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CELL CULTURE TECHNIQUES: A STANDARD OPERATING PROCEDURE

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We use this protocol and it's working

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Abstract

This protocol presents a comprehensive, beginner-friendly standard operating procedure (SOP) for core cell culture techniques, including thawing, subculturing, cryopreserving cell lines, and performing the MTT cytotoxicity assay. The protocol provides step-by-step instructions to guide users through handling, maintaining, and evaluating mammalian cell cultures under sterile conditions. Following the workflow, users can consistently recover, expand, and preserve healthy cell lines while minimizing contamination. The MTT assay protocol yields quantitative measures of cell viability and enables generation of reproducible dose–response curves and IC_{50} values. Together, these procedures ensure reliable cell culture performance and robust experimental outcomes suitable for research and training environments.

Guidelines

Please follow the notes and tips provided in the steps for each of the stages.

Materials

The materials and equipments needed for each stage of the process are as follows:

THAWING

- Frozen cell vial (stored in liquid nitrogen or at -80°C)
- Complete growth medium (specific to the cell line)
- Sterile pipettes and tips
- Sterile centrifuge tubes (15 mL or 50 mL)
- Water bath set at 37°C
- Biosafety cabinet
- CO₂ incubator (37°C, 5% CO₂)
- Hemocytometer or automated cell counter
- Steril phosphate-buffered saline (PBS)
- Personal protective equipment (PPE)

SUBCULTURING

- Cell culture flasks or dishes
- Complete growth medium
- Sterile PBS
- Trypsin-EDTA solution (for adherent cells)
- Centrifuge and centrifuge tubes (for suspension cells)
- Cell counter (hemocytometer or automated cell counter)
- Biosafety cabinet
- 70% ethanol (for sterilization)

MTT CYTOTOXICITY ASSAY

- Cells in culture (adherent or suspension)
- Complete growth medium
- MTT reagent (stock solution: 5 mg/mL in PBS)
- Dimethyl Sulfoxide (DMSO)
- 96-well plate
- Microplate reader (with a 570 nm filter)
- Sterile PBS
- Trypsin-EDTA (for adherent cells)
- Positive and negative controls (e.g., untreated control, known toxic agent)
- Incubator (37°C, 5% CO₂)

CRYOPRESERVATION

- Cells in culture (adherent or suspension)
- Cryopreservation medium: 10% DMSO + 90% Fetal Bovine Serum (FBS) or commercial cryopreservation medium (e.g., CryoStor)
- Sterile cryovials (labelled)
- Centrifuge and centrifuge tubes
- Trypsin-EDTA (for adherent cells)
- PBS
- Biosafety cabinet
- Freezing container (e.g., Mr. Frosty) or programmable freezer
- Liquid nitrogen storage

Safety warnings

 Critical warnings when handling the liquid nitrogen (LN₂):

- Avoid any skin contact with LN₂ or cold vapors to prevent instant frostbite. This could be achieved by wearing proper cryogenic PPE
- Nitrogen displaces oxygen, therefore, always handle in well-ventilated areas
- Prevent splashes and spills of LN₂ by slow handling, avoid overfilling, and remove gloves/shoes immediately if LN₂ enters them.

Thawing Cell Lines

- 1 Thawing cell lines is a critical process in cell culture that requires careful handling to ensure cell viability and recovery.

The materials and equipments needed for the thawing process:

- Frozen cell vial (stored in liquid nitrogen or at -80°C)
- Complete growth medium (specific to the cell line)
- Sterile pipettes and tips
- Sterile centrifuge tubes (15 mL or 50 mL)
- Water bath set at 37°C
- Biosafety cabinet
- CO_2 incubator (37°C , 5% CO_2)
- Hemocytometer or automated cell counter
- Steril phosphate-buffered saline (PBS)
- Personal protective equipment (PPE)

- 2 During the preparation, ensure that all materials are sterile and ready for use before thawing the cells.

Pre-warm the complete growth medium to 37°C . Prepare enough medium to dilute the cells after thawing (e.g., 5-10 mL per vial).



The growth medium is immersed in 37°C water.

3 Thawing process

- 3.1 Retrieve the frozen cell vial from liquid nitrogen or -80°C storage. Avoid prolonged exposure to air as this may affect cell viability.
- 3.2 Quickly thaw the vial in a 37°C water bath: Immerse the vial in the water bath until about 80% of the contents are thawed (approximately 1-2 minutes). Gently swirl the vial occasionally to help with uniform thawing.
- 3.3 Do not fully submerge the vial; ensure the cap remains above water to prevent contamination.

