps

1. Prepare 0.5% agar by mixing 3.5 ml of 3% Noble agar with 16.5 ml of RPMI 1640 containing 20% fetal bovine serum.
2. Add 500 µl of this agar mixture to each well of a 24-well plate. Then refrigerate the plate at 4°C for 10 min.
3. Resuspend cells in a mixture containing 0.4% agar in RPMI 1640 containing 20% fetal bovine serum at a final concentration of 10^4 cells/ml.
4. Add 1 ml of the cell suspension to each well containing the hardened underlayer.
5. Incubate the plate at 37°C in a 5% CO_2 atmosphere for 24 h.

Assay Procedure

Steps

1. Add sodium azide at a final concentration of 4×10^3 µg/ml to the contol wells (cells without drug).
2. Add the drug to all the remaining wells at the concentrations planned.
3. Incubate cells + drug for 72 h.
4. At the end of this incubation, layer 5 µ Ci of [^{3}H]-thymidine over each well.
5. Incubate the plate for an additional 24 h.
6. Transfer the agar layers from each well to 15-ml centrifuge tubes and bring the volume to 13 ml adding PBS to each tube.
7. Boil the tubes for 30 min and then centrifuge at 1000 rpm for 5 min.
8. Aspirate the supernatants and wash each pellet two times with cold PBS.
9. Centrifuge the tubes and collect the precipitates. Then wash each pellet with 5% TCA.
10. Dissolve each pellet by adding 0.3 ml of 0.075 *N* KOH and pipetting up and down to completely solubilize cells.
11. Transfer each solubilized cell solution into a scintillation vial containing 5 ml scintillation liquid.
12. Count the radioactivity of each vial in a LS 6500 Beckman liquid scintillation counter.

Comments

When used for cells in suspension the assay may be modified to obtain several time points, e.g., 5, 10, 20, 40, and 60 min, thus generating a rate of thymidine incorporation into DNA and more quantitative data.

III. CLONOGENIC ASSAYS

One of the most important methods for the assessment of survival is the measurement of the ability of a single cell to form colonies. This is usually done by simple dilution after generating a single cell suspension and counting the colonies that arise from single cells. For effective and correct counting, a lower threshold, such as five or six doublings (32 or 64 cells/colony), is quantitated, taking into account the doubling time. Thus the effect of a concentration of a drug on cell survival may be measured with this assay.

In addition to counting colonies, as some drugs may have a delayed effect on cell proliferation, it might be necessary to do colony size analysis. This can be done by counting the cells per colony, by measuring the diameter, or by measuring the absorbance of colonies stained with 1% crystal violet.

The clonogenic assay for tumor colony-forming cells has applicability to a broad spectrum of cell lines and fresh cells obtained from human tumors and has provided information on the biology, clinical course, and chemosensitivity of human cancers

A. Monolayer Cloning

In this method, adherent cells are plated onto a plastic or glass surface and colonies formed are stained and counted. The method is straightforward and useful for cell lines that grow on plastic if a reasonable percentage of cells generate colonies.

Materials and Instrumentation

25-cm^2 flasks (Corning, Cat. No. 430639)
Phosphate-buffered saline (GIBCO, Cat. No. 10010-023)
Trypsin (GIBCO, Cat. No. 25200-056)
Petri dishes (Falcon, Cat. No. 1007, 60 × 15 mm)
Methanol (J. T. Baker, Cat. No. 9069-03)
1% crystal violet
Hemocytometer (Reichert-Improved Neubauer, Cat. No. 132501)

Steps

1. Prepare replicate 25-cm^2 flasks, two for each concentration of drug and two for controls.
2. Add the drug to the test flask and solvent to the control flask when the cells reach the required growth phase (usually 24 h after plating) and incubate for 1 h at 37°C.
3. Remove the drug, rinse the monolayer with PBS, and prepare a single cell suspension by trypsinization (desirable).
4. Count the cells and dilute the cell concentration to give 100–200 colonies per 6-cm petri dish. The cell number used per dish depends on the efficiency of plating and the effect of the drug. Plate setup should contain at least two different cell concentrations: one for lower concentrations of the drug and one for higher drug concentrations.
5. Plate out the appropriate number of the cells and incubate at 37°C with 5% CO_2 until colonies grow. This time varies according to the doubling time of the cells, but generally ranges from 10 to 21 days. The colonies should grow to 1000 cells or more on average for the survival assays.
6. Rinse dishes with PBS, fix in 1% methanol or 0.5% glutaraldehyde, and stain with 1% crystal violet. Rinse in running tap water, distilled water, and dry. Count colonies above threshold and calculate as a fraction of control. Plot on a log scale against drug concentration.

