PROTOCOL FOR THE CULTIVATION AND PRESERVATION OF HUMAN DIPLOID CELL STRAINS

Leonard Hayflick
The Wistar Institute
36th and Spruce Streets
Philadelphia 4, Pennsylvania

I. CULTIVATION

REAGENTS

Media

Eagle's Basal Medium
10% Calf Serum
100 µg Streptomycin/ml } or 50 µg Aureomycin/ml
100 units Penicillin/ml }

Media should be prewarmed to 37°C before use. The final pH should be brought to 7.2 before serum addition. The pH of the media after equilibration of the culture at 37°C must be less than 7.5. This is vital!

Aureomycin (Lederle Product #4691-96, intravenous) is bottled in 500 mg amounts. Reconstitute in 50 ml of warm (37°C) sterile, distilled water. Agitate to insure a clear amber solution. Prepare 5 ml aliquots and store at -20°C. Use one 5 ml aliquot per liter of medium. Final concentration is therefore 50 µg/ml. Five thousand cell cultures grown in Aureomycin in lieu of Penicillin and Streptomycin have been found, upon testing, to be free of Mycoplasmas for periods of over 3 years.

Trypsin

0.25% Trypsin in Phosphate Buffered Saline (PBS) or any BSS plus Penicillin and Streptomycin (or Aureomycin in lieu of both).

The trypsin must be prewarmed to 37°C before use. The final pH should be at least 7.5.

SUBCULTIVATIONS

1. The bottle you will receive contains a nearly confluent sheet of cells. The bottle is completely filled with medium to prevent loss of cells in transit. Decant all of the medium upon arrival. (It can be saved and used).

2. Add enough prewarmed trypsin to cover the sheet. Allow it to stand at room temperature for 1 minute. Discard all of the trypsin. Allow the bottle to stand for 15 to 30 minutes more at room temperature or at 37°C. Incubation during this period of time at 37°C will hasten the trypsinization process.

3. The trypsinization process will be completed when the cell sheet appears to be loosened from the glass surface. This can be seen macroscopically by holding the bottle up to the light in a vertical position and observing the cell sheet sloughing off of the glass surface.

4. After this has occurred, add a small amount of fresh medium and splash it over the cell sheet. All of the cells should be removed. Aspirate the medium plus the cells with a pipette onto the glass surface to remove all remaining cells. It is absolutely essential that this aspiration be done as completely as possible so as to obtain single dispersed cells. This is one of the most crucial steps, for if the cells are not broken up, the new culture will contain numerous microcolonies of "explants" that will ultimately lead to the early death of the culture.

5. Add sufficient fresh medium to the aspirated suspension so that the total volume will cover the glass surface of two bottles, each having the same surface area as the original bottle (or use a single bottle having twice the floor area of the original bottle). This is a 2:1 split. (The old bottle can be reused without washing).

6. Incubate the bottle (or bottles) at 37°C. No intervening culture feedings are necessary.

7. When making 2:1 splits, subcultivation of these cultures should be done on a rigid 3 or 4 day schedule, at which time confluent sheets should occur. Surplus cells can be stored at -70°C. The procedure for cell preservation at -70°C follows below. It is important to point out at this time that the pH of the medium is of extreme importance. The final pH of the medium must not exceed about 7.4 after equilibration of the culture at 37°C. A higher pH may result if too few cells are contained in the culture or if the original pH of the medium is too high, or thirdly, and most importantly, if the gas phase of the culture vessel is too large. If the latter is the case, it is then essential that the Eagle's medium be prepared in Hanks' balanced salt solution and not in Earle's balanced salt solution in which it is ordinarily prepared.

8. The passage number of the strain is indicated by the encircled number. Increase this number by one at each subcultivation.

9. By making repeated 2:1 splits (twice a week) it can be seen that the number of culture bottles 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.

10. The strain has a "passage potential" of about 50 subcultivations at which time the cells will cease to divide and eventually die.