4 Dilution and Neutralization

- 4.1 Once the cells are thawed, immediately transfer the contents of the vial to a sterile centrifuge tube containing 5-10 mL of pre-warmed complete growth medium.



The thawed cells are transferred into a centrifuge tube using an aspirator.

4.2 Gently mix the tube by inverting it several times to ensure even distribution of cells.

5 **Centrifugation**

5.1 Centrifuge the tube at low speed (e.g., 1000 rpm for 5 minutes) to pellet the cells.



A benchtop centrifuge is used for collection of the cells.

- 5.2 After centrifugation, carefully aspirate the supernatant without disturbing the cell pellet. This removes most of the dimethyl sulfoxide (DMSO).



Removal of the supernatant is done using an aspirator.

6 **Resuspension**

- 6.1 Resuspend the cell pellet in an appropriate volume of fresh complete growth medium (usually 5-10 mL), depending on the desired cell density.
- 6.2 Gently pipette up and down to ensure the cells are fully resuspended without creating bubbles.

7 **Cell Counting and Viability Assessment**

- 7.1 Take a small aliquot of the cell suspension and use a hemocytometer or automated cell counter to determine the cell count and viability.



- 7.2 Assess cell viability using trypan blue exclusion method: Mix the cell suspension with an equal volume of trypan blue and count live (unstained) and dead (stained) cells under the microscope.



A bright field microscope is used to examine the cells during the cell counting.

8 Plating Cells

- 8.1 Based on the cell count and desired seeding density, transfer the appropriate volume of the cell suspension to a culture flask or plate.



- 8.2 Incubate the cells in a CO₂ incubator at 37°C.



An example of an incubator suitable for placement of cell lines in culture flasks.

9 Monitoring and Maintenance

9.1 After thawing, monitor the cells for confluence and morphology regularly.



9.2 Change the medium every 2-3 days or as necessary, depending on the growth characteristics of the cell line.

Note

- Be sure to handle the cells gently throughout the thawing process to avoid damaging the cells.
- It's recommended to use PPE and work in a sterile environment (biosafety cabinet) to minimize contamination risks.
- Always keep accurate records of thawed cell lines, including passage number and any treatments applied.

9.3 TIPS:

- Always use pre-warmed medium when resuspending thawed cells to ensure optimal recovery.
- Minimize the exposure of cells to DMSO, as it can be toxic at room temperature.
- Keep cryovials on ice when handling to prevent thawing before freezing.

- Cryopreservation allows you to maintain a reliable stock of cells for future experiments, reducing the need to frequently re-culture cells.

Subculturing Cell Lines (Adherent Cell Lines)

- 10 Subculturing (or passaging) cell lines is essential for maintaining healthy and viable cells for experiments. It involves transferring cells from a high-density culture to a new vessel with fresh growth medium.

Here are the materials and equipments needed for subculturing:

- Cell culture flasks or dishes
- Complete growth medium (pre-warmed)
- Sterile PBS
- Trypsin-EDTA solution (for adherent cells)
- Centrifuge and centrifuge tubes (for suspension cells)
- Cell counter (hemocytometer or automated cell counter)
- Biosafety cabinet
- 70% ethanol (for sterilization)

11 Preparation of Materials and Equipment

- 11.1 Pre-warm the medium and trypsin by placing the complete growth medium and trypsin-EDTA in a 37°C water bath.
- 11.2 Sterilize the work area by wiping down the cell culture hood with 70% ethanol. Also sterilize any materials (pipettes, flasks, etc.) before use.



Sterilization of the work area must be done thoroughly to avoid contamination of the cells.

12 Observation of Cell Morphology and Confluence

- 12.1 Check confluence under the microscope. Cells should be about 70-80% confluent (logarithmic growth phase) for optimal subculturing. Look for signs of over-confluence (crowding) or contamination.



