A REPRODUCIBLE PROCEDURE FOR PRIMARY CULTURE AND SUBSEQUENT MAINTENANCE OF MULTIPLE LINES OF HUMAN SKIN FIBROBLASTS

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ABSTRACT

Cultured fibroblasts are a valuable tool to study many cellular processes and their modification by aging. Fibroblasts are a useful cell type in which to study many diseases, including those of the nervous system, in which a strong genetic component is suspected. Fibroblasts permit the study of multiple, dynamic processes in living cells, while avoiding the effects of the dying process and post-mortem artifacts that limit other approaches. For results to be comparable across time in one laboratory or consistent between laboratories, the detailed culture techniques require meticulous care and replicability. Lack of attention to detail in initial stages can lead to selection of different cell populations. Small variations in other variables such as batches of serum can significantly alter growth rates and comparisons of cells from controls and Alzheimer patients. The aim of this paper is to present a detailed protocol for comparison of multiple cell lines from many patients. An example of using this approach to study growth and phase out (i.e., senescence) of cells from Alzheimer patients is presented. This procedure represents a modification of an earlier protocol (Cristofalo and Charpentier, 1980).

KEY WORDS. Fibroblasts, Alzheimer's disease

INTRODUCTION

Fibroblasts are the least specialized cells in the connective tissue family, a cell lineage that has an unusual extent of interconvertability (Alberts et al., 1989). Fibroblasts are selected from multiple cell types in a skin biopsy by the use of selective media. The cytoskeletal structure and attachment factors for fibroblasts have been studied extensively (Gauss-Muller et al., 1980). Cultured fibroblasts have been used to determine basic mechanisms of aging. As fibroblasts age in culture, their

ability to divide decreases. In addition, fibroblasts from aged donors do not grow as well as those from young donors (Houck et al., 1971; Cristofalo and Pignolo, 1993).

Utilization of fibroblasts has many advantages for studying disease, particularly those disorders that have a strong genetic component. Any effects of the diet or drugs that the patient may be taking are diluted hundreds of times by the passage of the cells. Fibroblasts can even be used to study many neurological diseases with a significant genetic component. If a protein is expressed in brain and in fibroblasts, a genetic defect in that protein would be expected to be present in both tissues. The primary biochemical deficit of a number of neurological disorders has been discovered in studies with fibroblasts (Stanbury et al., 1983). Recent results showing a strong genetic component in Alzheimer's disease have increased the interest in these cells (Huang et al., 1991, 1993, 1994, 1995) and the need for a standardized protocol.

Although fibroblasts are extraordinarily easy to keep alive in culture, the difficulty in using fibroblasts for studying disease is that the comparison of multiple cell lines from numerous patients is challenging. The first requirement for a reproducible method is that the biopsy is taken the same way each time. The initial steps in the primary culture have to be the same. Lack of attention to detail in early stages appears to lead to selection of different cell populations. Each cell line then has to be maintained in the same way for multiple passages. Small variations in other variables such as batches of serum can significantly alter growth rates and comparisons of cells from controls and Alzheimer patients. If all of these conditions are rigorously controlled, differences between cell lines are likely to be due to genetic differences.

The purpose of this paper is to present a protocol that has allowed us to grow multiple fibroblast cell lines replicably. The current protocol modified the procedure of Cristofalo and Charpentier (1980) to allow comparisons of multiple cell lines, to minimize loss in case of contamination and to update the equipment and sup-

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plies available. To demonstrate the usefulness of this procedure, growth curves to phase out (i.e., senescence) are presented for 25 cell lines derived from control and Alzheimer patients.

MATERIALS AND METHODS

Although we have not documented that the following products are the best, we know that the use of these products ordered repeatedly over several years has given highly replicable results.

Cells

All of the cells that were used in these investigations are available to any investigator from the Coriell Cell Repository. Cells were chosen from sporadic cases of Alzheimer diseases and from a Nova Scotian kindred that has a presenilin-1 mutation.