11. Although the strain will be eventually lost as a continuously passaged strain, it will not be lost for use since frozen ampules can be obtained at almost every passage and thus the strain can be restored to continuous passage again, up to a cumulative total of about 50 passages. By repeating this procedure, the number of cells that can be obtained is almost unlimited for all practical purposes.

12. Using split ratios higher than 2:1 results in the advantage of minimizing the number of manipulations necessary to obtain a specific cell density or number of bottle cultures. Since human diploid cell strains pass through a finite number of generations <u>in vitro</u>, of the order of 50, it is necessary to keep a record of the number of generations that have elapsed. With a 2:1 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 4:1 would yield 2 doublings per 4:1 split; a 10:1 split ratio would yield 3.25 doublings per 10:1 split. In order to have knowledge of the approach of Phase III it is essential to keep records of the number of elapsed generations.

13. Since human diploid cells multiply by fission, the increase in population may be expressed per cell as:

1	2	4	8	16 .... number of cells
0	1	2	3	4 ...n number of generations

one generation — one generation

Expressed exponentially, the population after n generations is 2^n per cell in the inoculum, or the total population N is the initial population, X_o, multiplied by 2^n or:

$$(1) \qquad N = X_o \, 2^n$$

The data needed to determine the number of generations, n, will be the number of cells per unit volume in the inoculum, X_o, at time, t_o, and the final population, N, at time = t_2. The number of generations, n, can be most readily evaluated by expressing equation (1) in logarithmic form. Using logarithms to the base 10, this equation becomes:

$$(2) \qquad \log N = \log X_o + n \log 2$$

or rearranging

$$(3) \qquad n = \frac{\log N - \log X_o}{\log 2}$$

since log 2 = 0.301:

(4) $n = 3.32 (\log N - \log X_o)$

Logarithms to the base 2 should be used for biological systems because an increase of one logarithmic unit would correspond to one doubling or one generation. If this is done, the log 2 drops out of the denominator of equation (3). Natural logarithms (base e, written ln) may also be used. Regardless of the base of the logarithms used, the equation will take the same form, and conversion from one form to another can be made by multiplying by a constant, i.e., log 2 = 0.301 or 1/log 2 = 3.32, so $\log_2 = 3.32 \log_{10}$.

The multiplication rate, r, or number of generations per unit time can be obtained for equation (4) by dividing by the time interval between inoculation, t_o, and the time at which the final population, N, was taken, i.e., t_2.

Therefore the multiplication rate, r, is:

(5) $r = n/(t_2 - t_o)$

or

(6) $$r = \frac{3.32 (\log N - \log X_o)}{t_2 - t_o}$$

One must specify the units of r, both the base of the logarithms used and the units of time -- usually in days (units of 24 hours) for tissue culture systems, i.e., doublings in population per 24 hours. To write formulae (2) and (5) in general form, one may determine the number of generations or multiplication rate over any interval in which the initial count is X_1, at any selected time t_1, and the final count X_2, at time t_2. Thus:

(7) $$n = \frac{(\log X_2 - \log X_1)}{\log 2} = 3.32 (\log X_2 - \log X_1)$$

or

(8) $$n = 3.32 \log X_2/X_1 \quad \text{or} \quad n = \left(\frac{\log X_2}{\log X_1}\right)$$

and

(9) $$r = \frac{3.32 \log X_2/X_1}{t_2 - t_1}$$

Since the generation time, g, is the time for the population to double, it is the reciprocal of the doubling per unit time,

(10) $g = 1/r$

generation time = time elapsed per doubling in number.

14. General References

(1) Hayflick, L. and Moorhead, P. S. 1961, The Serial Cultivation of Human Diploid Cell Strains. Exp. Cell Research 25:585-621.

(2) Hayflick, L., Plotkin, S. A., Norton, T. W. and Koprowski, H. 1962, Preparation of Poliovirus Vaccines in a Human Diploid Cell Strain. Am. J. of Hyg. 75:240-258.

