Protocol: Cell Culture

1. CAUTION: When working with human blood, cells, or infectious agents, all appropriate bio-safety practices must be followed.

2. NOTE:
   
   All solutions and equipment coming into contact with living cells must be sterile and aseptic techniques should be used accordingly.

   All culture incubations should be performed in a humidified 37°C, 5% CO2 incubator unless otherwise specified.

MATERIAL

- 70% ethanol (v/v)
- Primary cultures of cells
- Sterile 1X PBS
- Trypsin/EDTA, 37°C
- Complete medium with serum: e.g., supplemented DMEM with 10% to 15% (v/v) FBS, 37°C
- Sterile Pasteur pipets
- 37°C warming tray or incubator
- Tissue culture plasticware or glassware, including sterile pipets and flasks or Petri Dishes
- Cryovials, FBS and DMSO for freezing cells
- Hemacytometer with coverslip
- Hand-held counter
- 0.4% trypan blue (w/v) in PBS

METHOD

I. Starting Cell Culture from Frozen Cells

   Note: It is important to properly thaw cells to maintain the viability of the culture and enable the culture to recover quickly. Please note that some cryoprotectants (i.e. DMSO) are toxic above 4°C. Thus, it is vital that cultures are quickly thawed and diluted in culture medium.

   1. Collect the cells from liquid nitrogen storage wearing appropriate protective equipment.

   2. Place frozen screw-cap vial in a water bath at the appropriate temperature for your cell line (i.e. 37°C for mammalian cells). Thaw for 1-2 minutes with constant agitation to quickly thaw the cells. Wipe the vial with 70% alcohol before transferring to the cell culture hood.

   3. Slowly, drop by drop, dilute cells in a 10-fold volume of pre-warmed growth medium in a culture dish or flask. *For cells frozen with DMSO, please see below for our recommendation.
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4. Incubate at the appropriate temperature and concentration of CO₂ for the species. Change medium after a few hours or overnight incubation, i.e. until cells have attached to the culture dish or flask.

5. Examine cells microscopically (phase contrast) after 24 hours and sub-culture as necessary.
   *We recommend washing the cells with media to remove the DMSO before adding to the culture dish or flask.
   **Use early passages to freeze cells. It is recommended to freeze a few vials of each vial of cells thawed.

II. Subculture of Adherent Cell Lines

Note: Adherent cell lines must be sub-cultured once confluent and before all nutrients in the media are exhausted in order to prevent the cells from dying. To subculture the cells they need to be brought into suspension. The degree of adhesion varies but in the majority of cases trypsin (or other proteases) is used to release the cells from the flask. However, this may not be appropriate for some lines where exposure to proteases is harmful or where the enzymes used remove membrane markers or receptors of interest. Cells may also be brought into suspension by adding a small volume of media and using cell scrapers, to physically detach the cells from the surface.

1. View cultures using an inverted microscope to check confluence and confirm the absence of bacterial and fungal contaminants. Remove the spent medium.

2. Wash the cell monolayer with PBS, use approximately half the volume of culture medium. Repeat this wash step if the cells are strongly adherent. All serum must be removed before the addition of trypsin.

3. Add trypsin/EDTA (1 ml per 25 cm² of surface area) to the washed cell monolayer. Rotate flask to cover the entire surface with trypsin. Return the flask to the incubator and incubate for 2-10 minutes or until cells are detached. Prolonged exposure of the cells to trypsin may result in damage to cell surface receptors.

4. Examine the cells using an inverted microscope to ensure that the cells are detached and floating. You can gently tap the sides of the flask or dish to release adherent cells. Resuspend the cells in a small volume of fresh serum-containing medium to inactivate the trypsin.*

5. Incubate as appropriate for the cell line. Repeat sub-culturing as required.
   * Trypsin should be neutralized with serum before seeding cells into new flasks. We recommend removing the trypsin by washing the detached cells with medium and centrifuging before seeding into a new flask or dish.

III. Subculture of Suspension Cell Lines

Note: Cells taken from blood, ex. lymphocytes, are usually grown in suspension. Cells that grow as single cells are much easier to dilute and subculture than cells that aggregate and grow in clumps. Cells that aggregate must be diluted and gently resuspended to a single cell suspension by pipetting. Passage cells at a density of 1-3 X 10^6 viable cells.

1. Determine cell density and viability. Calculate the split ratio required to seed a new shaker flask with 3 X 10^5 cells/ml depending on the desired final volume of the new shaker flask.

2. Transfer the required number of cells to the new shaker flask with the appropriate final volume of new media. Gently pipette cells to eliminate aggregation.
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3. Incubate as appropriate for cells and repeat as required.

IV. Cryopreservation of Cells

1. View cultures using an inverted microscope to check confluence and confirm the absence of bacterial and fungal contaminants. Remove the spent medium and wash with PBS. Trypsinize cells and centrifuge to remove medium.

2. Resuspend cells with freezing medium at a concentration of 2-4 million cells per ml. Add 1 ml of cells into labeled cryovials.

3. Place cryovials on ice for one hour before transferring to the -80°C freezer overnight. For most cell cultures, the appropriate rate of cooling is between -1°C and -3°C per minute.

4. Transfer the vials to liquid nitrogen for long term storage.

V. Determining cell number and viability with a hemacytometer and trypan blue staining

Note1: Determining the number of cells in culture is important in standardization of culture conditions and in performing accurate quantitation experiments.

Note 2: When using the hemacytometer, a maximum cell count of 20 to 50 cells per 1 mm² is recommended.

Prepare cell suspension

1. For cells grown in monolayer cultures, detach cells from surface of dish using trypsin.

2. Dilute cells as needed to obtain a uniform suspension. Disperse any clumps.

Load hemacytometer

3. Use a sterile Pasteur pipet to transfer cell suspension to edge of hemacytometer counting chamber. Hold tip of pipet under the coverslip and dispense one drop of suspension.

*The suspension will be drawn under the coverslip by capillary action.

4. Fill second counting chamber.

Count cells

5. Allow cells to settle for a few minutes before beginning count. Blot off excess liquid.

6. View slide on microscope with 100X magnification

Position slide to view the large central area of the grid, this area is bordered by a set of three parallel lines. The central area of the grid should almost fill the microscope field.

7. Use a hand-held counter to count cells in each of the four corner and central squares

Repeat counts for other counting chamber. Five squares (four corners and one center) are counted from each of the two counting chambers for a total of ten squares counted. Count cells touching the middle line of the triple line on the top and left of the squares. Do not count cells touching the middle line of the triple lines.
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on the bottom or right side of the square.

Calculate cell number

8. Determine cells per milliliter by the following calculations:

\[
\text{cells/ml} = \frac{\text{average count per square} \times \text{dilution factor} \times 10^4}{10}
\]

\[
\text{total cells} = \text{cells/ml} \times \text{total original volume of cell suspension from which sample was taken.}
\]

Stain cells with trypan blue to determine cell viability

9. Determine number of viable cells by adding 0.5 ml of 0.4% trypan blue, 0.3 ml HBSS, and 0.1 ml cell suspension to a small tube. Mix thoroughly and let stand 5 min before loading hemacytometer. Nonviable cells will take up the dye, while live cells will be impermeable to dye.

10. Count total number of cells and total number of viable (unstained) cells. Calculate percent viable cells: \(\% \text{ viable cell} = \left(\frac{\# \text{ of unstained cells/total # of cells}}{100}\right)\)

11. Clean coverslip and hemacytometer by rinsing with 70% ethanol followed by deionized water.
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