Equipment

A room with the following equipment should be reserved for mycoplasma free fibroblasts. The cell culture incubator (Forma model 3860; 5%CO₂, 95% humidity) maintains CO₂ by adding CO₂ instead of the more expensive way of buying a CO₂-air gas mixture. This incubator also returns the CO₂ to initial levels very quickly after the door has been opened. The Laminar-flow hood (Biological Class II, Forma Model 1100) should be routinely accredited to make sure that flow rates are appropriate to protect both the cells and the investigator. The hoods should meet Federal Standard 209E and include the following: velocity profile (100 ft/min 6 inches from the filter face), particle counts (at 0.5 μ cannot have more than 100 counts and at 0.3 μ not more than 300 counts) and no hepafilter leak as determined with emory 3004.

The microscope (Model CK2 Olympus, inverted, phase contrast, with long working-distance condenser) is used for cell counts, examining the extent of harvesting and the health of the cells. A Beckman TJ6 centrifuge is used for spinning the cells. A Coulter Counter, Model ZF, is used to expedite cell counting of multiple cell lines. A pH meter is reserved for the preparation of solutions that are described below. A second room with similar equipment should be used for cultures that have not yet been tested for mycoplasma.

Glass and Plasticware include the following: vented tissue culture flasks with caps that allow complete air exchange through a filter that blocks any other contaminants [175 cm² (Falcon# 3112), 75 cm² (Falcon#3111), 25 cm². (Falcon#3109).], 35 mm petri dishes (Falcon#1008), polystyrene pipettes [1 ml (Falcon#7520), 2 ml (Falcon#7507), 5 ml (Falcon#7543), 10 ml (Falcon#7551), 10 ml, for primary culture (Falcon#7504), 25 ml, (Falcon#7525), 50 ml (Falcon#7550)], centrifuge tubes [15 ml (Falcon#2099), 50 ml (Falcon#2098)], Pasteur pipettes (VWR #14673-010), glass bottles and caps (Pyrex #1395) and cryotubes 2 ml (Corning #25-724-2).

Filters include: bottle top 150 ml (0.22 μ , cellulose nitrate (Falcon #7105), 115 ml (0.22 μ , cellulose nitrate; Nalgene #120-0020), 500 ml (0.22 μ , cellulose nitrate: Nalgene #156-4020)

Growth Medium components include Dulbecco's Modified Eagle Medium (DMEM) [low glucose - 1000 mg/L with L-glutamine, sodium pyruvate (110 mg/L) and without sodium bicarbonate (Gibco 430-1600EB)], sodium bicarbonate solution, 7.5%(w/v) (Sigma S8761; opened bottles stored refrigerated), sterile fetal bovine serum (100 ml quantities; performance tested, mycoplasma tested and virus tested; see below for selection of lot and supplier), penicillin/streptomycin (Gibco #600-5140) and fungizone (Gibco# 600-5295).

Dissociation medium components include trypsin solution [10X in 2.5% in Hank's Saline without Ca⁺² and Mg⁺² (ordered in 100 ml quantities; GIBCO 15090-046). Store this solution frozen. At first thawing aliquot in 5.5 ml or 10.5 ml portions and refreeze at -20°C.], sodium chloride (cell culture tested, Sigma S5886), potassium chloride (cell culture tested; Sigma P5405), sodium phosphate monobasic (cell culture tested; Sigma S5011), glucose (dextrose, cell culture tested; Sigma G7021), MEM Amino Acids [50X, Flow/ICN 16-011-49; Store these at 4°C; when opened, aliquot 20.5 ml in 50 ml centrifuge tubes and store at 4°C.], MEM Vitamins [100X, Flow/ICN 16-014-49; Stored at 4°C. When opened, aliquot 10.5 ml in 15 ml centrifuge tubes and refreeze.], phenol red [0.5% solution, Flow/ICN 16-900-49; Store at 4°C. When opened, aliquot 10.5 ml in 15 ml centrifuge tubes and store at 4°C].

Miscellaneous items include 70% Ethanol, Isoton (azide-free, Coulter #8546720; Curtin Matheson, NJ), #10, #11 Scalpel blades, glycerol, (J.T. Baker FW 92.1 #4043-00), fine line straight hemostats (5.5").

PREPARATION OF SOLUTIONS

All preparation of solutions and manipulation of cells should be done with latex gloves that have been thoroughly cleaned with alcohol. The preparation date for each solution should be logged in a notebook.