(3) Hayflick, L. 1963, Human Diploid Cell Strains as Hosts for Viruses. In Perspectives in Virology III, M. Pollard, Editor, Chapter 13, Hoeber Medical Division, Harper & Row.

(4) Hayflick, L. 1963, A Comparison of Primary Monkey Kidney, Heteroploid Cell Lines and Human Diploid Cell Strains for Human Virus Vaccine Preparation. The American Review of Respiratory Diseases 88, No. 3, Part 2, Sept. 1963.

(5) Hayflick, L. and Moorhead, P. S. 1964 (in press), The Limited In Vitro Lifetime of Human Diploid Cell Strains. In Symposia of the International Society for Cell Biology, Vol. 3, Academic Press, N.Y.C., N.Y.

(6) Hayflick, L. 1965 (in press), The Limited In Vitro Lifetime of Human Diploid Cell Strains. Exp. Cell Research.

II. PRESERVATION

REAGENTS

Trypsin

0.25% Trypsin in Phosphate Buffered Saline or BSS. Final pH 7.5

Glycerol Medium

10% Calf Serum
10% Glycerol (Sterilized by autoclave) (Reagent grade)
80% Basal Medium Eagle's

Prewarmed to 37°C before use and prepared exactly as growth medium plus the addition of 10% Glycerol.

PREPARATION OF THE CELLS FOR FREEZING

1. A mature bottle (or bottles) of cells is selected.

2. The cell sheet is removed by trypsinization according to the methods outlined above. After the cells are aspirated well in a known small volume of glycerol medium, they are counted in a hemocytometer.

3. The cell concentration is adjusted to 1.5-2 x 10^6 cells per ml. If the cell concentration is too dilute, then the suspension must be centrifuged and resuspended in the proper amount of glycerol medium.

4. The cells which are suspended in glycerol medium are dispensed into 1.2 ml artificial insemination ampules, * 1 ml per ampule. The ampules are then sealed and placed in the refrigerator at 4°C overnight or at least for 3 hours.

5. The temperature of the contents of the ampule must then be lowered under optimum conditions at a rate of 1°C/minute throughout the range of +4°C to -25°C after agitation of the ampule. A number of methods have been described to achieve this. Two mechanical systems are available and can be obtained from Canalco, Bethesda, Maryland, or from Linde Co., 270 Park Avenue, New York 17, N. Y. It is also possible to achieve these conditions in an ordinary dry ice chest. By plotting the rate of temperature drop with a toluene thermometer in various parts of the dry ice chest, an area may be found that will closely approximate these conditions, thus obviating the necessity of purchasing any mechanical device. We have learned that temperature drop rates of the order of -3°C/minute from +4°C to -25°C in a dry ice chest are satisfactory.

6. After the temperature has reached -25°C the rate of the temperature drop to -70°C (which is the minimum temperature at which cells should be stored) can be done very quickly. Storage of the ampules must be at -70°C or colder. We prefer a storage temperature of -190°C which is achieved with liquid nitrogen. In any event it is absolutely essential that the temperature of the <u>contents of the ampule</u> be -70°C or colder at all times until reconstitution.

III. RECONSTITUTION

1. An ampule that is to be reconstituted is removed from the -70°C refrigerator or the liquid nitrogen refrigerator and immediately plunged into water at 37°C.

* Kimble Glass Co., Article #12090.

2. After about 15 minutes the ampule is opened and the contents placed in a milk dilution bottle or Petri dish.

3. Sufficient fresh media is added to the bottle or Petri dish to cover the surface. The bottle is then closed with sterile aluminum foil and either the Petri dish or milk dilution bottle is placed in a CO_2 incubator at 37°C.

4. Within 24 hours, the medium is changed and the culture refed periodically until the cell growth is confluent.

5. The culture is then subcultivated as described above.