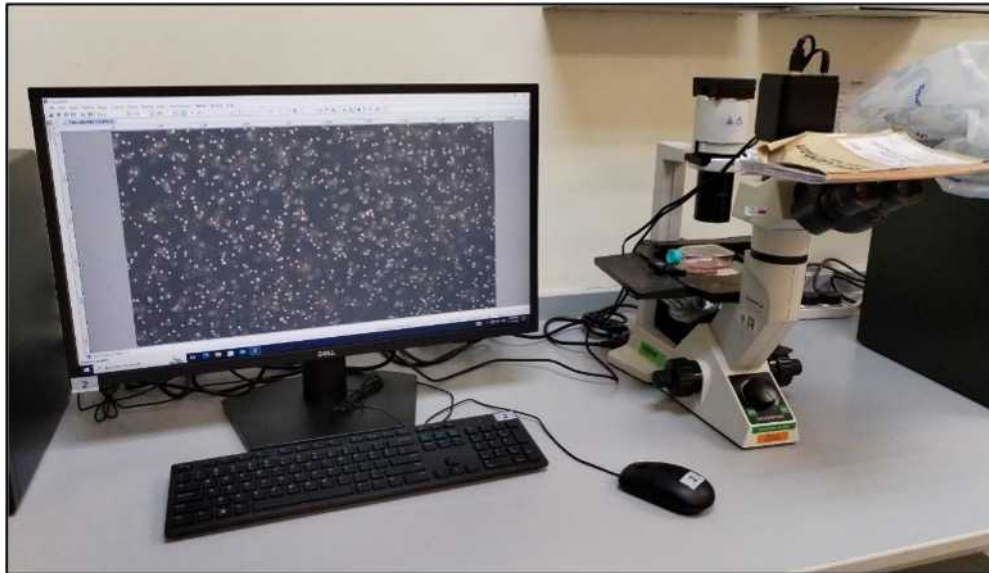
An image of how 70-80% cell confluency appears under the light microscope.

13 **Aspiration of Old Medium**

- 13.1 Aspirate spent medium inside a biosafety cabinet by using a sterile pipette without disturbing the cells.
- 13.2 Add sterile PBS (enough to cover the cells, typically 2-5 mL) to wash away residual medium and serum that can inhibit trypsin activity. Gently aspirate the PBS.

14 **Detachment of Cells**

- 14.1 Add enough trypsin-EDTA to cover the cells (e.g., 1-3 mL for a T25 flask).
- 14.2 Incubate at 37°C for 2-5 minutes, or until the cells round up and begin to detach.
- 14.3 Monitor under a microscope to avoid over-trypsinization.



An image of how the cells appear upon incubation with trypsin.

15 Trypsin Neutralization and Resuspending Cells

- 15.1 Add an equal volume (e.g., 5 mL) of pre-warmed complete growth medium to the flask to neutralize trypsin.
- 15.2 Gently pipette the medium up and down to resuspend the detached cells into a single-cell suspension.
- 15.3 For cell counting, take an aliquot of the cell suspension and dilute it using a hemocytometer or automated cell counter.
- 15.4 For seeding of cells into new flasks, dilute the cells based on your desired split ratio (e.g., 1:3, 1:5, or 1:10), then calculate how much cell suspension to transfer to a new flask.
- 15.5 Add the calculated volume of cell suspension to a new flask containing fresh pre-warmed complete medium (typically 5-10 mL for a T25 flask).
- 15.6 Gently swirl the flask to ensure even distribution of the cells. Place the new flask into a 37°C incubator with 5% CO₂.
- 15.7 Check the cells under the microscope after 24 hours to ensure they are adherent and growing well.

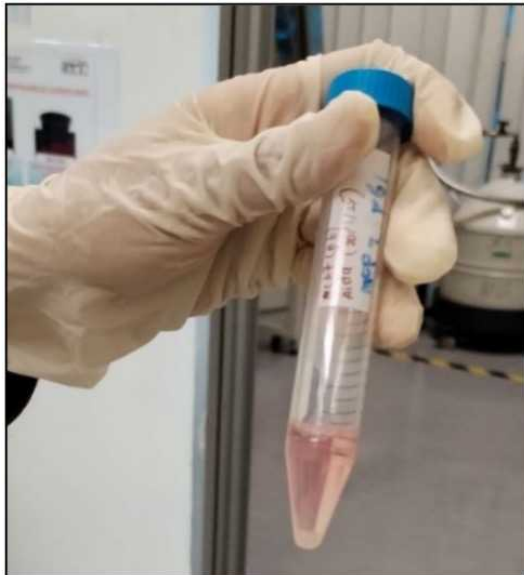
Subculturing Cell Lines (Suspension Cell Lines)

- 16 Pre-warm the medium by placing the complete growth medium in a 37°C water bath. Wipe down the biosafety cabinet and sterilize all materials.
- 17 Check cell density and viability under the microscope to ensure the cells are healthy and in the exponential growth phase.

Note

Suspension cells are subcultured based on density (usually $2-5 \times 10^5$ cells/mL).

- 18 For cell counting, take an aliquot from the cell suspension, and use a hemocytometer or automated counter to determine cell density.
- 19 Transfer the cell culture to a centrifuge tube and centrifuge at 1,000-1,500 rpm (200-300g) for 5-10 minutes to pellet the cells.
- 20 Carefully aspirate the old medium, leaving the cell pellet undisturbed. Then, add fresh, pre-warmed complete medium to the cell pellet and gently pipette up and down to resuspend the cells.