DMEM Growth Medium. Solutions should be made three days before use to allow testing for contamination. They should not be stored more than two weeks at 4°C. Prepare DMEM by dissolving a one liter package of powdered DMEM in 900 mls of deionized distilled water. Add 49.3 mls of 7.5% sodium bicarbonate. Adjust pH to 7.1 with 5 N HCl. Bring the volume to one liter with deionized distilled water. Under the tissue culture hood, filter 250 mls through a bottle top filter (0.22 cellulose nitrate), into a clean, autoclaved, Pyrex bottle. Add 27 mls of fetal bovine serum (see precautions below) and then remove about 5 mls of the media. Place this ~5 ml aliquot into a 15 ml centrifuge tube and incubate at 37°C for three days to check for contamination before the media is used with

cells. Cap the bottles of complete media and store them at 4°C. Store unopened bottles, sealed at 4°C.

Each lot of fetal bovine serum must be evaluated. This testing minimizes the drift seen in tissue culture over the years. Fetal bovine serum is purchased in 1-2 year quantities (about 50 L) in 100 or 500 ml bottles and stored frozen. Once thawed it is not re-frozen. If it has been thawed for more than one week, it is not used. Samples of a number of lots are obtained from multiple suppliers. The ability of these different lots of serum and their ability to support growth is tested with one cell line. The cell line we arbitrarily chose was GM O3652A, which happens to be from a young individual. The growth of this cell line in the different media is compared after three transfers and the lot that produces growth rates closest to that of the currently used lot is selected. The final CPDL for the three transfers is used to determine growth rate and thus to choose the appropriate lot.

DMEM Primary Culture Growth Media. This media should be prepared at least three days before use and checked for contamination by incubating an aliquot at 37°C (see above). Make DMEM growth media as described above. Filter sterilize 75 mls of DMEM through a bottle top filter (0.22 μ cellulose nitrate). Add 20 mls of fetal bovine serum. Add 2.5 mls penicillin/streptomycin. Add 2.5 mls fungizone. Store at 4°C for up to two weeks.

Freeze Media. This media should be prepared at least 3 days before use and checked for contamination by incubating an aliquot at 37°C. Filter sterilize 75 mls of the DMEM Growth media without serum described above through a 0.22 μ cellulose nitrate filter. Add 15 mls of fetal bovine serum. Add 10 mls of sterilized (0.1 μ nylon filter) DMSO. Store at 4°C for up to two weeks.

Ca⁺² & Mg⁺² Free MEM. Dissolve the following into 900 mls of deionized distilled water: NaCl (6800 mg), KCl (400 mg), NaH₂PO₄ (anhydrous; 120 mg), Glucose (1 g), 50X MEM Amino Acids (20 mls), 100X MEM Vitamins (10 mls) and 0.5% solution Phenol Red (10 mls).

Bring the volume to 1 L with glass distilled water and sterilize by vacuum filtering using a 0.22 μ cellulose nitrate filter. Transfer 40 ml aliquots to 50 ml centrifuge tubes and store at 4°C.

Trypsin Solution (0.25%). Make this solution at the time that it is to be used. To a 40 ml aliquot of MEM without Ca⁺² & Mg⁺², add 5 mls of trypsin (from the prefrozen 5.5 ml aliquots described above) and 5 mls of 7.5% sodium bicarbonate. Adjust the pH to 7.1 at room temperature with 5-N HCl (pH usually rises 0.1 to 0.3 units upon filtration). Filter sterilize using a 0.22 μ cellulose nitrate filter unit.

Viability Dye: Erythrosin-B Stock Solution. Dissolve the following in 95 mls of glass distilled water: 0.4 g Erythrosin B, 0.81 g sodium chloride, 0.06 g Potassium phosphate monobasic, 0.05 g Methyl p-hydroxy benzoate. Adjust the pH to 7.4 with NaOH. The compounds will not go into solution until the pH is near 7.4. Bring the volume to 100 ml with glass distilled water.

PROCEDURES

Obtaining the primary culture. Skin biopsy is a simple procedure that causes minimal pain. The practice for obtaining informed consent, recording histories and coding the patients so as to protect their identity are very important, but requirements vary among institutions and their Internal Review Boards. Consistency in obtaining the biopsy sample is critical for comparisons between individuals. The patient lays on his/her back with the left arm over their head and resting on the table. The biopsy site is taken one third of the way from the elbow to the axilla along the medial surface of the upper arm. This is an area that does not normally receive intense exposure to the sun. Clean the area well with alcohol wipes. Pinch the skin at the biopsy site with a fine line hemostat. This is the most painful part of the procedure. Pinching the skin with the hemostat reduces the pain of the biopsy, restricts blood flow and helps to induce clotting. With a sterile #10 scalpel cut downward and remove a superficial piece of skin measuring approximately 1/8 inch by 1/8 inch. While one person removes the clamp and applies a band-aid, a second person immediately transfers the biopsy to culture media (see below).

