Subculture of Adherent Cell Lines

Aim

Adherent cell lines will grow in vitro until they have covered the surface area available or the medium is depleted of nutrients. At this point the cell lines should be sub-cultured in order to prevent the culture dying. To subculture the cells they need to be brought into suspension. The degree of adhesion varies from cell line to cell line but in the majority of cases proteases, e.g. trypsin, are used to release the cells from the flask. However, this may not be appropriate for some lines where exposure to proteases is harmful or where the enzymes used remove membrane markers/receptors of interest. In these cases cells should be brought into suspension into a small volume of medium mechanically with the aid of cell scrapers.

Materials

cRF10 – pre-warmed to room temperature
- cRF5 for CHO cells, cRF10 for most other cells

FBS
Anti-biotic solution (i.e. penicillin-streptomycin)
L-glutamine
HEPES
PBS without Ca2+/Mg2+
70% ethanol in water
TrypLE Select

Equipment

Personal protective equipment
Microbiological safety cabinet at appropriate containment level
CO2 incubator
Pre-labeled flasks
Marker Pen
Pipettes
Ampule Rack
15 ml tubes
Pipettes and pasture pipettes

Procedure

1) View cultures using an inverted microscope to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants.
2) Make complete RPMI media with 5% fetal bovine serum (FBS) (cRF10 or cRF5) by adding 46ml of RPMI, 2.5ml of FBS, and 0.5ml of penicillin-streptomycin, 0.5ml of L-glutamine, 0.5ml of HEPES to a small, sterile tube
3) View cultures using an inverted microscope to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants.
4) When all materials and reagents are ready, then begin the subculture.
5) Remove spent medium.
6) Wash the cell monolayer with PBS without Ca2+/Mg2+ using a volume equivalent to half the volume (5ml) of culture medium. Repeat this wash step if the cells are known to adhere strongly.
7) Make sure the cells are still adhering to the flask and remove the PBS.
8) Add 0.5ml of TrypLE Select to a T25 flask (scale up for larger flasks)

9) Examine the cells using an inverted microscope to ensure that all the cells are detached and floating. The side of the flasks may be gently tapped to release any remaining attached cells.
10) Add 1.5ml (for T25) of cRF5 to neutralize trypsin
11) Re-suspend the cells by pipetting up and down several times
12) Transfer cells to a new flask or aliquot the appropriate amount of cells to the current flask
13) Incubate as appropriate for the cell line (37C, 5% CO2).

Key Points

 Some cultures whilst growing as attached lines adhere only lightly to the flask, thus it is important to ensure that the culture medium is retained and the flasks are handled with care to prevent the cells detaching prematurely.

 Trypsin is inactivated in the presence of serum. Therefore, it is essential to remove all traces of serum from the culture medium by washing the monolayer of cells with PBS without Ca2+/Mg2+.

 Cells should only be exposed to trypsin/EDTA long enough to detach cells. Prolonged exposure could damage surface receptors or **kill** the cells. Maximum of 10 min in diluted Trypsin.

 Trypsin should be neutralized with serum prior to seeding cells into new flasks otherwise cells will not attach.

 Trypsin may also be neutralized by the addition of soybean trypsin inhibitor (Prod. No. T6414), where an equal volume of inhibitor at a concentration of 1mg/ml is added to the trypsinised cells. The cells are then centrifuged, resuspended in fresh culture medium and counted as above. This is especially necessary for serum-free cell culture.

* Modified Sigma protocol

Useful info Cell Number for Various Sizes of Cell Culture Dishes, Plates, Flasks

You will get useful cell numbers in various sizes of tissue cell culture dishes, plates and flasks. We provides useful numbers, such as, growth surface area, volumes of dissociation EDTA-Trypsin solution, culture medium, seeding density and cell numbers at 100% confluent, are given below for Greiner Bio One and Nest Biotechnology cell culture dishes, cell culture plates and cell culture flasks. This can be a reference for cell culture dishes, plates, flasks from other companies, such as Corning, TPP, ThermoFisher Scientific, Sigma, etc according to the same cell growth surface area.