After centrifugation, the cells accumulated at the bottom of the tube, forming a pellet.



- 21 Based on the cell count or split ratio, transfer the required volume of cell suspension into a new culture flask. Add fresh medium to achieve the desired cell concentration (typically $2-5 \times 10^4$ cells/mL). Gently swirl the flask to evenly distribute the cells in the medium.
- 22 Incubate the cells in the incubator at 37°C, 5% CO₂. Observe cell growth over the next 24-48 hours to ensure healthy proliferation.

Note

- Subculture cells at the right confluence (70-80% for adherent cells, appropriate density for suspension cells).
- Minimize exposure to trypsin to prevent damage to adherent cells.
- Use fresh, pre-warmed medium for optimal cell recovery and growth.
- Maintain sterile techniques throughout the process to avoid contamination.

MTT Cytotoxicity Assay

- 23 The MTT assay is a colorimetric assay used to measure cell viability and cytotoxicity. The principle of the assay is based on the reduction of the yellow tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a purple formazan product by metabolically active cells.

Here are the materials and equipments needed for the assay:

- Cells in culture (adherent or suspension)
- Complete growth medium
- MTT reagent (stock solution: 5 mg/mL in PBS)
- DMSO
- 96-well plate
- Microplate reader (with a 570 nm filter)
- Sterile PBS
- Trypsin-EDTA (for adherent cells)
- Positive and negative controls (e.g., untreated control, known toxic agent)
- Incubator (37°C, 5% CO₂)

- 24 **Day 1: Cell Seeding**

- 24.1 For adherent cells, harvest cells using trypsin-EDTA and resuspend them in fresh complete medium.
For suspension cells, resuspend cells directly from the culture flask into fresh medium.
- 24.2 Count the cells using a hemocytometer or automated cell counter to ensure the correct cell density.
- 24.3 In a 96-well plate, seed 5,000–10,000 cells per well in 100 μ L of complete growth medium.
- 24.4 Leave some wells as blank (with medium only) to serve as a background control. Set three replicates for each condition (including controls and treatment groups).



- 24.5 Incubate the plate at 37°C, 5% CO₂ for 24 hours to allow the cells to attach and grow.

25 Day 2: Treatment with Test Compounds

- 25.1 Prepare a range of concentrations of the test compound in complete growth medium (e.g., serial dilutions of an anticancer drug). Include a positive control (e.g., a compound known to be cytotoxic) and a negative control (untreated cells).
- 25.2 Prior to the treatment to the cells, remove the old medium from the cells (if necessary) and replace it with 100 μ L of the test compound solution at the desired concentration.
- 25.3 For untreated control wells, add 100 μ L of fresh medium into the wells.

25.4 Incubate the cells with the test compounds at 37°C, 5% CO₂ for 24-72 hours, depending on the experiment.

26 **Day 3: MTT Addition (After Treatment Incubation)**

26.1 Prepare MTT stock solution (5 mg/mL in PBS) and filter-sterilize it.

26.2 Dilute the stock solution in complete medium (1:10 ratio) to obtain a final concentration of 0.5 mg/mL MTT in the wells.

26.3 Remove the medium from the cells (optional for some protocols) and add 10 µL of MTT solution to each well.

26.4 Incubate the plate at 37°C, 5% CO₂ for 3-4 hours. During this time, metabolically active cells will reduce the yellow MTT to purple formazan crystals.

27 **Day 4: Dissolving Formazan Crystals and Measuring Absorbance**

27.1 After incubation, carefully remove the medium without disturbing the formazan crystals formed at the bottom of the wells.

27.2 Add 100 µL of DMSO to each well to dissolve the formazan crystals. DMSO will turn the crystals into a purple-colored solution.



27.3 Shake the plate gently for 5-10 minutes to ensure complete dissolution of the crystals.

27.4 Use a microplate reader to measure the absorbance at 570 nm (with a reference wavelength of 630 nm, if available). The intensity of the color correlates with the number of viable, metabolically active cells.

