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A Simplified Method for the Cloning of Heteroploid and Diploid Mammalian Cells.* (29242)

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To study metabolic or morphologic differences between individual cells, it is essential to be able to derive clones from a single cell. Although this is true for established heteroploid as well as diploid cells, it is particularly applicable to primary cells. Cloning of such primary cells offers an opportunity to develop lines derived from cells which in conventional mass cultures might be overgrown by more vigorous types.

Methods for isolating a single cell for cloning include: Sanford's isolation of single cells in a capillary tube(1), implantation by Lwoff of single cells in drops of medium under mineral oil(2), trypsinization of a clone within a glass or plastic well by Puck(3), and isolation of single cells on glass squares by Schenck and Moskowitz(4).

All of these methods have been used successfully for cloning heteroploid cells with an efficiency approaching 100% in various media. With the exception of Puck's report of 25-60% efficiency for fibroblastic cells(5), however, the cloning of diploid cells is reported to be much less efficient, ranging from one clone per thousand to one clone per million cells(6). It is perhaps for this reason that certain workers clone diploid cells by diluting to a point where a single clone grows (6). Under these conditions it is apparent that a high degree of selection has been applied to the original population.

A simplified method is presented herein for the cloning of heteroploid and diploid cells. The following points are covered:

1. Procedures which help assure that the clones are derived from single cells. 2. A modified Schenck method for planting cells on small pieces of broken cover slips which facilitates their detection and isolation. 3. A description of media and conditions which provide average plating efficiencies from 5% to 50% for the diploid strains and from 5% to 100% for the heteroploid strains studied. 4. A tabulation of cloning efficiency as a function of population. 5. A tabulation of cell line derivation efficiency as a function of population.

Materials and methods. Media. All cloning was carried out in a medium consisting of Eagle's Basal Medium in Earle's Balanced Salt Solution, supplemented with 2 mM glutamine, 0.1 mM non-essential amino acids, 1 mM pyruvate and 10% dialyzed calf serum or 10% whole fetal calf serum. All media were obtained from Microbiological Associates and had been certified by their Quality Control Department as having 90% cloning efficiency for HeLa S3 cells.

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Preparation of cell suspensions. Heteroploid cells were grown in suspension culture. If, as determined by cell count, a culture had been in log phase for 3 consecutive days, a 10 to 15 ml aliquot was removed and placed in a test tube for 5 to 15 minutes, allowing larger cell clumps to settle out. The top few ml of supernatant fluid were removed for counting and plating.

Diploid cell strains were prepared by removing the growth medium from a monolayer culture which had been refed the day before and replacing with fresh cloning medium, containing 2% serum and 0.25% trypsin. After one minute this was poured off and the cells were allowed to incubate at 37°C for an additional 10 to 15 minutes. Removal of the cell sheet, if not complete, was assisted by scraping with a rubber policeman. The cells were resuspended in fresh cloning medium, pipetted vigorously, and placed in a tube for five minutes to allow settling of clumps.

Primary cells were prepared by trypsinization with 0.25% trypsin in cloning medium containing 2% serum. After an initial discard, 3 cell collections were made at 15-minute intervals and the pooled cells were stored at 0°C until the final collection was made. The final cell pool was centrifuged at 800 rpm for 8 minutes and resuspended vigorously in cloning medium. A 5-minute settling out period was allowed before removal of the planting aliquot.

Counting of cells. The counting of cells was performed in a hemocytometer with trypan blue as a vital stain. Total viable cells and total viable clone forming units (CFU), *i.e.*, single cells, doublets, or triplets were counted. The cell distribution obtained will be discussed later.

Planting of cells. The planting of cells was carried out by a 2-man team. One member made the cell counts on the original aliquot, while the second man made the necessary serial dilutions based on the estimate of the cell concentration. As soon as the dilutions were completed, 1 ml of cell suspension was added to 5 ml of cloning medium in a 60 mm tissue culture petri dish. Dishes were planted so as to contain approximately 10, 100, and 1000 cells. The number of clone forming units per dish which had actually been planted was then calculated, based on the viable count of the original aliquot. For example, a series of dishes may have been planted at 6 CFU, 60 CFU, and 600 CFU. The important reasons for this simultaneous count and plant will be discussed later.

Two sets of plates were planted, consisting of dishes with and without broken cover slips. The dishes with broken cover slips were used for the actual isolation and selection of clones; the other dishes were stained at periodic intervals to yield information on cell distribution, cloning efficiency or generation time.

The cloning dishes were incubated at 37°C in an atmosphere of 5% CO_2 in air with a humidity of about 70%.

Preparation of cover slips. The cover slips were prepared by placing #1 cover glasses in a mortar and pestle and grinding to a size that would pass a 10 mesh but not a 20 mesh screen. The pieces were then washed in running tap water for 5 minutes, followed by 3 rinses in 70% ethyl alcohol and one in ether. After a preliminary air drying, the pieces were oven dried in an open tube, distributed into glass petri dishes, and autoclaved before being used.

