Tissue Culture Requirements and Methods

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I. MAINTENANCE

- Cultures should be examined daily, observing the morphology, the color of the medium and the density of the cells.
- A tissue culture log should be maintained that is separate from your regular laboratory notebook.
- The log should contain: the name of the cell line, the medium components and any alterations to the standard medium, the dates on which the cells were split and/or fed, a calculation of the doubling time of the culture (this should be done at least once during the semester), and any observations relative to the morphology, etc.

A. Growth pattern.

- Cells will initially go through a quiescent or lag phase that depends on:
  - the cell type
  - the seeding density
  - the media components
  - and previous handling
- The cells will then go into exponential growth where they have the highest metabolic activity.

A. Growth pattern

- The cells will then enter into stationary phase where the number of cells is constant, this is characteristic of a confluent population (where all growth surfaces are covered).
- Cells are harvested when the cells have reached a population density which suppresses growth.

B. Harvesting.

- Ideally, cells are harvested when they are in a semi-confluent state and are still in log phase.
- Cells that are not passaged and are allowed to grow to a confluent state can sometime lag for a long period of time and some may never recover.
- It is also essential to keep your cells as happy as possible to maximize the efficiency of transformation.

Mechanical

- A rubber spatula can be used to physically remove the cells from the growth surface.

This method is quick and easy but is also disruptive to the cells and may result in significant cell death.

This method is best when harvesting many different samples of cells for preparing extracts, i.e., when viability is not important.

Proteolytic enzymes

- Trypsin, collagenase, or pronase, usually in combination with EDTA, causes cells to detach from the growth surface.
- This method is fast and reliable but can damage the cell surface by digesting exposed cell surface proteins. The proteolysis reaction can be quickly terminated by the addition of complete medium containing serum
- EDTA - EDTA alone can also be used to detach cells and seems to be gentler on the cells than trypsin

The standard procedure for detaching adherent cells is as follows:

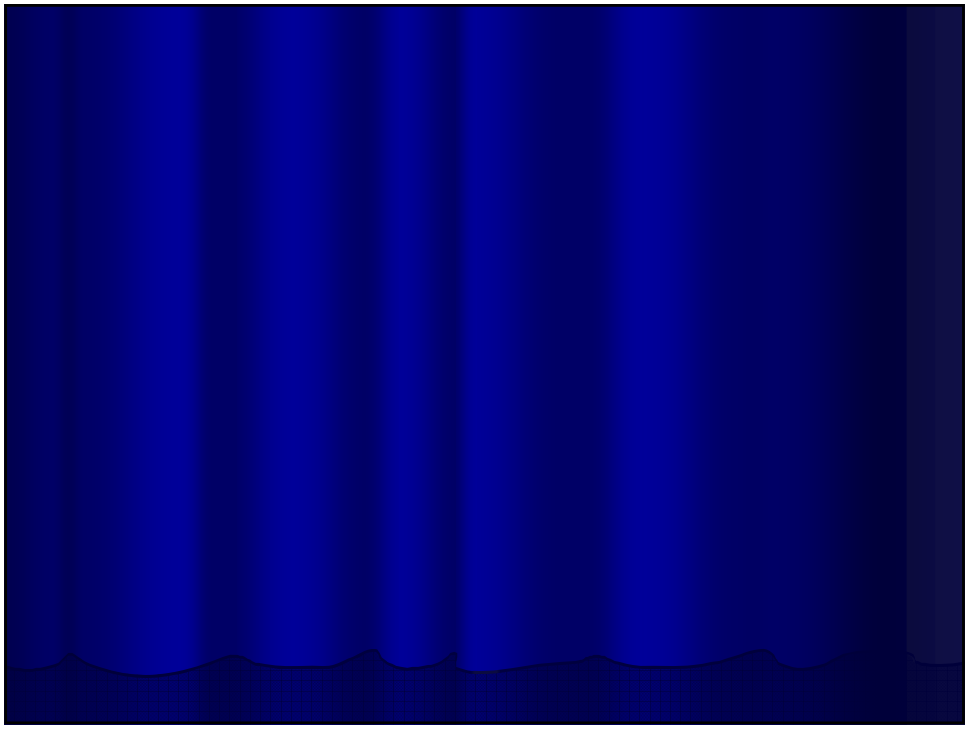
a. Wash once with a buffer solution.
b. Treat with dissociating agent
c. Observe cells under the microscope. Incubate until cells become rounded and loosen when flask is gently tapped with the side of the hand.
d. Transfer cells to a culture tube and dilute with medium containing serum.
e. Spin down cells, remove supernatant and replace with fresh medium.
f. Count the cells in a hemacytometer, and dilute as appropriate into fresh medium.

