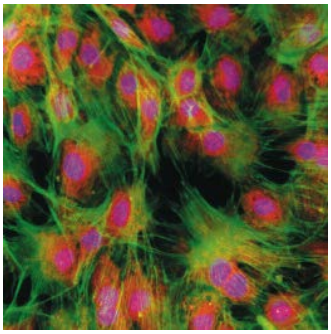
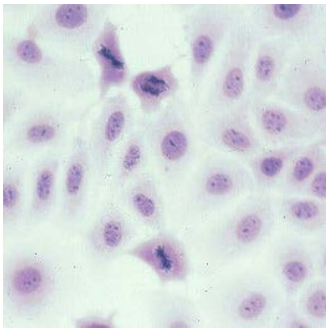
Subculturing Monolayer Cell Cultures

Protocol

CORNING





Introduction

Most animal cell lines and primary cultures can be grown as a single thickness cell layer or sheet attached to a plastic, glass, or other appropriate substrate depending on cell type. Once the available substrate surface is covered by cells (a confluent culture), growth slows and then ceases. Thus, in order to keep the cells healthy and actively growing, it is necessary to subculture them at regular intervals. Usually, this subcultivation process involves breaking the bonds or cellular "glue" that attaches the cells to the substrate and to each other by using proteolytic enzymes such as trypsin, dispase, accutase, or collagenase. Occasionally, these enzymes or dissociating agents are combined with divalent cation chelators such as EDTA (binds calcium and magnesium ions). The loosened cells are then removed from the culture vessel, counted, diluted, and subdivided into new vessels. Cells then reattach, begin to grow and divide, and, after a suitable incubation period (depending on the initial inoculum size, growth conditions and cell line), again reach saturation or confluency. At this point, the subcultivation cycle can be repeated.

The following protocol covers the basic techniques that are suitable for subculturing many cell lines.

Supplies

Nonsterile

- Pipetting aids
- Disposal tray or bucket for pipets
- Bottle of 70% alcohol for wiping down work area
- Paper towels
- Marking pen
- Inverted phase contrast microscope
- Ice bucket or Corning® CoolBox™ Module
- Liquid waste container
- Hemacytometer or Corning Cell Counter
- 37°C incubator

Sterile

- Flask of actively growing cells that are 80% to 90% confluent
- Cell culture medium. This should contain all of the additives (fetal bovine serum, glutamine, etc.) required by the above cell line.
- Calcium- and Magnesium-free Phosphate-Buffered Saline CMF-PBS (10 mL). This simple salt solution is used to maintain proper pH and osmotic balance while the cells are being washed to remove protease inhibitors that are found in most animal sera.
- 0.1% Trypsin solution: Trypsin is normally used in concentrations ranging from 0.05% to 0.25%. Working concentrations are usually determined by using the lowest trypsin concentration that can remove the cells from the substrate and give a single cell suspension in a relatively short time (5 to 10 minutes). Trypsin solutions are often supplemented with other enzymes (collagenase) or chelating agents (EDTA) to improve its performance.
- 15 mL disposable screw cap centrifuge tubes (Corning Cat. No. 430055)
- Appropriate culture vessels

- 1, 5, 10 and 25 mL pipets (Corning 4485, 4487, 4488, and 4489)
- Sterile 0.04% Trypan blue solution for viability staining
- 20 µL pipettor (Corning 4072[1])
- Pipet tips
- Laminar flow hood

Procedure

1. Examination

Prior to subcultivation, it is important to examine your cultures daily. Using an inverted phase contrast microscope (100 to 200X), quickly check the general appearance of your culture. Look for signs of microbial contamination. Many cells round up during mitosis, forming very refractile (bright) spheres that may float free of the surface when the culture is disturbed. Dead cells often round up and become detached but are usually not bright or refractile.

NOTE: A phosphate buffered saline is used for both rinsing and the trypsin solution since it maintains a physiological pH without requiring a closed system (required by buffers based on Hanks' saline) or gassing with carbon dioxide (required by buffers based on Earle's saline). Calcium and magnesium are omitted because these play a role in cell attachment.

2. Cell harvesting

This step removes the cells from the plastic substrate and breaks cell-to-cell bonds as gently as possible. When using enzymatic dissociation: a) the old medium is removed and discarded; b) the cell monolayer is gently rinsed; c) the enzyme solution is added and the culture incubated until the cells are released. There are many variations of this protocol; the following is a commonly used approach.

NOTE: Do not forget to examine the culture vessel with the unaided eye to look for small fungal colonies that may be floating at the media-air interface (especially near the vessel neck) and thus not visible through the microscope.

- Using a sterile pipet remove and discard the culture medium. All materials and solutions exposed to cells must be disposed of properly. Medium can be left in the pipets if they are placed in disinfectant.
- For a T-75 flask, wash the cell monolayer by adding 5 to 10 mL of 1X CMF to the flask, and then slowly rock it back and forth to remove all traces of serum. Remove and discard the wash solution. Failure to remove traces of serum is frequently responsible for failure of the trypsin solution to remove the cells from the vessel. Proportionally reduce or increase the volumes used in this protocol for smaller or larger culture vessels.

NOTE: Two CMF-PBS washes and/or rinsing with trypsin can be used for removing more difficult cells.

