# A Definitive Cloning Technique for Human Fibroblast Cultures.* (31423)

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Dilution plating techniques of cloning(1) are essential if one is required to investigate large numbers of colonies. However, such techniques cannot always be relied upon to give definitive clones—that is to say, colonies which were definitely derived from a single cell. In our experience, this is especially the case with cultures of diploid human fibroblasts, which have low plating efficiencies (<1-30%) and form "loose," poorly defined colonies because of the extensive regional migration of the fibroblasts. More serious is the fact that cells in mitosis readily float off into the medium, especially with the mechanical agitation which occurs when the medium is changed. We therefore have developed a modified coverslip cloning technique(2,3) which has proven quite successful in providing definitive clones from such cell cultures. In our hands, it is more convenient, rapid and efficient than techniques involving capillary tubes(4) or drops of medium covered by mineral oil(5).

*Methods and materials.* Strains of human skin fibroblasts were established by explant techniques and maintained in Waymouth's medium(6) containing penicillin (50 units/ml), phenol red (0.05%) and approximately 10% newborn calf serum previously heated to 56°C for 30 minutes. The medium for cloning contained penicillin (100 units/ml), streptomycin (50 mg/ml), phenol red (0.05%), Na pyruvate (1 mM), non-essential amino acids (L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, L-serine and glycine, 0.1 mM) in addition to the amounts already present in Waymouth's medium(6) and 23% fetal calf serum. Lots of sera, obtained from Colorado Serum Company, Denver, were pre-selected by dilution plating cloning tests using standard strains of diploid human skin fibroblasts.

Sub-confluent bottles (6 oz. prescription) of fibroblasts were trypsinized(7), the cell suspension diluted with cloning medium, counted with a hemocytometer and plated in 35 mm plastic petri dishes ("Gold Label," Falcon Co., Los Angeles, Calif.) to give 3,000, 1,500 and 750 cells per dish in 2 ml of medium. Each petri dish had been previously loaded with approximately 30 3 mm square coverslips (No. 3 thickness, custom made by Corning Glass Co., Corning, N.Y.). The coverslips had been washed in 50% nitric acid, exhaustively rinsed in tap water followed by at least 6 changes of single distilled water, after which they were blotted dry with clean bibulous filter paper and sterilized in glass petri dishes at 165°C for 2 hours.

After an overnight period of incubation in a continuous flow $CO_2$ incubator, during which the cells were permitted to attach to the glass, the petri dishes were examined with an inverted microscope in order to select those dishes with coverslips containing only one or a few cells. One by one, the coverslips were transferred to a previously warmed (37°C hot plate) 35 mm plastic petri dish containing 2 ml serum-free Waymouth's medium buffered at pH 7.4 with 0.02 M tris-HCl(7) instead of bicarbonate. Using the inverted microscope and a sterile #27 gauge needle (attached to a syringe), all but one cell were "brushed" off a given coverslip. With a few days' practice, this procedure can be carried out with considerable speed and accuracy. The coverslips with only single attached cells were transferred to 1/2-oz prescription bottles previously warmed and equilibrated with 5% $CO_2$ in a $CO_2$ incubator. The coverslips were attached to the inner flat surfaces of the prescription bottles with a small amount of silicone grease, 1.5 ml of cloning medium added and the caps sealed. (The cardboard liners of the screw-caps had been replaced with silicone-rubber liners.) After 14 days, another 0.5 ml of medium was added. Observations on the cloning efficiency were made at 21 days. The

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TABLE I. Efficiency of Cloning from Single Cells Isolated from Established Cultures of Human Skin Fibroblasts. DEYM = Foreskin, normal newborn. McC "A" = Foreskin, Down's Syndrome Mosaic, newborn. McC "B" = Skin of back, Down's Mosaic, age 4 mo. 66–14 = Skin of knee, Scheie's Syndrome, age 10. 66–15 = Skin of knee, Scheie's Syndrome, age 8. MART = Foreskin, normal newborn. R = Strain had been stored at —75°C for 2-3 years prior to experiments. SC = Sub-clone.

| Exp No. | Strain | Estim. prior cell doublings | No. cells isolated | No. clones obtained | % Cloning efficiency |
|---|---|---|---|---|---|
| 1 | DEYM | 3- 7 | 50 | 5 | 10.0 |
| 2 | McC "A"—R | 9-13 | 45 | 5 | 11.0 |
| 3 | McC "B"—R | 12-16 | 31 | 6 | 19.3 |
| 4 | DEYM SC | 24-28 | 17 | 4 | 23.5 |
| 5 | " | 24-28 | 23 | 5 | 21.7 |
| 6 | " | 24-28 | 16 | 4 | 25.0 |
| 7 | 66–14 | 3- 7 | 8 | * 2 | 25.0 |
| 8 | 66–15 | 5- 9 | 24 | * 2 | 8.3 |
| 9 | 66–14 | 5- 9 | 28 | * 6 | 21.4 |
| 10 | MART—R | 9-13 | 30 | 13 | 43.3 |
| | | Mean = 13.8 | Totals = 272 | 52 | Mean = 19.1 |

* Slow, sparse growth.