Trypsin-EDTA

a. Remove medium from culture dish and wash cells in a balanced salt solution without Ca^{++} or Mg^{++}. Remove the wash solution.
b. Add enough trypsin-EDTA solution to cover the bottom of the culture vessel and then pour off the excess.
c. Place culture in the 37°C incubator for 2 minutes.
d. Monitor cells under microscope. Cells are beginning to detach when they appear rounded.
e. As soon as cells are in suspension, immediately add culture medium containing serum. Wash cells once with serum containing medium and dilute as appropriate (generally 4-20 fold).

EDTA alone

a. **Prepare a 2 mM EDTA solution in a balanced salt solution (i.e., PBS without Ca^{++} or Mg^{++}).**
b. **Remove medium from culture vessel by aspiration and wash the monolayer to remove all traces of serum. Remove salt solution by aspiration.**
c. **Dispense enough EDTA solution into culture vessels to completely cover the monolayer of cells.**
d. **The coated cells are allowed to incubate until cells detach from the surface. Progress can be checked by examination with an inverted microscope. Cells can be gently nudged by banging the side of the flask against the palm of the hand.**
e. **Dilute cells with fresh medium and transfer to a sterile centrifuge tube.**
f. **Spin cells down, remove supernatant, and resuspend in culture medium (or freezing medium if cells are to be frozen). Dilute as appropriate into culture flasks.**



Viable cell counts

- Using a hemocytometer to determine total cell counts and viable cell numbers:

- Trypan blue is one of several stains recommended for use in dye exclusion procedures for viable cell counting. This method is based on the principle that live cells do not take up certain dyes, whereas dead cells do.

1. Prepare a cell suspension, and combine 20 µl of cells with 20 µl of trypan blue suspension (0.4%). Mix thoroughly and allow to stand for 5-15 minutes.

Viable cell counts

2. With the cover slip in place, transfer a small amount of trypan blue-cell suspension to both chambers of the hemocytometer by carefully touching the edge of the cover slip with the pipette tip and allowing each chamber to fill by capillary action. Do not overfill or underfill the chambers.

3. Starting with 1 chamber of the hemocytometer, count all the cells in the 1 mm center square and four 1 mm corner square. Keep a separate count of viable and non-viable cells.

Viable cell counts

4. If there are too many or too few cells to count, repeat the procedure either concentrating or diluting the original suspension as appropriate.

5. The circle indicates the approximate area covered at 100X microscope magnification (10X ocular and 10X objective). Include cells on top and left touching middle line. Do not count cells touching middle line at bottom and right. Count 4 corner squares and middle square in both chambers and calculate the average.

Viable cell counts

6. Each large square of the hemocytometer, with cover-slip in place, represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is equivalent to approximately 1 ml, the total number of cells per ml will be determined using the following calculations:

Cells/ml =

average cell count per square x dilution factor x 10^4

Total cells =

cells/ml x the original volume of fluid from which the cell sample was removed

% Cell viability =

total viable cells (unstained)/total cells x 100

Thawing frozen cells.

a. **Remove cells from frozen storage and quickly thaw in a 37°C water bath by gently agitating vial.**
b. **As soon as the ice crystals melt, pipette gently into a culture flask containing pre-warmed growth medium.**
c. **Log out cells in the "Liquid Nitrogen Freezer Log" Book.**

Freezing cells.

- Harvest cells as usual and wash once with complete medium.
- Resuspend cells in complete medium and determine cell count/viability.
- Centrifuge and resuspend in ice-cold freezing medium: 90% calf serum/10% DMSO, at 10^6 - 10^7 cells/ml. Keep cells on ice.
- Transfer 1 ml aliquots to freezer vials on ice.

Freezing cells

- Place in a Mr. Frosty container at room temperature that has sufficient isopropanol.
- Place the Mr. Frosty in the -70°C freezer overnight. Note: Cells should be exposed to freezing medium for as little time as possible prior to freezing
- Next day, transfer to liquid nitrogen (DON'T FORGET) and log in the "Liquid Nitrogen Freezer Log" Book.


Thank You