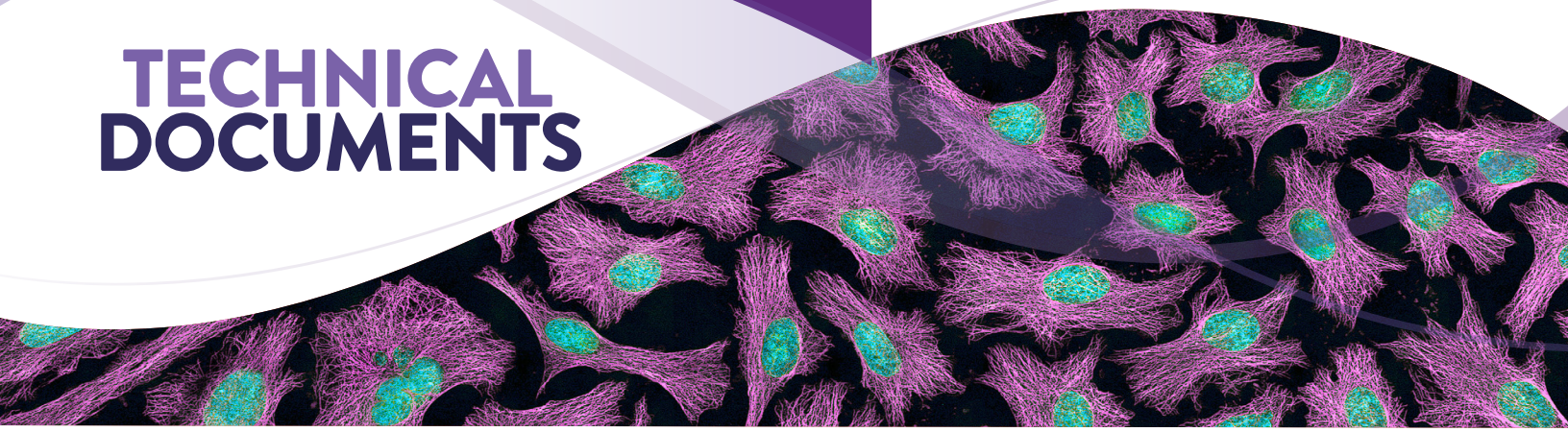
ATCC

TECHNICAL DOCUMENTS



GUIDE TO SUBCULTURING CELL LINE MONOLAYERS

Most cell lines and primary cell cultures grow as a single thickness cell layer (monolayer) or sheet attached to glass or specially treated plastic substrates. In order to keep cultures healthy and actively growing it is usually necessary to subculture them at regular intervals.

The most common methods of subcultivation involve the breakage of both intercellular and cell-to-substrate connections by the use of proteolytic enzymes such as trypsin or collagenase. After the cells have been dissociated into a suspension consisting primarily of single cells, they are diluted and transferred into fresh culture vessels. There they can reattach and begin to grow and divide and after a period of incubation again reach near confluency. At this point they can again be subcultured or used for experiments.

The following guide describes the basic principles involved in the routine subcultivation and maintenance of a typical monolayer cell culture. For consistent results, maintaining good records is important. Records should include date of cell passaging and passage number.

EXAMINE CULTURES

It is good practice to routinely and carefully examine cultures to determine their status and health. First, examine the contents of the culture vessel with the unaided eye for macroscopic evidence of microbial contamination, such as unexpected pH shifts or turbidity and particles in the medium. Also look for small fungal colonies that may not be readily visible through the microscope. These colonies may be floating at the medium-air interface, especially around the edges of the vessel.

Second, check the general cellular morphology and growth patterns using an inverted microscope. Carefully look for any microscopic evidence of microbial contamination. In some cell lines, cells floating in the medium are a sign of cellular death. However, many cells round up during mitosis, forming very refractile (bright) spheres that may float free if the culture is physically disturbed. Dead cells often round up and become detached but are usually not refractile.

In addition to these daily examinations, culture the cells periodically for fungi and bacteria and test for mycoplasma contamination. There are several methods that can be used to check for these contaminants. Refer to the ATCC website for information on mycoplasma detection kits and services.

PREPARE MEDIUM

Prepare fresh culture medium that is recommended for the cell line and label the culture vessels appropriately. Be sure to add the supplements and equilibrate the pH. In a 75-cm^2 flask, use approximately 12 to 15 mL of medium. Adjust this volume accordingly for other culture vessels.