Starting Primary Cultures. Place the skin biopsy in sterile 15 ml centrifuge tube (FALCON 209) containing 10 mls of DMEM Primary Culture Growth Media. Store samples at 4°C and place into culture within 24 hour of excision. Wash each piece of skin in 4 different baths of Primary Culture Growth Media (5-8 mls each). Make transfers from each bath with a sterile forceps. After washing the skin, place it into a sterile 35 mm petri dish containing 1 ml of Primary Culture Growth Media (see above). Using two sterile scalpels (#11 blades) and a crisscrossing motion cut (shred) the skin into small pieces, not greater than 1 mm X 1 mm. Prewet a wide tip pipette with Primary Culture Growth Media and pull media and tissue pieces into the pipette and transfer the suspension into a T25 vented flask. Add one ml more media to the petri dish to rinse it and then transfer this additional media/tissue to the T25. The total volume should now be 2 mls. Place the T25 in a 5% CO₂ incubator at 37° for 24 hours. Then gently invert flask (cells and tissue on the top), and leave it in that position for 24 hours. Then reinvert the flask (cells and tissue on the bottom). Change the media weekly until the fibroblast layer in the flask is confluent (see definition of confluence below).

Maintenance of the Cell Lines. Placing an aliquot of each solution in the incubator before use minimizes contamination. Keeping an aliquot of each solution in the incubator after the solution is used helps to trouble shoot sites of contamination. In addition, one T25 flask from each group is not harvested but is kept as a backup. In the event that a cell line is contaminated only that single week of work is lost.

General Procedures for Transferring the Cells.

Preparation of the working area. Leave the hood on or turn it on for at least one hour before introducing any cells. Immediately before use, wipe the working area with 70% ethanol. Spray or wipe all items including gloves with a cotton square or kimwipe saturated with 70% ethanol before putting them under the hood.

Checking the flask for confluence. Put the Olympus IMT microscope on 4X. Examine six different areas of each flask to gain a general impression of cell density. Confluence is defined as when "all of the cells are in contact all around their periphery with other cells and no available substrate (i.e. the bottom of the flask) is left uncovered" (Freshney, 1987). Although this examination is primarily to determine confluence, the general shape of the cells and the pattern of growth should also be carefully noted. Draw a picture or take a photograph if the cells look abnormal.

If a particular cell line does not reach confluence after one week, save one flask as a backup and change the media in the remaining flasks. To change the media, remove all of the media and replace it with the appropriate amount of new media. Check for confluence after one more week.

Phase out. Normally, cell lines are harvested and re-seeded weekly. If the cells are not confluent after one week, remove all the media and replace with fresh media. If after three weeks of re-feeding the cells are not confluent, harvest the line and count the cells. If the population doubling level is not at least one, consider the line phased out.

Trypsinizing the Cells. Warm the Trypsin solution to 37°C. Use a separate aliquot of trypsin for each cell line or for flasks of the same cell line at different passages or CPDLs to avoid confusion and the possibility of cross contamination. Process different cell lines or flasks of the same cell line at different passages or CPDLs at different times. Take the flasks out of the incubator, wipe or spray them with 70% ethanol, and transfer them to the tissue culture hood. Remove the lid of each flask as you work with it. The lid should not be placed on the tissue culture hood surface. While holding the flask and the lid in the same hand, do the removal or additions with a pipette in your opposite hand. If you are working with multiple flasks of the same line, then loosen all of the lids and only remove the lid of the flask you are working on. Remove the media from the flasks. Take the fluid from the side of the flask opposite to the cell growth surface. With another serum-free pipet, rinse the monolayer with 4 mls of trypsin solution and discard. Repeat with another 4 mls of trypsin and remove the last bit with a 1 ml pipette. Add 2 mls of trypsin solution (T25 or T75) or 5 ml (T175), screw the lid back on the flask, and incubate at 37°C for eight minutes. Check under the microscope to see if cells are rounding up and detaching. The flask can be tapped to speed up detachment process, but care should be taken to not splash media. If most cells have

detached, proceed to "Harvesting and Counting the Cells". If not, return the flask to the incubator and check every two minutes. Never allow the trypsin treatment to go beyond 15 min.