Comments

Longer drug exposures can also be assessed by incubating cells with drug for different times, removing media and adding fresh media.

B. Cloning by Limiting Dilution

Puck and Marcus (1955) first established this method. It is more useful for suspension cultures. To improve plating efficacy, modifications for improving the yield of harvested cells, such as using a rich medium that has been optimized for the cell type in use, may be necessary. Cells in log phase should be selected for this method. Also, where serum is required, fetal bovine serum is generally better than calf or horse serum. Sometimes changing the conditions may be useful for obtaining high colony efficiency such as filtering the media or incubating cells for a further 48 h.

Materials and Instrumentation

DMEM, high glucose (Life Technologies, Inc., Cat. No. 10313-021 or equivalent)
Fetal bovine serum (Gibco-Invitrogen, Co. Cat. No. 10437-028 or equivalent)
L-Glutamine (Gibco-Invitrogen, Co. Cat. No. 25030-081 or equivalent)
Hybridoma cloning factor (Fisher, Cat. No. IG50-0615)
50-ml sterile centrifuge tubes (Falcon, Cat. No. 2070)
15-ml sterile centrifuge tubes (Falcon, Cat. No. 2099)
24- and 96-well culture plate (Falcon 353047-0413 and Falcon 353072-0664)
Hemocytometer (Reichert-Improved Neubauer, Cat. No. 132501)
Trypan blue, 0.4% (Sigma Chemicals, Co. Cat. No. 72K2328)
Multichannel pipetter (Thermo Labsystem, Cat. No. 4610050) and sterile tips (Denville Scientific, Cat. No. P-3950-200)
Reagent reservoir (Labcor, Inc., Cat. No. 730-004)
HT (Life Technologies, Inc., Cat. No. 11067-30)

Steps

1. Refeed cells in 24-well plates or flasks with fresh medium 24 h before cloning.

2. Prepare the cloning media by using 10% hybridoma cloning factor, 20% FBS, 4 m*M* L-glutamine, and DMEM.

3. Resuspend the cells to be cloned in 15-ml sterile tubes; use the trypan blue dye exclusion method to determine viability. Viability should be greater than 80%.

4. For each cell line calculate the dilutions to give 4, 2, and 1 cell/ml in cloning medium. Using 50-ml tubes, serially dilute to contain 4, 2, and 1 cell/ml. The final dilution tube should contain 50 ml of cloning medium at 1 cell/ml.

5. Pour each of the dilutions into a sterile reservoir. Plate 250 µl/well into 96-well plates (one plate with 4 cells/ml, one plate with 2 cells/ml, and two plates with 1 cell/ml). Complete dilutions and plating for each cell line.

6. Incubate all plates at 37°C with 8–10% CO_2 for 5–7 days. At the end of this time, examine all plates microscopically to ensure cloning and plating efficiency before refeeding the plates.

7. Count colonies

C. Soft Agar Clonogenic Assay

Another useful method for cytotoxicity studies is the soft agar technique. It is particularly useful for cells that grow in suspension, but may also be used for cells that attach to glass or plastic. The cells are treated with drug, washed, instead of creating a growth curve as in the outgrowth method, the cells are cloned in soft agar as described next. Agar solution, medium, and cell suspensions are the three basic components in the cloning technique.

Materials and Instrumentation

Noble agar (Agar-Noble Difco Lab)
Fetal bovine serum (Gibco-Invitrogen, Co. Cat. No. 10437-028 or equivalent)
RPMI 1640 medium (GIBCO, Cat. No. 11875-093)
Large culture tubes (Daigger, Cat. No. EF4003)
24-well plate (Falcon, Cat. No. 3487)

Use the following steps for preparing the agar.

Steps

1. Weigh 0.11 g Noble agar and put into a dry flat-bottom bottle that can hold 50 ml.
2. To the 0.11 g agar, add 5.2 ml distilled water. In adding the water, be sure that the water runs in gently so that the agar does not explode.
3. Autoclave 15 min, slow exhaust, and remove immediately upon completion of sterilization.

Use the following steps for preparing the medium.