HARVEST CELLS

Most cell cultures grow best if they are subcultured before they have reached confluency (while there is still some growing space available). This will help keep the cells in the active log phase of growth. Any unusual observations should always be noted on the record sheets that are maintained for each cell line. The harvesting step is designed to remove the cells from their substrate and to break the intercellular bonds as gently as possible. For most cell cultures this requires using chemical or enzymatic dissociating agents in a buffered saline solution.

Trypsin is the most common dissociating agent and is normally used in concentrations from 0.05% to 0.25%. Optimal working concentrations are usually determined by using the lowest concentrations of trypsin that will remove the cells from the substrate and give single cell suspensions in a relatively short period of time (a 10- to 15-min incubation). Some cells are more difficult to detach than others, so trypsin solutions are frequently supplemented with enzymes, such as collagenase, or chelating agents, such as EDTA, to improve results.

Cells grown in medium containing mammalian-derived serum must first be washed to remove the serum since trypsin is inhibited by its presence. Residual serum is frequently responsible for failure of the trypsin solution to dissociate the cells from the substrate and each other. There are a variety of buffered saline solutions available. The most popular are based on modifications of Hanks' buffered saline solution, Earle's buffered saline solution, or Dulbecco's phosphate-buffered saline solution. Usually these formulations are modified to be calcium- and magnesium-free (CMF-PBS) if they are to be used for dissociating cells since these two ions play an important role in cell-to-cell and cell-to-substrate attachment.

STEPS FOR HARVESTING A CELL MONOLAYER:

1. Remove and discard the culture medium.
2. Rinse the cell sheet with 5 mL of the dissociating solution and remove. (Amounts used in this protocol are for a 75-cm^2 flask; reduce or increase amount proportionally for cultures in other vessels.) If the dissociating solution contains trypsin, it is very important to remove all traces of serum, which contains trypsin inhibitors.
3. Add 2 to 3 mL of the dissociating solution. Check the progress of the enzyme treatment every 5 minutes with an inverted phase-contrast microscope. Monolayers that are particularly difficult to detach can be placed at 37°C to facilitate dispersal. Prewarming of the enzyme solution will also decrease the exposure period. To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach.
4. Add 6 to 8 mL of growth medium to the cell suspension using a pipette, and wash any remaining cells from the bottom of the culture vessel. At this point a quick check on the inverted microscope should show the cell suspension to consist of at least 95% single cells. If this is not the case, more vigorous pipetting may be necessary.
5. Collect the cell suspension, count or divide the inoculum as necessary, and dispense into the prepared culture vessels. For some cell lines and enzyme solutions (collagenase), it is necessary to remove the enzymes by gentle centrifugation (5 min at 125 × *g*) prior to dispensing (plating) the cells.

COUNT CELLS OR SPLIT SUSPENSION

To determine growth rates or set up cultures at known concentrations, it is necessary to count the cells in suspension. Hemocytometers or electronic cell counting devices can be used. Hemocytometers have the advantage of being less expensive and allowing viability determinations at the same time.

Mix the cell suspension gently and remove a 0.5 mL aliquot for counting. To this add 0.5 mL of a vital stain for cell count such as trypan blue or erythrosin B. Mix well, withdraw a sample, and carefully load a clean hemocytometer.

Determine the actual concentration of your cell suspension (number of cells/mL) and the percent viability, and calculate the volume of suspension that is required to seed each subculture at appropriate density. Instead of counting cells, the suspension is often split among a number of culture vessels. For example, a 1:2 split means dividing the cell suspension of one vessel into two new vessels, usually of equivalent surface area. This method is appropriate for routine maintenance of cell lines where keeping track of exact cell densities is not important.

PLATE CULTURES

Dispense appropriate aliquots of the cell suspension into the prepared vessels (adding concentrated cell suspensions to empty culture vessels can result in uneven cell attachment and growth). Faster-growing cultures are usually set up at lower concentrations. Some cultures do not grow well unless a minimum concentration of cells is initially added. However, most cultures will thrive at initial concentrations ranging from 10^3 to 10^4 cells/cm^2 or higher (7.5×10^4 to 7.5×10^5 cells per 75-cm^2 flask).