Selection of clones. Using the stained dishes as a guide to cloning efficiency, a planting level was established which yielded from 5 to 50 clones per dish. Beginning with the third day after planting, the dishes containing glass chips were screened for clones of a size which, based on generation time, would be expected to have been derived from a single cell. For example, a cell with a generation time of 24 hours produces a clone of not more than 16 cells in 4 days.

Clone containing chips were removed with a sterile forceps. The isolated clone was introduced into 0.3 ml fresh medium in a 5 ml screw cap vial then left undisturbed except for feeding once weekly.

Building up of clone. In the case of heteroploid cells, the floor of the vial became filled with daughter clones derived from floating cells. As the number of cells increased, refeeding was increased to twice a week. The

TABLE I. Cloning Efficiencies Obtained for Heteroploid, Diploid, and Primary Cells.

Cell line	Percent cloning efficiency: Range of CFU per plate <10	25–75	50–150	250–750	500–1500	5000–15,000
Primary diploid						
Human emb skin/muscle	—	54	—	35	—	TM*
Monkey kidney, rhesus adult	0	5	6	7	—	—
Mouse kidney, adult	5	—	4	—	5	—
Whole mouse embryo	0	—	8	—	7	—
Rabbit kidney, adult	0	—	3	—	7	—
Human emb kidney	15	—	8	—	5	—
Serial diploid						
MA† 5-11 human emb skin	33	—	26	—	—	TM
MA† 7 human emb lung	20	—	12	—	—	TM
WI 26 human emb lung	7	—	12	—	11	—
Heteroploid						
Adult rhesus monkey spleen, MA† 107	0	—	5	—	4	—
HeLa S3 (Puck)	100	—	100	—	—	—
MA 106 human emb lung	—	—	100	—	TM	—

* TM = Too many to count.
— = Not done.
† MA = Microbiological Associates.

cells were eventually scraped, planted into 4 oz Brockway prescription bottles, and subsequently handled by conventional mass culture methods. Diploid cells, however, needed help in spreading. When the clone completely filled the glass chip and had begun to spread on the floor of the vessel, it was trypsinized gently and allowed to resettle on the same surface, forming many new centers of growth. This was repeated until the bottom was about 75% sheeted, at which time the culture was trypsinized and transferred to a 4 oz Brockway bottle.

Results. Cloning efficiency based on a minimum size of 10 cells in 7 days is listed in Table I. The results are expressed as the mean efficiency for a minimum of 3 plates in each of 2 separate experiments. In the case of primary monkey kidney and HeLa S3, the results are a mean of multiple experiments. The range in all cases fell within ± 20% which would be expected from figures originally based on hemocytometer counts.

The optimal number of CFU per plate was 100. Planting concentrations below that figure resulted in too few clones per plate, although there did not appear to be any population effect. Cloning efficiencies of less than 10% in a 100 CFU plant would probably not produce any clones at a 10 CFU level due to dilution. It seems that either 10 or 1000 CFU per 5 ml of medium is below the population level required for enhanced plating efficiency.

The clones derived from a single cell differed morphologically. They can be divided into 4 groups based on 2 characteristics. These are: 1. Epithelial, forming closely knit clones with no migration or repulsion of cells. 2. Fibroblastic, forming similar clones to type 1. 3. Epithelial, forming loose or broken clones by migration or repulsion. 4. Fibroblastic, forming similar clones to type 3. Type 1 is typical of heteroploid cells and primary kidney, Type 2 is unusual for these cells.

Type 4 is typical of diploid fibroblastic cells; Type 3 is unusual for these cells.

In addition, there are individual morphological cell characteristics which seem constant within a clone. For example, some clones consist of giant cells, some of vacuolated cells. These morphological differences indicate the clone, if not derived from a single cell, is derived from a reduced population which results in different colonial morphology.

The ability to plate clones at an efficiency of 5% to 50%, however, does not mean that cell lines can be derived from these clones.

TABLE II. Cell Line Derivation Efficiency as a Function of Size—Cell Line No. 3884.

Size of clone	No. clones selected	No. cell lines derived	Cell line derivation efficiency*
(cells)			(%)
0- 5	41	0	0
6- 10	9	1	11
11- 15	6	1	17
16- 20	10	4	40
21- 25	4	1	25
26-100	5	2	40

* Cell line derivation efficiency is based upon number of clones selected.

As shown in Table II, even the trauma-reducing method of removing the entire clone on a glass slip results in a low efficiency of cell line 3884 derived from a hamster tumor induced by the Schmidt-Puppin strain Rous Sarcoma virus. No clone selected with fewer than 6 cells was able to survive. Clones of larger size yielded from 11% to 40% cell line derivation efficiency. In this case there appears to be a definite population effect which has a cut-off point in the vicinity of 10 cells.