The estimates (Estim.) of the numbers of total cell doublings which had accrued prior to cloning include approximately 3-7 doublings estimated to occur during establishment of the cell culture from explants.

confluent clones were then fed twice weekly with 2 ml of cloning medium for 1-2 weeks after which time they could be transferred by trypsinization to 4 oz prescription bottles.

*Results and discussion.* The results of 10 independent experiments are summarized in Table I. Confluent clones were obtained in all 10 experiments involving 6 biopsies from 5 individuals. The cloning efficiencies ranged from 8.3 to 43.3%. With the exception of the clones derived from 2 sisters with Scheie's Syndrome (Mucopolysaccharidosis Type V) (8), all grew well and could be easily established as mass cultures. Fifty-two clones were obtained from a total of 272 individually isolated cells giving an average cloning efficiency of 19.1%.

The parental populations of strains McC "A" and "B" included cells with 46, 47 and 48 chromosomes, disomic, trisomic and quadrisomic for a G group acrocentric. The clones were examined cytogenetically and enzymatically and are the subject of another communication. While pure trisomic clones were isolated, the majority of the clones showed a few disomic and quadrisomic cells in addition to a major population of trisomic cells, thus proving the occurrence of non-disjunction *in vitro*. This illustrates an important application of the method.

Sub-clones could readily be obtained from the one strain in which this was attempted. It was estimated that the cells which were recloned had previously completed approximately 24-28 cell doublings, including the 3-7 cell doublings estimated for establishment of the strain from explants.

In view of the restricted *in vitro* lifespan of diploid human fibroblast strains(9), it is important to establish clones early in the history of a culture. In this connection, Mr. Curtis Sprague of our laboratory has recently successfully employed a modification of the method described in this paper to obtain primary or near-primary clones. Explants were attached to 3 mm square coverslips. As soon as a few fibroblasts had migrated, the tissue fragment and all but a single cell per coverslip were removed and the coverslips transferred to half-ounce prescription bottles as described above. The technique is being utilized for quantitative studies of fibroblast culture "senescence"(9) both as a function of the tissue of origin and the age of the donor.

*Summary.* Established human fibroblast cultures were cloned by isolating single cells on 3 mm square coverslips and growing them in medium containing pre-tested fetal calf serum. Coverslips with single cells were readily obtained by dilute plating combined with mechanical removal of surplus cells. The

mean cloning efficiency was 19.1%. Sub-clones were obtained with a mean frequency of 23%.

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## Tryptophane Transport in Cultures of Human Fibroblasts.* (31424)

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Cystinuria(1), Hartnup Disease(2) and certain forms of hyperglycinuria(3) appear to involve defects in amino acid transport. It is possible that the mutations responsible for these biochemical lesions are expressed only in certain specialized cell types, such as the renal tubular epithelium and the intestinal mucosal cells. An alternative hypothesis is that all somatic cells are affected, although possibly to varying degrees. An investigation of amino acid transport in cultured skin fibroblasts from patients and controls would provide a simple test of this hypothesis. In this paper we describe a method suitable for such studies, together with the results of tryptophane transport studies on a group of control cultures.

*Materials and methods. Cell strains.* Human skin fibroblast cultures were established by explant techniques and maintained in Waymouth's medium(4) supplemented with 10% newborn calf serum (heated to 56°C for 30 minutes) and penicillin (50 units/ml). Table I lists the strains employed in this study. All of the subjects were caucasian. The biopsy of CHAM was obtained 18 hours after this child died of a bronchopneumonia; skeletal anomalies of the skull and pachygyria were observed. Chromosome studies have not yet been carried out on these strains. With the possible exception of CHAM, they are almost certainly diploid, since numerous cytogenetic studies of such strains developed in our laboratory have revealed stable diploidy with a small (less than 4%) population of tetraploids in most cultures.

*Preparation of monolayers.* Trypsinized cells(5) (approximately 100,000 cells/ml × 2 ml) were allowed to attach to 22 mm square coverslips ("Gold Label," No. 2 thickness, Clay Adams Co., New York) in 35 mm sterile plastic petri dishes ("Blue Label," Falcon Co., Los Angeles, Calif.). After a 24-hour period of incubation at 37°C in a continuous flow carbon dioxide incubator (5% $CO_2$, 95% air), the coverslips were transferred to Columbia staining jars containing 10 ml fresh medium and permitted to incubate for an additional 24 hours. The use of staining jars provided a common medium for replicate cultures(6) with better temperature control during subsequent manipulations of the cultures. At 48 hours, when pulsing experiments were performed, the monolayers were still subconfluent with the exception of small confluent patches in the para-central areas of the coverslips.

*Pulsing technique.* Immediately before incubation in radioactive medium, the monolayers were rinsed in Hanks' balanced salt solution(7) buffered with 0.02 M tris (hy-

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