In mammalian tissue cell culture, confluence is commonly used to estimate the number of adherent cells in a culture dish, plate or a flask, referring to the proportion of the surface which is covered by cells. For example, 70% confluence means roughly 70 percent of the growth surface is covered by cells, and there is still room for cells to grow. 100% confluence means the cell growth surface is completely covered by the cells, and no more room is left for the cells to grow as a monolayer.

Different cell lines exhibit differences in growth rate. Most cells are typically passaged before becoming fully confluent in order to maintain their proliferative phenotype. Some cell lines are not limited by contact inhibition, such as immortalized cells, may continue to divide and form layers on top of the parent cells. To achieve optimal and consistent results, experiments must be conducted at certain confluence, depending on the cell type. The cell number listed in the growth chart here is based on Hela cells, and provided as a reference. For your specific cell type you may have to gain empirical numbers.

Dishes	Surface Area (cm^2)	Seeding Density $x10^6$	Cell No. At Confluency	Trypsin-EDTA (ml)	Medium (ml)
35mm	9	0.3	1.2	1	2
60mm	21	0.8	3.2	2	3
100mm	55	2.2	8.8	3	10
150mm	152	5.0	20.0	8	20

Cell Culture Plates

Plates	Surface Area (cm^2)	Seeding Density $x10^6$	Cell No. At Confluency	Trypsin-EDTA (ml)	Medium (ml)
6-well	9.6	0.3	2	2	2 ~ 5

12-well	3.9	0.1	1	1	2 ~ 4
24-well	1.9	0.05	0.5	0.5	0.5 ~ 1.5
48-well	1.0	0.025	0.25	0.3	0.5 ~ 1.0
96-well (F-Bottom)	0.3	0.01	0.1	0.1	0.025 ~ 0.34

Cell Culture Flasks

Flasks	Surface Area (cm^2)	Seeding Density $x10^6$	Cell No. At Confluency	Trypsin-EDTA (ml)	Medium (ml)T-25
T-25	25	0.7	2.8	3	3 ~ 5
T-75	75	2.1	8.4	5	8 ~ 15
T-182	182	5.0	20.5	10	15 ~ 30

https://greenbioresearch.com/cell-number-density-percentage-confluency-cell-culture-dish-plate-flask/

TAGS: Cell Culture

Cell Counting with a Hemocytometer: Easy as 1, 2, 3

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- 8th December, 2014

Many biological applications such as microbiology, cell culture, blood work and many others that use cells require that we determine cell concentration for our experiment.

Cell counting is rather straightforward and requires a counting chamber called a hemocytometer, a device invented by the 19th century French anatomist Louis-Charles Malassez to perform blood cell counts. A hemocytometer consists of a thick glass microscope slide with a grid of perpendicular lines etched in the middle. The grid has specified dimensions so that the area covered by the lines is known, which makes it possible to count the number of cells in a specific volume of solution.

Figure 1. A Classic Hemocytometer

The most common type of hemocytometer has an "H" shape engraved in the middle that encloses two separate mirror-like polished grid surfaces and provides the cover slip mounting area (Figure 1).

Loading the hemocytometer

Before starting ensure that both the hemocytometer and its coverslip are clean by removing any dust particles with lens paper. Coverslips that are used for mounting on hemocytometers are specially made to be thicker than the conventional microscopy coverslips because they must be able to overcome the surface tension of a drop of liquid.

Make sure to first place the coverslip over the counting surface before loading the cell suspension. Then place the pipette tip with your sample into one of the V-shaped wells, as in Figure 2, and gently expel the sample. The area under the coverslip fills by capillary action. Enough liquid should be introduced so that the mirrored surface is just covered, usually around 10 µl, but do not overfill the surface. You can load two samples on one hemocytometer, one into each of the two grids.

