

Guide to Subculturing Cell Cultures

Morphology Each cell line or cell type expresses different characteristics in terms of growth and appearance in culture. Many cell lines grow as a single sheet monolayer attached to both themselves, and the culture vessel. Other cell types exist as single cells or clumps of cells suspended in the growth medium. Both adherent and suspension cultures must be maintained regularly to prevent overgrowth and accelerated cell death from exhausted medium and to promote the growth of the next generation of cells.

Cell Dissociation Viable subcultures may be obtained by transferring a particular volume of cells to new culture vessels with fresh medium. These fresh cultures are allowed to grow and divide as normal until such time the culture reaches confluence and the cells are used for experiments or subcultured. To do this correctly, obtain a single-cell suspension first. For adherent cell types, proteolytic enzymes, such as trypsin (MT Catalog Numbers 25-050, 25-052, 25-053, and 25-054), are used to break cell-cell and cell-substrate bonds and create a suspension from which new cultures may be split. For cultures already growing in suspension, this enzyme step is not necessary.

Harvesting describes the detachment of adherent cell lines to prepare a cell suspension for counting. During this step, the intercellular and intracellular (cell-substrate) bonds are broken, allowing the cells to separate into a single cell suspension. Depending on the cell type and the culture environment, this is achieved by using enzymatic or non-enzymatic dissociation solutions, such as Cellstripper™, catalog number 25-056.

Growth Phases Cell growth typically exhibits a consistent pattern comprising three main phases, including an initial lag phase, a period of logarithmic growth, and a final stationary phase. See Figure 1. This growth pattern continues for each subculture despite the cell type. The initial lag phase occurs at the beginning of a subculture as the cells become accustomed to the new environment, during which they do not divide. The length of the log phase is determined by cell conditions prior to subculturing, as well as the seeding density and changes in the growth medium. The log (logarithmic) phase is a period of active proliferation, during which the number of cells increases exponentially. The length of the log phase is determined by many factors, including the seeding density and rate of normal cell growth, as well as factors affecting the lag phase. The final stationary phase occurs when the rate of cell proliferation slows down. During this phase, the rate of cell division may be balanced by the rate of cell death, thus showing no change in cell density.

Subculturing is usually performed during the log phase when the cells are at their healthiest and are able to adapt to the new environment most efficiently. This is also the best time for cryopreservation and functionality studies. Check for cultures that appear at least 70% confluent.

Culture Examination Before handling, it is good practice to observe cultures both microscopically and macroscopically. Visual observations of the culture flask and medium may indicate evidence of microbial contamination including pH fluctuations and turbidity, as well as fungal colonies. The monolayer may also be viewed macroscopically to obtain a general idea of confluence. See Figures 2 and 3 below. This is most easily performed by viewing the culture vessel against a light source. Further microscopic observation may substantiate abnormal cell appearance and confirm microbial contamination. Rounding cells may indicate mitosis, especially if the cells are very refractive, or bright. Dead cells do not express this same brightness.

Figure 1. Growth Phases of Cells in Culture

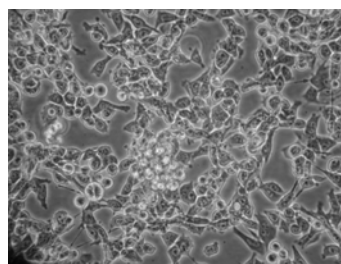
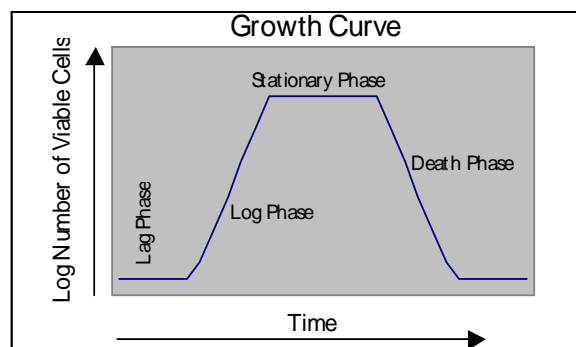


Figure 2. Monolayer Confluent Monolayer. This culture is ready for splitting.

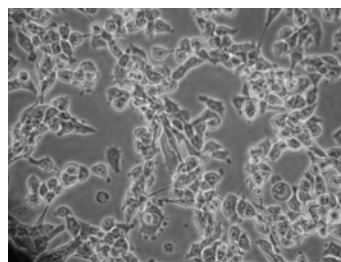


Figure 3. Monolayer This monolayer appears less confluent than the monolayer in Figure 1. A cell count would determine if maximum cell density has been reached.

Routine Subcultivation & Maintenance

Procedure The following procedure describes the basic principles involved in the routine subcultivation and maintenance of cell cultures. **It is imperative that aseptic technique is maintained throughout this procedure.**

1. Visually examine cultures, as discussed above.
2. Harvest adherent cell lines using a dissociating agent. For suspension lines, this step is not required. Proceed to Step 7.
3. Aspirate and discard the culture medium.
4. Rinse the monolayer with the dissociation solution or a buffered salt solution without calcium and magnesium. This rinsing step removes any residual serum from the monolayer that could inactivate the trypsin. Use about 3ml for 25-cm² flasks and 5ml for 75-cm² flasks. Be sure to add the solution on the side of the flask opposite the monolayer to prevent cell loss. Swirl the solution gently across the cell sheet. Remove and discard the solution.
5. Repeat the washing step, this time using the dissociation solution. For more fastidious cell lines, the flask can be placed in a 37°C incubator to facilitate the enzyme. The flask may also be observed microscopically to monitor progress and prevent over-exposure of the cells to the enzyme activity. Non-enzymatic solutions, such as Cellstripper™, will require longer incubation times than when using enzymes like trypsin, but are more gentle to the cells.
6. Once the cells are detached, add the desired amount of growth medium to the flask, creating a cell suspension. When pipetting the growth medium into the flask, be sure to “wash” the sides of the flask to ensure all cells become suspended. It is not unusual for a small amount of cells to remain attached to the flask or substrate. However, more vigorous pipetting may be necessary to break up cell clumps or to aid in the removal of attached cells.
7. Using the cell suspension, determine the appropriate inoculum for subculturing the particular cell line. This seeding density may be determined by performing growth rate studies, or by counting. When simply passing a culture, when tracking exact cell densities is not necessary, a split according to a suspension ratio is commonly used. For example, a 1:2 ratio indicates that the cell suspension may be split in half between two culture flasks of equal surface area. Counting, however, requires a hemacytometer or other cell counting device. A hemacytometer is used in conjunction with a stain, such as trypan blue (25-900-CI). Simply remove a small amount of the cell suspension, such as 500µl, and mix with an equal amount of trypanblue. Using a clean hemacytometer, determine the number of cells/ml in the suspension and calculate the volume of suspension required to seed the desired density for each subculture.
8. Dispense these aliquots into clean, sterile, labeled culture vessels. Add the desired amount of culture medium to the vessels and pipette to ensure equal distribution of cells.
9. Return cultures to their appropriate environment. Most mammalian cell lines require a 37°C growth environment including a carbon dioxide level of 5%. The type of culture medium may alter the type of environment required for cell growth.
10. After about 24hours, observe the culture for reattachment and active growth. Note any unusual observations. Change medium as needed and subculture when necessary.

References:

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4. Freshney, R.I. *Culture of Animal Cells: A Manual of Basic Technique, 4th Ed.* Wiley Liss, New York, (2000).