- Add 5 mL of the trypsin solution (in CMF) to the flask and place the flask back in an incubator at 37°C to increase the activity of the enzyme solution. (Prewarming of the enzyme solution to 37°C will decrease the required exposure period.) Proportionally reduce or increase the volumes used in this protocol for smaller or larger culture vessels.
- With an inverted phase contrast microscope, check the progress of the enzyme treatment every few minutes. Some cell types detach very quickly. Customers should follow advice from cell line manufacture as to length and type of enzyme exposure. Once most of the cells have rounded up, gently tap the side of the flask to detach them from the plastic surface. Then add 5 mL of growth medium containing serum to inactivate the enzyme to the cell suspension and, using a 10 mL pipet, vigorously wash any remaining cells from the bottom of the culture vessel. At this point a quick check on the inverted microscope should show that the cell suspension consists of at least 95% single cells. If this is not the case, more vigorous pipetting or trituration may be necessary.

NOTE: For difficult to break up cell clumps, try holding the pipet tip tight against and perpendicular to the side of the flask, and then forcibly expel its contents. This will create a strong shearing force that should break up cell clumps.

- Collect the suspended cells in a 15 mL centrifuge tube and place it on ice. At this point, some dissociating agents should be removed by centrifugation to prevent carry over which can cause poor cell attachment or toxicity. However, the trypsin in the cell suspension will be inactivated by the serum and does not absolutely need to be removed. If removal is desired, spin the cell suspension at 100 x g for 5 minutes. Then, remove the trypsin-containing medium and resuspend the cells in fresh medium.

NOTE: Storing cells on ice will slow cell metabolism. This will improve cell viability and reduce cell clumping.

3. Cell Counting

To determine growth rates or set up cultures at known concentrations it is necessary to count the cell suspension. Hemacytometers or electronic cell counting devices can be used. The hemacytometer has the added advantages of both being less expensive and allowing cell viability determinations to be made during counting. The Corning Cell Counter can be used for counting cells.

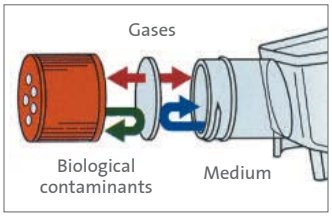
- Gently vortex the cell suspension, and remove a 0.5 mL sample, and place in a tube for counting. To this add 1 mL of the vital stain trypan blue (0.04%). Mix well by vortexing, withdraw a 20 µL sample with a wide tip pipet and carefully load a clean hemacytometer. DO NOT OVERFILL.
- Do a viable cell count and calculate the number of viable cells/mL and the total cell number.

NOTE: Frequently, instead of counting the cells in the suspension, the suspension is split among a number of culture vessels. For example, a 1:2 split would divide the cell suspension of one vessel into two new vessels of equivalent surface area. This is a quick and easy method for the routine maintenance of cell lines.

NOTE: Corning recommends using 0.2 to 0.3 mL of medium for every square centimeter of growth area.

Culture dishes

U-shaped culture flasks



Flasks with vented caps isolate the container it is placed on from the environment while allowing gas exchange and reducing spills.

4. Plating

After making the appropriate dilutions, add the correct amount of cells to each culture vessel. Then add fresh medium to bring the culture vessel to its recommended working volume (see chart below). Be sure to label all vessels accurately; write on the sides of flasks and around the outer edge of the dish tops so as not to interfere with microscopic observation.

Typical Cell Yields and Recommended Medium Volumes for Corning® Flasks and Dishes

	Average Cell Yield*	Recommended Medium Volume (mL)
Corning Flasks		
25 cm^2	2.5 x 10^6	5 - 7.5
75 cm^2	7.5 x 10^6	15 - 22.5
150 cm^2	1.5 x 10^7	30 - 45
175 cm^2	1.75 x 10^7	35 - 52.5
225 cm^2	2.25 x 10^7	45 - 67.5
Corning Dishes		
35 mm	9.0 x 10^5	1.6 - 2.4
60 mm	2.1 x 10^6	4.2 - 6.3
100 mm	5.5 x 10^6	11 - 17
150 mm	1.52 x 10^7	34 - 45
245 mm (square)	5.0 x 10^7	100 - 150

*Assumes an average yield of 1 x 10^5 cells/cm^2 from a 100% confluent culture. Yields from many cell types can be lower or higher than this.

5. Incubation

Most mammalian cell cultures do best at a temperature between 35°C and 37°C. In addition to maintaining constant temperature, some incubators also maintain high humidity levels and CO_2 concentrations. The high humidity cuts down evaporation losses in open systems such as Petri dishes and microplates that would otherwise result in hypertonic culture medium and stressed cells. The elevated CO_2 concentrations (usually 5% to 10%, depending on bicarbonate concentrations in the medium) help maintain the proper pH (7.4 ± 0.2) when used with the correct bicarbonate buffer system. In order for this type of buffer system to work it is necessary to allow gas exchange by using unsealed dishes and plates or flasks with gas permeable (vented) caps.

- Leave caps on flasks slightly loosened (or use vented caps on the flasks for extra protection against spillage and the outside environment) and place on a shelf in a 37°C, humidified CO_2 incubator.
- Examine cultures daily, and change medium as needed.

Acknowledgements

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References

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2. McAteer JA and Douglas WHJ. (1979). Monolayer Cell Culture Techniques. Methods in Enzymology, Vol. 58: Cell Culture. WB Jakoby and IH Pastan, Eds. Academic Press, New York, 132-140.

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