

Subculturing Procedures for Mammalian Cells

NOTE: Not applicable to Clonetics™ and Poietics™ Primary Human or Animal Cells.

In cell culture there is frequently the need to subculture cells. In doing so, cells can be propagated for the purposes of increasing cell numbers or providing cells in a culture vessel suitable to one's needs. There are a number of ways to remove cells from one culture vessel and pass them to another vessel. Cells may be removed from surfaces on which they are attached by:

- Mechanical means (scraping)
- Chelating agents, ethylenediaminetetraacetic acid (EDTA)
- Enzymes (trypsin, pronase, collagenase)

Enzymes and chelating agents are often used in combination. Trypsin is an aqueous crude extract prepared from porcine pancreas. It is the most common means used for removal of cells from surfaces and from intact tissue. Trypsin is, to some extent, a misnomer because in addition to trypsin, the preparation contains other proteases, lipases, and carbohydrases. The multitude of digestive enzymes produced by the pancreas would be expected to be found in trypsin preparations. Pure crystalline trypsin can be used, but it is more expensive than crude trypsin and often does not work as well, especially when preparing cells from intact tissue.

The optimum conditions for trypsin activity are a pH range of 7.6–7.8 and a temperature of 37°C. The effect of trypsin is to break down the intracellular matrix that binds cells to each other or to a substrate surface.

There are no chemical standards for trypsin activity. We conduct quality assurance tests on trypsin to determine its capacity to detach cells from a substrate surface in a standard time period without damage. This is in addition to the usual tests for sterility.

Trypsin is typically used at concentrations between 0.05% and 0.25%, although some applications may require concentrations outside this range. Versene® (EDTA) enhances trypsin action, and therefore lowers the required trypsin concentration for effective performance. Concentrated trypsin (2.5%, Cat. No. 17-160) should be diluted in calcium- and magnesium-free balanced salt solution (BSS) (Hanks' BSS, Cat. No. 10-543; or Dulbecco's Phosphate Buffered Saline, Cat. No. 17-512). Dilution in water is not recommended since the solution will be hypotonic and produce cell damage. Dilution in saline alone is also damaging to cells.

Trypsinization Procedure

Cell cultures are normally subcultured ("split") when the cultures are at or near confluency. As a general rule, the longer the time frame between when confluency is first achieved and subculturing, the longer it will take for the trypsin to act.

1. Decant medium from the culture vessel. Serum inhibits trypsin activity, so complete removal of serum-containing medium is necessary.
2. Rinse the cell sheet with BSS without calcium and magnesium before addition of Trypsin/Versene® (Cat. No. 17-161). The monolayer should be thoroughly covered with BSS. This rinse is instantaneous but the BSS can remain on the cell sheet for up to 4 hours, if desired.
3. Pour off rinse medium. Trypsin/Versene® is to be added to each vessel as follows:

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|-----------------------------------|--------------------|
| 75 cm ² flask | 2.5 mL to 5.0 mL |
| 150 cm ² flask | 5.0 mL to 10.0 mL |
| 850 cm ² roller bottle | 10.0 mL to 20.0 mL |

4. Cover the monolayer thoroughly with Trypsin/Versene®. Since different lots of Trypsin/Versene® may vary in strength, it is acceptable to monitor the trypsinization process at room temperature for the first 30 seconds. This will ensure that the trypsinization process is not occurring too rapidly.
5. The culture vessel should then be moderately hit against the palm of the hand to see if the cells are being dislodged. Hold the vessel up to a light in a vertical position and look for signs of the cell sheet sloughing off of the surface. If the entire monolayer is dislodged, proceed to step #6. If not, incubate at 37°C and observe the vessel every minute for dissociation. The culture vessel should again be hit against the palm of the hand to ensure all cells have been dislodged. Remove culture vessel from the incubator.

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6. Immediately transfer dissociated cells to a vessel containing medium supplemented with 10% serum. All of the cells should be removed. Aspirate the medium plus cells with a pipette onto the surface to remove all remaining cells. It is essential that this aspiration be done as completely as possible with a small bore pipette so as to obtain individual, dispersed cells. If the cells are not separated, the new culture will contain numerous microcolonies. Cells added to the vessel should be stirred with a magnetic stir bar at a speed that avoids vortexing (approximately 100–200 rpm), or agitated frequently. It is important at this point to add medium containing serum at least 10 times the volume of Trypsin/Versene® used. This will ensure that the digestive agent is inhibited.
7. Add sufficient fresh medium to the aspirated suspension so that the total volume will cover the surface of two culture vessels, each having the same surface area as the original culture vessel (or use a single culture vessel having twice the floor area of the original vessel). This is a 1:2 split. Other split ratios can be used for vigorously growing cell populations.
8. Incubate the culture vessel (or vessels) at 37°C.
9. When making 1:2 splits, subculturing of human diploid cell cultures should be done on a rigid 3 or 4 day schedule, at which time confluent sheets should occur. Surplus cells can be frozen and stored in liquid nitrogen.
10. Populations that can be cultivated indefinitely can be subcultured serially each time confluency is reached. If the culture is a diploid population with a finite doubling capacity, increase the population doubling level (PDL) number by one at each 1:2 subculturing (split).
11. By making repeated 1:2 splits (twice a week) it can be seen that the number of culture vessels can be built up geometrically (1, 2, 4, 8, 16, 32, 64, etc.) in a short period of time for the production of large quantities of cells for various purposes.
12. Although the line will be eventually lost as a continuously passaged line, it will not be lost for use since frozen ampoules can be obtained at almost every passage and thus the line can be restored to continuous passage again, up to a cumulative total of about 50 population doublings. By repeating this procedure, the number of cells that can be obtained is almost unlimited for all practical purposes.

13. A human embryonic diploid line has an in vitro life span of about fifty 1:2 subcultivations, or population doublings, at which time the cells will cease to divide and eventually die.
14. Using split ratios higher than 1:2 results in the advantage of minimizing the number of manipulations necessary to obtain a specific cell density or number of culture vessels. Since human embryonic diploid cell lines pass through a finite number of population doublings in vitro, it is necessary to keep a record of the number of population doublings that have elapsed. With a 1:2 split ratio this is achieved by simply adding “1” to each split since this ratio yields one population doubling. Larger split ratios can be used. For example, a split ratio of 1:4 would yield 2 doublings per 1:4 split; a 1:8 split ratio would yield 3 doublings per 1:8 split. In order to have knowledge of the approach of cessation, it is essential to keep records of the number of elapsed population doublings.
15. Since human diploid cells multiply by fission, the increase in population may be expressed per cell as follows:

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|---|---|---|---|----|-------------------------------|
| 1 | 2 | 4 | 8 | 16 | ... Number of cells |
| 0 | 1 | 2 | 3 | 4 | ... Population Doubling Level |

References

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