Subculturing Monolayer Cell Cultures

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Introduction

Most animal cell lines and primary cultures grow as a single thickness cell layer or sheet attached to a plastic or glass substrate. 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, 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

1. Pipetting aids
2. Disposal tray or bucket for pipettes

3. Bottle of 70% alcohol for wiping down work area
4. Paper towels
5. Marking pen
6. Inverted phase contrast microscope
7. Ice bucket
8. Liquid waste container
9. Hemacytometer
10. 37ºC incubator

Sterile

1. Flask of actively growing cells that are 80 to 90% confluent
2. Cell culture medium. This should contain all of the additives (fetal bovine serum, glutamine etc.) required by the above cell line.
3. Calcium- and Magnesium-Free Phosphate-Buffered Saline CMF-PBS (10mL). 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.
4. 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.
5. 15mL disposable screw cap centrifuge tubes (Corning® Catalog # 430055 or 430788)
6. Appropriate culture vessels
7. 1, 5, 10 and 25mL pipettes - Corning Catalog # 4485, 4487, 4488 and 4489
8. Sterile 0.04% Trypan blue solution for viablity staining
9. 20µL pipettor Corning Catalog # 4961 or 4462 (1)
10. Pipette tips
11. Laminar flow hood

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.

Procedure

1. **Examination**:
   It is important to examine your cultures daily and always prior to subcultivation. 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.

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.

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.

a) Using a sterile pipette, remove and discard the culture medium. All materials and solutions exposed to cells must be disposed of properly. Medium can be left in the pipettes if they are placed in disinfectant.

> Two CMF-PBS washes and/or rinsing with trypsin can be used for more difficult to remove cells.

b) For a T-75 flask, wash the cell monolayer by adding 5 to 10mL of CMF-PBS to the flask and then slowly rock it back and forth to remove all traces of fetal bovine serum. Remove and discard the wash solution. Failure to remove traces of fetal bovine 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.

c) Add 5mL of the trypsin solution (in CMF-PBS) to the flask and place the flask back in an incubator at 37ºC to increase the activity of the enzyme solution. (Prewarming of the enzyme solution to 37ºC will decrease the required exposure period.)

> For difficult to break up cell clumps, try holding the pipette 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.

d) Check the progress of the enzyme treatment every few minutes with an inverted phase contrast microscope. Once most of the cells have rounded up, gently tap the side of the flask to detach them from the plastic surface. Then add 5mL of growth medium to the cell suspension and, using a 10mL pipette, 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 may be necessary

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

e) Collect the suspended cells in a 15mL centrifuge tube and place on ice. Some dissociating agents should be removed at this point 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 100xg for 5 minutes. Then remove the trypsin-containing medium and replace with fresh medium.

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.

> 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.

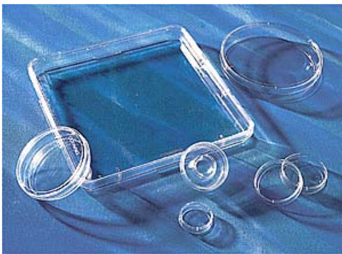
a) Vortex the cell suspension and remove a 0.5mL sample and place in a tube for counting. To this add 1mL of the vital stain trypan blue (0.04%). Mix well by vortexing, withdraw a 20µL sample with a wide tip pipettor and carefully load a clean hemacytometer. (Do not overfill!)

b) Do a viable cell count and calculate the number of viable cells/mL and the total cell number.

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

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.

Corning cell culture flasks



Corning cell culture dishes

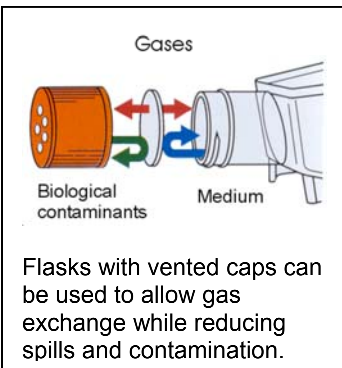
Typical cell yields and recommended medium volumes for Corning flasks and dishes

Corning Flasks	Average cell yield*	Recommended medium volume (mL)	Maximum working volume (mL)**
25 cm^2	2.5×10^6	5 – 7.5	10
75 cm^2	7.5×10^6	15 – 22.5	60
150 cm^2	1.5×10^7	30 - 45	210
162 cm^2	1.6×10^7	32 - 48	175
175 cm^2	1.75×10^7	35 – 52.5	250
225 cm^2	2.25×10^7	45 – 67.5	370
Corning Dishes			
35mm	8.0×10^5	1.6 – 2.4	NA
60mm	2.1×10^6	4.2 – 6.3	NA
100mm	5.5×10^6	10 - 15	NA
150mm	1.48×10^7	30 –45	NA
245mm (square)	5.0×10^7	100 - 150	NA

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

**Maximum working volume is the amount a flask can hold in the horizontal position when filled to the neck.

5. **Incubation**:
Most mammalian cell cultures do best at a temperature between 35º and 37ºC. 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.



Flasks with vented caps can be used to allow gas exchange while reducing spills and contamination.

a) Leave caps on flasks slightly loosened (or use vented caps on the flasks for extra protection against spillage and contamination) and place on a shelf in a 37ºC, humidified CO_2 incubator.

b) Examine cultures daily and change medium as needed.

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Acknowledgements

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