PROCEDURE NO. 49200

A STANDARD PROCEDURE FOR CULTIVATING HUMAN DIPLOID FIBROBLASTLIKE CELLS TO STUDY CELLULAR AGING

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SUMMARY: Human diploid cultures are widely used for the study of aging. We have developed quantitative cell culture procedures that include optimization of culture conditions and determination of replicative age by direct cell count. Using these procedures we are able to determine reproducibly the stage in the replicative life history of diploid cell cultures.

Key words: aging; diploid cells; population doubling level; life span; cell culture.

I. INTRODUCTION

It has been well documented that human diploid fibroblastlike cultures, derived from donors ranging from the fetal to the adult stage of development, have a limited proliferative capacity in vitro (1,2). This finite replicative life span is characterized by a period of rapid proliferation followed by a decline in the rate of proliferation, after which the cultures can no longer be propagated (1-3). This decline in proliferative capacity has been interpreted as an expression of aging at the cellular level (1-3). Proliferative rate and the stage in the life history of a culture can be monitored best by well controlled and reproducible cell culture procedures and by recording the cumulative number of actual population doublings (CPDL). This paper describes the standard subcultivation procedures we use for the study of aging in normal human diploid fibroblastlike cell cultures.

II. MATERIALS

A. Growth medium components

Auto-Pow minimal essential medium Eagle, modified, with Earle's salts, without glutamine, without sodium bicarbonate, No. 11-100-22 Flow[1]
Sodium bicarbonate, 7.5% solution; No. 17-163 M.A. Bioproducts[2]
L-Glutamine, 200 m*M*; sterile in 100-ml quantities, No. 16-801-49[1]
Fetal bovine serum; sterile in 100-ml quantities, No. 29-101-49[1]
Vitamins for basal medium Eagle (BME), 100X; sterile in 100-ml quantities, No. 16-004-49[1]

B. Dissociation medium components

10x Trypsin solution, 2.5% in Hanks' saline without Ca^{+2} and Mg^{+2}; sterile in 100-ml quantities, No. 16-893-49[1]
NaCl, No. 3632-1 Baker[3]
KCl, No. 3040-1[3]
$NaH_2PO_4 \cdot H_2O$, No. 3818-1[3]
Glucose (dextrose), No. 4893-2[3]
MEM amino acids (50X solution), No. 16-011-49[1]
MEM vitamins (100X solution), No. 16-014-49[1]
Phenol red (0.5% solution), No. 16-900-49[1]

C. Plastic tissue culture items

Tissue culture flasks, T75, No. 3024 Falcon[4]
Other plasticware may be used if designated as tissue culture quality (special growth surface treatment)

D. Glassware

Sterile cotton-plugged Mohr pipettes (1-, 2-, 5-, 10-ml)
Prescription bottles, 32-oz, screw capped
Sterile 100-ml serum bottles, screw capped
Sterile 25-, 50-ml graduated cylinders
Sterile 250-ml Erlenmeyer flasks
Beakers, 250-ml
Sterile screw-cap test tubes (120 × 50 mm)

E. Equipment

Laminar flow hood. Sterilgard hood, Model No. VBM 400[5]
Coulter counter, Model ZB Coulter[6]
Incubator, e.g., Forma Model No. 7172 Forma[7]

F. Miscellaneous items

70% Ethanol
Isoton (azide-free), No. 75-46719[6]

0271-8057/80/0117-0121 $1.50/0

Special gas mixture; 95% air:5% CO_2, Ohio[8]
Sterile cotton-plugged $CaCl_2$ drying tubes
Nalgene filter unit 0.20 μm, 500 ml, No. 09-740-25A Fisher Scientific[9]

III. PROCEDURES

A. Storage of medium components

All should be prepared sterile.

1. L-Glutamine: purchase in 100-ml bottles and store frozen (−20° C). At first thawing, prepare 20 aliquots (5 ml each) in screw-cap test tubes and refreeze. Thaw immediately before use.
2. 10X Tryspin solution: keep frozen (−20° C); aliquot at first thawing as for L-glutamine.
3. Sodium bicarbonate, 7.5% solution: keep refrigerated (4° C) after opening.
4. Vitamins (BME, 100X): store frozen (−20° C) and protect from direct light.
5. Fetal bovine serum: store frozen (−20° C); once thawed, do not refreeze. Test each lot (before purchase) by determining proliferation rate and harvest density for three consecutive weeks.