28 **Data Analysis**

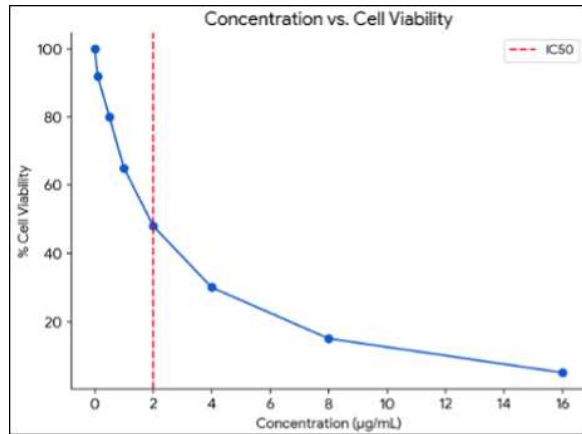
28.1 For blank correction, subtract the background absorbance (from blank wells containing medium but no cells) from all absorbance values.

28.2 For each concentration of the test compound, calculate the percentage cell viability relative to the untreated control (considered as 100% viability) using the formula:

$$\text{Cell Viability (\%)} = (\text{Absorbance of treated cells} / \text{Absorbance of control cells}) \times 100$$

28.3 Plot a graph of cell viability (%) on the y-axis against the log of the test compound concentration on the x-axis. This will give you a dose-response curve that shows the effect of the compound on cell viability.

28.4 From the dose-response curve, if needed, determine the IC_{50} value, which is the concentration of the compound that inhibits 50% of cell viability.



Cryopreserving Cell Lines

- 29 Cryopreserving cell lines is an essential technique to store cells for long periods without losing their viability or functionality.

Here are the materials and equipments needed for cell lines cryopreservation:

- Cells in culture (adherent or suspension)
- Cryopreservation medium: 10% DMSO + 90% Fetal Bovine Serum (FBS) or commercial cryopreservation medium (e.g., CryoStor)
- Sterile cryovials (labelled)
- Centrifuge and centrifuge tubes
- Trypsin-EDTA (for adherent cells)
- PBS
- Biosafety cabinet
- Freezing container (e.g., Mr. Frosty) or programmable freezer
- Liquid nitrogen storage

30 Preparation of Adherent Cells for Cryopreservation

- 30.1 Cells should be in the logarithmic growth phase, typically around 70-80% confluence.



An image of how 70-80% cell confluency appears under the light microscope.

30.2 Aspirate the culture medium and wash the cells once with sterile PBS to remove excess serum and debris.

30.3 Add trypsin-EDTA to detach the adherent cells. Incubate for 2-5 minutes (or until cells are rounded and detached) at 37°C.

30.4 Add complete culture medium (with FBS) to neutralize the trypsin and resuspend the cells.

31 **Preparation of Suspension Cells for Cryopreservation**

31.1 To harvest the cells, collect the cells by pipetting them into a sterile centrifuge tube. If the culture is dense, dilute it to avoid overloading the cells.

31.2 Centrifuge the cells at 1,000-1,500 rpm (or 200-300g) for 5-10 minutes to pellet the cells.

31.3 Remove a small aliquot of the cell suspension to count the cells using a hemocytometer or an automated cell counter.

31.4 Ensure the cell density is typically around 1-5 million cells/mL for cryopreservation.

- 31.5 Centrifuge the cell suspension again at 1,000-1,500 rpm (200-300g) for 5-10 minutes to pellet the cells.
- 31.6 After centrifugation, carefully aspirate and discard the supernatant without disturbing the cell pellet.

Note

- To prepare the cryopreservation medium, use a solution of 90% FBS + 10% DMSO, or a commercially available cryopreservation medium.
- DMSO is essential as it prevents the formation of ice crystals that can damage the cells.



- 31.7 Gently resuspend the cell pellet in cold cryopreservation medium at a concentration of 1-5 million cells/mL. Use a cold medium to reduce thermal shock to the cells.
- 31.8 To make aliquots, dispense 1 mL of cell suspension into pre-labelled cryovials. Ensure proper labelling with cell line name, date, passage number, and any other relevant information.



An example of cell suspension in a labelled 1 ml cryovial.

- 31.9 If a freezing container (e.g., Mr. Frosty) is used, place the cryovials inside and fill the container with isopropanol. This allows for a controlled freezing rate of approximately -1°C per minute.



Alternatively, if available, use a programmable freezer set to cool at -1°C per minute until the temperature reaches -80°C .

- 31.10 Transfer the cell suspension-filled freezing container or cryovials to a -80°C freezer and leave overnight.



- 31.11 An example of a liquid nitrogen tank used for storing cell lines. For long-term storage of the cells, the cryovials that have been frozen at -80°C overnight must be transferred to a liquid nitrogen storage tank for long-term preservation at -196°C . Vials can be stored indefinitely in liquid nitrogen if handled properly.



An example of a liquid nitrogen tank used for storing cell lines.