Figure 2. Loading the Hemocytometer

The loaded hemocytometer is then placed on the microscope stage and the counting grid is brought into focus at low power. Allow the sample to settle for a couple of minutes and avoid moving the coverslip as it might introduce air bubbles and make counting difficult.

Counting cells in a hemocytometer

The full grid on a hemocytometer contains nine squares, each of which is 1 mm^2 (Figure 3). The central counting area of the hemocytometer (Figure 3B) contains 25 large squares and each large square has 16 smaller squares. When counting, count only those cells on the lines of two sides of the large square to avoid counting cells twice (Figure 3G). Suspensions should be dilute enough so that the cells or other particles do not overlap each other on the grid, and should be uniformly distributed.

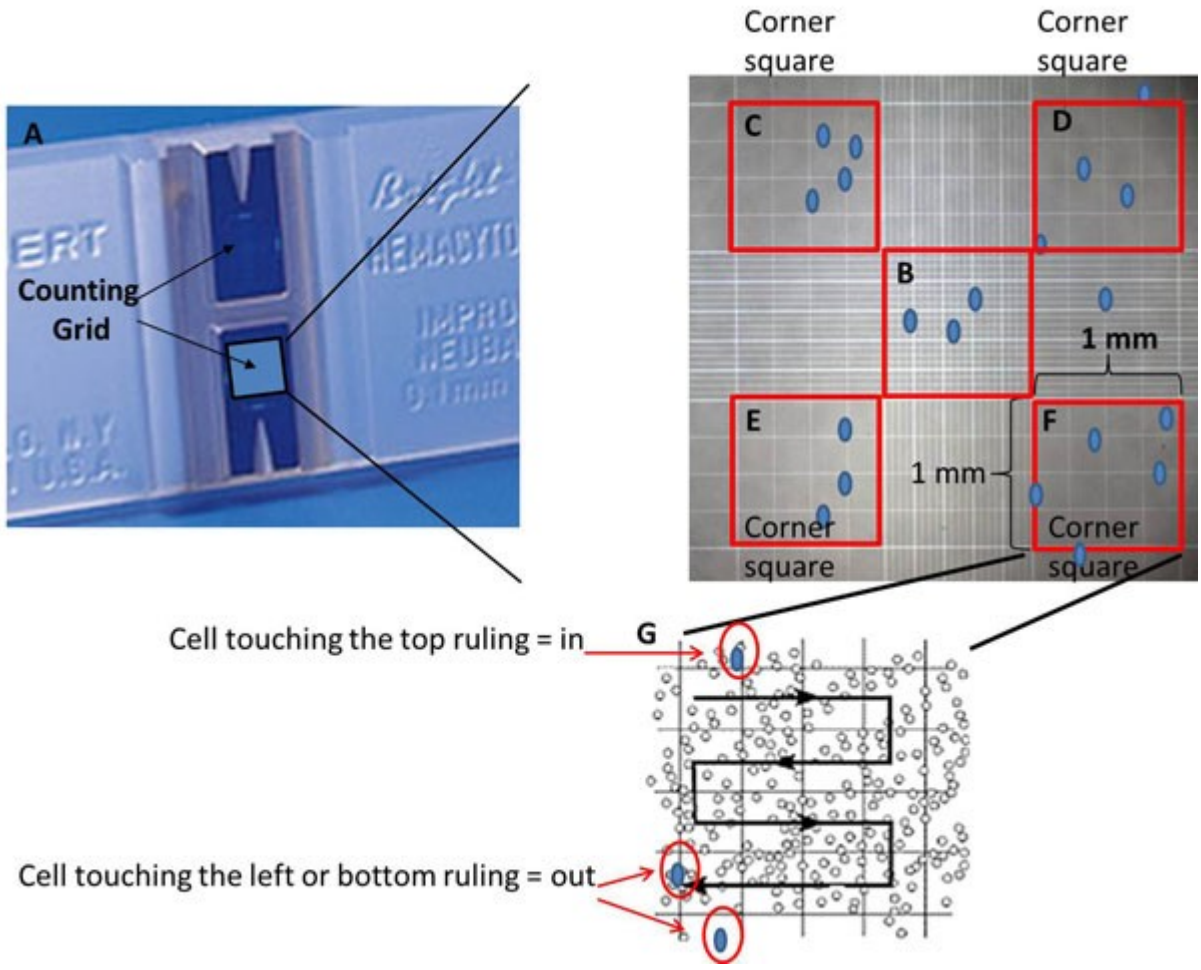

Figure 3. Counting Cells on the Hemocytometer.