B. Preparation of incomplete medium (1 liter)

1. To 854 ml deionized H_2O, add 9.4 g of Auto-Pow MEM Powder and 10 ml BME vitamins. Mix thoroughly.
2. Dispense 432 ml of incomplete modified MEM into each 1 liter bottle.
3. Screw the caps on loosely, apply autoclave tape to each bottle and autoclave for 15 min at 121° C.
4. When the sterilization cycle is finished, remove the bottles from the autoclave quickly, as prolonged heat destroys some medium components. With the caps still loose, place the vessels in the laminar flow hood and allow to cool to room temperature.
5. When cooling is complete, tighten the caps and store the vessels at 4° C in the dark.

C. Preparation of complete medium with 10% vol/vol fetal bovine serum.

1. This medium should be prepared fresh with each use, but may be stored at 4° C for up to 1 wk. If stored longer, add 1 ml more of L-glutamine (200 m*M*)/100 ml medium.
2. To a 500-ml bottle containing 432 ml of incomplete medium, add in the following order:
   a. 7.5% Sodium bicarbonate (13 ml)
   b. Fetal bovine serum (50 ml)
   c. 200 m*M* L-Glutamine (5 ml)

D. Preparation of Ca^{+2}-, Mg^{+2}-free MEM

1. To 900 ml distilled water, add in the order listed:
   a. NaCl, 6800 mg
   b. KCl, 400 mg
   c. $NaH_2PO_4 \cdot H_2O$, 140 mg
   d. Glucose, 1000 mg
   e. MEM amino acids, 50X, 20 ml
   f. MEM vitamins, 100X, 10 ml
   g. Phenol red, 0.5% solution, 10 ml
2. Dilute to 1 liter and filter sterilize (0.20 μm pore size), with house vacuum.

E. Preparation of trypsin solution (0.25%)

1. To 40 ml of MEM without Ca^{+2} or Mg^{+2}, add 5 ml of 7.5% sodium bicarbonate.
2. Add 5 ml of sterile 2.5% trypsin. Final pH is 7.4. Prepare fresh.

F. Trypsinization

1. Pour off the medium into a beaker by decanting the fluid from the side opposite the cell growth surface.
2. Rinse the cell sheet twice with 4 ml of the trypsin solution.
3. Aspirate the excess trypsin using a 1-ml pipette.
4. Add enough trypsin to just cover the cell sheet. (For a T75 flask, 2 ml is sufficient.)
5. Incubate the flask for approximately 15 min at 37° C. Check to see if the cells are detaching. The cells will round up as they lift off the growth surface. The process may be speeded up by gently tapping the sides of the flask. Do not splatter the cells against the top and sides of the flask, or errors in cell number will be introduced.

G. Harvesting the cells

1. When all the cells have detached from the growth surface, add enough complete medium to ensure that the entire population has been collected. Record total volume.

2. To pool suspensions from several T75 flasks, trypsinize and harvest the cells in each flask as described above and transfer the cell suspensions to a conical 50-ml centrifuge tube. Keeping the cells on ice during long procedures inhibits attachment to the substratum.

H. Dispersion of cell aggregates

1. Before seeding into the new flasks, break up clumps of cells by drawing up the entire suspension into a 10-ml pipette, and then allowing it to flow out gently against the wall of the vessel. Repeat this process three times.

2. Repeat the same procedure using a 5-ml pipette to obtain a single-cell suspension. Until the procedure becomes routine, check the suspension under the microscope.

3. For volumes larger than 10 ml, use a syringe with a 13- to 15-gauge needle.

I. Cell counting

1. Using sterile procedures, remove an aliquot from the suspension and dilute in Isoton in a counting vial.

2. The dilution should be made so that the number of cells registered by the counter is about 10,000. For samples containing more than 10,000 cells, a coincidence correction must be applied. A correction chart is provided with the Coulter counter.

J. Seeding

1. After determining the number of cells, resuspend the harvest.

2. Seed the cells at a density of 1×10^4 cells/cm² of growth surface area. Use a final ratio of medium volume to growth surface of 0.53 ml/cm² (Table 1).

3. Add the appropriate amount of growth medium to each new flask.

4. Withdraw an aliquot from the cell suspension and dispense it into the new flasks.

TABLE 1

Cell Densities for Various Culture Vessels

Vessel	Growth Area	Cell Inoculum	Final Medium Volume
	cm^2		ml
Large glass roller	1485	15×10^6	785
Glass roller (1/2 gal)	690	7×10^6	350
Large plastic roller	850	8.5×10^6	450
Plastic roller	450	4.5×10^6	250
Povitsky bottle	350	3.5×10^6	185
Blake bottle	180	1.8×10^6	95
T150 Flask	150	1.5×10^6	80
T75 Flask	75	0.75×10^6	40
T25 Flask	25	0.25×10^6	10[a]
100-mm Plastic TC dish	64	0.64×10^6	25[a]
60-mm Plastic TC dish	22	0.22×10^6	5[a]
35-mm Plastic TC dish	9.6	0.1×10^6	2[a]

[a] The optimal medium volume-to-surface ratio of 0.53 ml/cm² is used except where prohibited by the growth vessel architecture.