Steps

1. Measure 50 ml of medium plus serum into a bottle and store at 37°C. (The medium should contain serum in excess of the normal amount used for liquid cultures, such as 15–20%.)
2. For each condition being tested, prepare a culture tube 125 × 20 mm containing 9.0 ml of medium.
3. Treat the cells with drug and resuspend in 15–20% serum-supplemented medium. Cells will need to be diluted so that no more than 1.0 ml (tube cloning) and 0.5 ml (double-layer cloning) of cell suspension will contain the desired number of cells.

   Example: For L5178Y cells, a mouse leukemia cell line, the cloning efficiency is 88%. In order to get a cloning tube with 20 clones per tube, 10 ml of cell suspension is made having 120 cells in 10 ml. This is done in the tube containing 9.0 ml of medium, as described previously.

   Example: Cell stock after centrifuging is 2×10^4 cells/ml. Dilute 1:100; take 0.6 ml of the 1:100 dilution, and add to the 9.0 ml of medium. Bring the volume to 10 ml by adding 0.3 ml of medium and 0.1 ml of appropriate drug solution. Each condition tested will require a separate cell suspension, i.e., each cell suspension tube supplies cells for a maximum of five cloning tubes. Only four are generally used.

For Tube Cloning Procedure

1. Add the previously measured 50 ml of medium to the bottle of liquified agar solution. It should be cooled enough so that it can be held by hand comfortably.
2. Distribute 3 ml of agar-medium mixture to each cloning tube.
3. Add 2 ml of cell suspension in the large culture tubes, being sure they are well suspended.
4. Tighten the cap, and mix in the following manner: Hold the tube horizontally and rotate. At the same time, rock the tube to mix. Do this gently to avoid bubbles.
5. Place the tube upright in ice for 2 min.
6. Remove from ice and place in culture tube rack.
7. Keep at room temperature for 15 min.
8. Incubate in an upright position at 37°C.
9. Clones of fast growing lines, such as L5178Y and L1210, are counted on the 10th day. Others take longer, depending on the generation of time of the cancer cell line.

Double-Layer Soft Agar Clonogenic Assay Procedure

This method has some additional benefits compared to the monolayer agar method (Runge *et al.*, 1985). It is very useful for cell cultures whose cloning capacity is low and for fresh cells obtained from tumor biopsy samples.

Materials and Instrumentation

The materials are the same used in other agar-clonogenic assays mentioned earlier.

Steps

Plate 1 ml of underlyer (feeder layer) consists of 15–20% serum-supplemented RPMI 1640 medium and 0.5% Noble agar in 24-well culture plates. The underlayers have to be gelled at least 1 h prior to plating the 1 ml cell and drug(s) (based on design) containing upper layer. For each condition, a suspension is prepared with 4 ml of 20% supplemented RPMI 1640 medium, 0.5 ml of 3% agar solution (final concentration 0.3%), and 0.5 ml of cell suspension containing 5000 cells and appropriate concentration(s) of drug(s). Plate 1 ml of such suspension in each well with a gelled underlayer. Each condition is in quadruplicate. Place all double-layered plates at room temperature for 20 min and then incubate for 10–14 days at 37°C in 100% humidity in 5% CO_2 of atmosphere. For continuous exposure, leave the drug(s) in culture for the entire period of incubation. For time point exposures, such as 4 h, 24 h, 48 h, and even 7 days, incubate cells with drug in suspension culture, wash cells twice with PBS, harvest, resuspend, and finally clone per the procedure described earlier. After 10–14 days of incubation, count clones greater than 50 cells in each well under an inverted microscope (×40). Results are expressed as the mean of 4 well as the percentage of untreated control colony counts.

D. Use of Image Analysis System to Count Colonies

The clonogenic assay for tumor colony-forming cells has applicability on a broad scope of human tumors and has proved valuable in studies of biology, clinical course, and chemosensitivity of human cancers. However, visual counting of colonies has several problems: it is time-consuming and therefore very expensive, the size of colonies changes very rapidly, and there is variability in counting from one researcher to another, partly because of differences in criteria for what constitutes a colony and fatigue.

Bausch and Lomb Omnicon FAS-II image analysis system provides sufficient reliability to be used for counting human tumor colonies grown *in vitro* (Kressner *et al.*, 1980). In addition, the colony counter performed the petri dish counts 10 times faster than experienced technicians did and without associated operator fatigue (Salmon *et al.*, 1984)

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