Discussion. There are two major problems in the planting of clone cells. The first is the maintenance of a "physiological" environment in the surrounding medium. Large populations can adjust their environment to some extent. With small populations, however, unfavorable conditions result in extremely low cloning efficiency. The conditions to be maintained include temperature, CO_2 tension, pH, and humidity. For example, even with heteroploid HeLa S3 cells, it is difficult to clone in medium 199 in 5% CO_2 unless additional bicarbonate is added to the medium. Humidity is important as an increase in tonicity due to evaporation can be lethal.

The second major problem is contamination of the clone with other cell types. This is eliminated if a single cell is isolated and cultured, but the methods most likely to achieve this (Sanford, Lwoff) are also the most tedious. Puck's method, which is much simpler, also has disadvantages. There is considerable chance for a migrant cell to contaminate a clone in 7 days. Furthermore, in our hands it is difficult to work within the glass wells. It is also possible to lose a number of cells during the trypsinization process so that only large clones can be selected. The major disadvantage lies in reduced cell line derivation efficiency due to the trauma of trypsinization on a small cell population.

The process of diluting out a cell suspension until isolated clones are obtained in subculture may not produce true clones. In the case of diploid cells, the cloning vessel may contain many cells which as single cells cannot clone but are viable. As a nearby clone grows, these cells may become incorporated and may begin to divide as mass culture conditions are restored.

The glass chip method offers definite advantages. A clone can be isolated easily, regardless of its size and without the trauma of trypsinization. Ideally, a single cell should be selected, but single cells are difficult to see and examination of the plates at such an early time may cause a change of conditions which lead to cell death. Furthermore, it is difficult to derive a cell strain from a single diploid cell. It is, therefore, necessary to employ other safeguards to help assure that the clone is derived from a single cell.

The cell inoculum should consist mostly of single cells. Although this can be accomplished with ease for most cell lines, it is quite difficult for some cell strains. Using the methods previously described for preparing cell suspensions, cell distribution ranged from 80-100% single cells and from 0-20% doubled or triplets.

The number of cells planted per dish should be small enough that it is possible to isolate a clone. If there are more than 1000 cells in a 60 mm dish, it has been difficult to isolate a true clone. In our experiments for most cell lines tested, a plate containing 100 CFU is optimal.

The clone selected must be a size that is consistent with the generation time of the cell. There have been published pictures of clones which contain too many cells to have been derived from a single cell in the time period indicated. A cell with a generation time of 24 hours can produce a clone of 16 cells in 4 days but can not produce a clone of 32 or 64 cells.

In theory, it is best to select and isolate a single cell and to derive a cell from it. This is relatively simple for most heteroploid cells but very difficult for diploid cells. Larger clones have a better chance to grow into cell lines. This population effect demonstrates the need for suspending the newly isolated clone in as small a volume of medium as is practical.

Methods of increasing efficiency. This paper reports higher plating efficiencies for diploid cells than generally reported. The reason for this is not clear. The answer may lie in controlling conditions, including media, temperature, CO_2, pH, and humidity. There is also a possibility that there is a time element involved. If there is a 5-minute delay between the preparation of a cell dilution and its inoculation, the cell concentration in the dilution tube will be greatly diminished because diploid cells attach to glass very rapidly. In some cases, we have observed 50% of the cells attach within 5 minutes. To offset this, one person counts the cells while simultaneously a second makes the dilution and plants the cells. In this way the calculated number of cells is more closely related to the number actually planted.

The variation of plating efficiency of from 5%-100% for heteroploid and from 5%-50% for diploid cells cannot be due to variations in environment because each experiment had a HeLa S3 control. The variations may be due to differences in the condition of the cells when they were planted or they may represent differences between cell types.

Summary and conclusions. A method which appears a practical and easy way to produce cloned cell lines is presented, with some observations and problems to be considered when cloning. Although the method attempts to derive cell lines from single cells by taking many precautions, it cannot be definitely stated that this goal is actually accomplished. It has yet to be demonstrated satisfactorily that a single diploid cell can in fact be cloned. The method, however, definitely produces cell lines which are derived from such a reduced population that morphological differences cannot be demonstrated within a clone, but can be demonstrated between cells from different clones.

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Ethynyl Estradiol: A Potent Orally Active Contraceptive in Rats. (29243)

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The action of estrogens in producing a state of infertility in a large number of animal species has been well documented. Smith (1) demonstrated that estrin, an ovarian extract, produced sterility in rats. Pincus and Kirsch(2) obtained similar results when treating rabbits with estrone. Brown and coworkers(3) were able to inhibit ovulation in the human with daily doses of stilbestrol. More recently, ethynyl-estradiol- 3 methyl-ether, without addition of a progestagen, has been reported by Walker to be an effective oral contraceptive in humans (cited by Jackson; 4). The contraceptive action of estro-

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