K. Gaseous environment

1. Dissolved CO_2, in equilibrium with HCO_3^-, represents the principal buffer system in the medium other than serum. CO_2 is volatile and the gas phase must be adjusted to the proper pCO_2 to maintain a medium pH of 7.4.

2. Sterilize a 95% air:5% CO_2 mixture by passing through a sterile, cotton-filled $CaCl_2$ drying tube. From the drying tube, deliver the gas through a sterile Pasteur pipette into the gas phase of the flask.

3. Flush directly over the medium surface for a few seconds; close the flask tightly and incubate at 37° C.

L. Calculating the population doubling increases

1. Calculate the population doubling level (PDL) for each harvest directly from the cell count.

2. For example, 1 wk after seeding a T75 flask with the standard inoculum of 7.5×10^5 cells, 6.0×10^6 cells were obtained. Population doubling increase can be calculated using the formula:

$N_H/N_I = 2^x$ (where N_I = inoculum number, N_H = cell harvest number, x = population doublings)

We compute:

$$6.0 \times 10^6 \text{ cells}/7.5 \times 10^5 \text{ cells} = 2^x$$

or:

$$\log_{10}(6 \times 10^6) - \log_{10}(7.5 \times 10^5) = x \log_{10} 2$$

therefore: $x = 3$

3. Add population doubling increase to the previous population doubling level to give the cumulative population doubling level (CPDL).

4. Write this CPDL value on the newly seeded flask along with the seeding date. Thus, the flask is labeled with the population doubling level at seeding.

M. Designation of phase out

1. Subcultivate cell stocks on a weekly schedule; if cells do not reach confluence during this time, refeed by replacing spent medium with fresh, complete medium, and flushing with CO_2 mixture.

2. If, after 3 wk of refeeding, the cells are not confluent, harvest the flask anyway. If the population has not doubled during 3 wk (e.g., for a T75 flask, $N_H < 1.5 \times 10^6$ cells), then the cell line is considered to be phased out. PDL at phase out is calculated from the actual cell number harvested.

3. When WI-38 cells, for example, are subcultivated according to the above procedures, the majority of the cultures phase out between 60 to 70 CPDL's. Recent data summarizing the last 36 WI-38 cell sublines are shown in Table 2.

N. General information

1. Typically, when cultures are prepared directly from tissues (primary culture), the first confluent monolayer is designated PDL 1. This designation is subject to major errors, as it is virtually impossible to estimate cell doublings in primary culture.

2. Expect little or no increase in cell number within 24 h after subcultivation. Between 20 to 50% of the cells do not survive subcultivation.

TABLE 2

CPDL at Phase-Out for a Recent Series of WI-38 Sublines

CPDL at Phase Out	Number of Sublines
50 - 55	2
55 - 60	4
60 - 65	12
65 - 70	6
70 - 75	10
75 - 80	2

3. A temperature of 37° C and a pH of 7.4 is necessary for optimal growth.

4. All sterile work is performed in the laminar flow hood. Ethanol (70%) is used to wipe the outer surfaces of items to be used in the hood.

5. Representative flasks of each cell line should be screened for mycoplasmal contamination twice a month (4).

IV. DISCUSSION

This paper describes our standard laboratory procedures for the routine subcultivation of normal human diploid fibroblastlike cells. However, we use these procedures to subcultivate all cell types, including transformed cells. This quantitative approach (i.e., constant seeding inoculum and medium volume, controlled environment, and determination of life history by cell counting) standardizes cell growth conditions. This is essential in aging studies in which small differences are often measured. Standardization also facilitates the comparison of data among workers and over a period of years. When our procedures are followed, cell line histories are extremely reproducible.

There is often confusion between the terms CPDL and "passage." CPDL indicates the actual number of doublings that the cell population has achieved as determined by the cell count, whereas passage may designate the number of times the culture has been subcultivated. In some cases, population doublings are estimated by subcultivation ratios and reported as doublings (for example, a 1:8 subcultivation ≅ 3 "doublings" or passages). This is a crude estimate since the cell number at confluence is known to decrease with the age of the cells.

In the designation of CPDL, cell losses occurring at subcultivation are ignored. One can estimate this error by assuming that 0.5 to 1 doubling may not be counted for each subcultivation. In our procedure, there are, on the average, three doublings per subcultivation, and thus between 10 and 20 doublings of the surviving population are not counted over a full life history. For historical reasons (1) and for comparison with the data of others, we do not correct for this.

V. REFERENCES

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