RE-INCUBATE CULTURES

Place cultures back in the incubator. Most mammalian cell cultures do best at a steady temperature between 35°C and 37°C. In addition to maintaining constant temperature, incubators that are used for unsealed cultures such as dishes and multiple-well plates must also maintain high humidity and carbon dioxide levels. The high humidity cuts down evaporation losses in unsealed culture vessels, which would result in the culture medium becoming hypertonic and stressing the cells. The elevated carbon dioxide levels (usually 5% to 10%) help maintain the proper pH (7.0 to 7.6) if used with a buffer system containing the appropriate amount of bicarbonate.

In order for this type of buffer system to work it is necessary to allow gas exchange by using unsealed (loosened caps) or gas-permeable culture vessels. If CO_2 is not available, a buffer system can maintain appropriate pH in closed vessels.

Examine the culture the following day to ensure the cells have reattached and are actively growing. Change medium as needed; for most actively growing cultures two to three times per week is typical.

TROUBLESHOOTING

CELLS ARE DIFFICULT TO REMOVE

- The dissociating agent is too weak. Use a higher enzyme concentration or add EDTA solution to supplement the enzyme solution. Also try incubating cells at 37°C to increase activity of dissociating enzymes.
- Inhibitors (serum for trypsin) in the medium are inactivating the dissociating enzymes. Rinse the cell monolayer more thoroughly before adding the dissociating solution.
- Cells have been at confluent density for a long time and the cell-to-cell junctions are so tight they are preventing the enzyme from reaching the substrate-cell interface. Subculture cells before they are 100% confluent.

CLUMPS FORM AFTER DISSOCIATION

- DNA has been released from lysed cells during the procedure and has uncoiled and gelled in the medium, forming a viscous mass containing the cells; the dissociating procedure may have been too harsh (pipetting too vigorous, dissociating agent too strong or toxic). Add a drop of sterile DNase (1 mg/mL in water) to the cell suspension to break down the DNA strands. Treat the cells more gently during pipetting to prevent physical damage to their membranes.
- Cells are reaggregating; try chilling the cell suspension if there is a delay before the suspension is dispensed into medium.
- Cells were centrifuged too hard or too long. Use gentle centrifugation (5 min at 125 × *g*).

CELLS HAVE DIFFICULTY REATTACHING TO CULTURE VESSEL

- Heavy treatment with dissociating enzymes may have stripped necessary attachment proteins from cell surface. Treat cells more gently, use lower enzyme concentrations, shorten enzyme exposure, or incubate with enzymes at lower temperature.
- Too little serum or attachment factors are in medium (common with serum-free medium). Add attachment factors or use protein-coated plates (collagen, polylysine, gelatin, etc.).
- Dissociating agent was not removed or inactivated. Add specific enzyme inhibitors or remove enzyme by gentle centrifugation (5 min at 125 × *g*) followed by a medium change.

VIABILITY IS LOWER THAN EXPECTED

- Dissociating procedure is too harsh (pipetting too vigorous, dissociating agent too strong or toxic, cells were centrifuged too hard or too long).
- There are problems with the balanced salt solution such as pH or osmolality.
- Cell suspension is left too long at a high cell concentration prior to plating. Keep cells on crushed ice prior to plating.
- The medium is faulty. Use the recommended formulation and make sure it contains all the required additives. Due to the many different versions of commercial media listed in catalogs, it is easy to inadvertently purchase media lacking critical components, such as glutamine.

REFERENCES

1 Freshney RI. Culture of Animal Cells: A Manual of Basic Technique, 4th ed. New York: Wiley Liss, 2000.

2 Jacoby WB, Pasten IH (eds.). Monolayer culture techniques. In: Methods in Enzymology: Cell Culture. Vol. 58. New York: Academic Press, 1979.

3 Schaeffer WI, Waymouth C. Enzymes used for disaggregation of tissue to obtain viable cells. In: Cell Biology I: Biological Handbook. P.L. Altman and D.D. Katz, eds. Bethesda, MD: FASEB; 46-47,1976.

4 Waymouth C. Balanced salt solutions for animal tissues. In: Cell Biology I: Biological Handbook. P.L. Altman and D.D. Katz, eds. Bethesda, MD: FASEB; 61-63, 1976.

10801 University Boulevard
Manassas, Virginia 20110-2209

703.365.2700 703.365.2701 sales@atcc.org www.atcc.org

TD-112021-v05