To distinguish between dead and viable cells, the sample is often diluted with a particular stain, such as Trypan blue. This staining method, also known as dye exclusion staining, uses a diazo dye that selectively penetrates cell membranes of dead cells, coloring them blue, whereas it is not absorbed by membranes of live cells, thus excluding live cells from staining. When viewed under a microscope, dead cells would appear as dark blue (Figure 4)

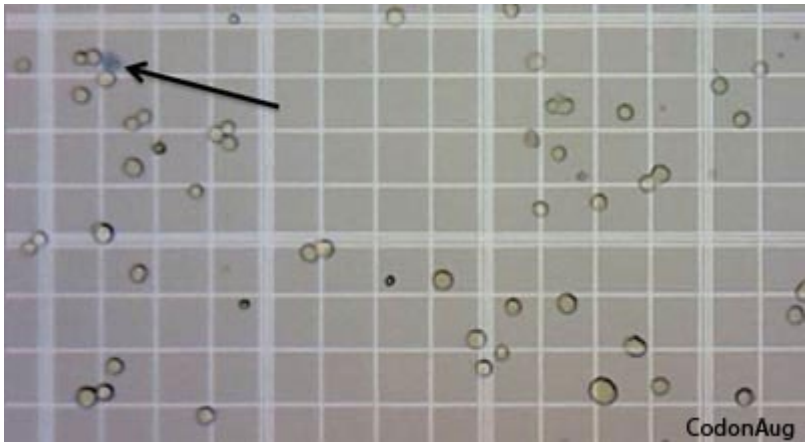

Figure 4. Trypan Blue Exclusion of Live Cells on the Hemocytometer. (Arrow indicates uptake of dye across the membrane of dead cells.)

To perform the count, determine the magnification needed to recognize the desired cell type and systematically count the cells in selected squares so that the total count is approximately 100 cells, a minimum number of cells needed for a statistically significant count.

For large cells, you can simply count the cells inside the four large corner squares (Figure 3C-F) and the middle one (Figure 3B). For a dense suspension of small cells you may wish to count the cells in the four outer and middle squares of the central square (Figure 3B) or make a more dilute suspension.

Remember if a cell overlaps a ruling, count it as “in” if it overlaps the top or right ruling, and “out” if it overlaps the bottom or left ruling (Figure 3G).

The area of the middle (Figure 3B) and each corner square (Figure 3C-F) is 1 mm x 1 mm = 1 mm^2: the depth of each square is 0.1 mm. The final volume of each square at that depth is 100nl.

Once you have obtained the total cell count, cell concentration can be calculated from the following formula:

$$\text{Total cells/ml} = \text{Total cells counted} \times \frac{\text{dilution factor}}{\text{\# of Squares}} \times 10{,}000 \text{ cells/ml}$$

So, for example, if you diluted your sample 1:1 with Trypan blue, and you counted 325 cells in 4 corner squares plus the central big square, total cells per ml =

$$325 \text{ cells} \times \frac{2(\text{dilution factor})}{5 \text{ Squares}} \times 10{,}000 \text{ cell/ml} = 130 \times 10^4 \text{ cells/ml}$$

If you want to know how many cells you have in your original sample, just multiply the cell concentration by total sample volume. For example, if your original sample volume is 5 ml, then your sample has a total =

$$130 \times 10^4 \text{ cells/ml} \times 5\text{ml} = 650 \times 10^4 \text{ cells}$$

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http://bitesizebio.com/13687/cell-counting-with-a-hemocytometer-easy-as-1-2-3/