Harvesting and Counting the Cells. Add 3 mls (T25 or T75) or 5 ml (T175) of DMEM growth media to the trypsinized flask and then place the 5 mls (T25 or T75) or 10 ml (T175) of cell suspension in a 50 ml centrifuge tube. Rinse the flask with 5 mls of complete media and add this volume to the centrifuge tube. Pool the suspension if there is more than one flask of the same cell line, which is also at the same CPDL. Triturate the suspension containing the trypsinized cells, 5 times with a 10 ml pipette and then 5 additional times with a 5 ml pipette. With a 1 ml pipette, take 0.5 ml of triturated cell suspension and add it to 19.5 mls (previously aliquoted) of Isoton II. Invert it twice and then take four counts on the Coulter Counter (settings: amplification 4; threshold 10; aperture 2). To obtain the number of cells/ml multiply the mean by 80, since this is a 1:40 dilution and the Coulter Counter only counts 0.5 mls. Counts over 10,000 or under 1,000 require special consideration.

Erythrosin-B exclusion Viability Assay (Phillips, 1973). Place the cover slip on the hemocytometer (Figure 1).

Figure 1

Triturate the cell suspension well and then take a 1 ml sample and place into a 1.5 ml centrifuge tube. Add 100 μ l of erythrosin-B to the tube and triturate to mix. Thus, the suspension is diluted by a factor of 1.1. Let it stand for 4 minutes, then gently triturate five times with a small transfer pipette, and then using a Pasteur pipette fill both chambers of the hemocytometer. Alternatively, the hemocytometer can be loaded with 12 μ l using a micropipette. Place the hemocytometer under 10x magnification and count non-viable cells (those stained) and viable cells (those unstained). Starting with one chamber of the hemocytometer, count all the cells in each of the four large corner squares. Each of these squares is 1 mm². The perimeter of each square is bordered by three parallel lines. Count the cells on top and left touching the middle line of the perimeter of each square (see Figure 1; open circles). Do not count cells touching the middle line at the bottom and right side (see Figure 1; closed circles). Record the total number of cells for each of the four squares. Move to the other chamber and repeat these counts. If the counts of the two chambers disagree by more than 5%, redo the count. Percent viability is calculated as the number of unstained cells divided by the total number of cells (stained and unstained) x 100%. If viability ever declines below 95%, the technique for harvesting needs to be reevaluated. Divide the total count by 8 to give the average count per square. Each square represents a total volume of 0.1 mm³ (0.1 μ l) or 10⁻⁴ cm³ (10⁻⁴ ml). The number of cells per ml will be determined using the following calculation:

$$\# \text{ cells/ml} = (\text{average cell count per square})(\text{dilution factor})(10^4)$$

For the procedure described above the dilution factor is 1.1. If the cell counts by this method and the Coulter counter do not agree, each step must be carefully reevaluated until they agree.

Seeding the flasks. Calculate the amount of cell suspension needed to seed the size flask needed at 1 x 10⁴ cells/cm² (Table 1). Triturate the cell suspension 5 times with a 5 ml pipette and then add the calculated amount of cell suspension. Cap vented flasks and place in a 5% CO₂ incubator. The area and the amount of complete media for the various containers are shown in Table 1.

Table 1. Volume of media for different containers.

Flask	Area	Total volume (ml)
T25	25 cm ²	13.3
T75	40 cm ²	40.0
T150	150 cm ²	80.0
T175	175 cm ²	93.0
33 mm Petri dish	9.6 cm ²	2.0
4-Well Chamber slide	1 cm ²	0.5

Add the appropriate amount of complete medium to every flask that is to be seeded with the same cell line at the same cumulative population doubling level (CPDL; see below). For example, the final volume for a T75 flask is 40 ml. Subtract the amount of cell suspension you will seed into each flask from the 40 ml. Adding the media first prevents unnecessary loss of cells due to a dam-

aged flask or an accidental break in aseptic technique. Then triturate the cell suspension and add an aliquot to the medium that is already in the flask. Seed an extra flask as a backup. (A T25 is fine for this.) This flask is kept with the current line until cells of the same week are harvested. The backup flask is moved to a backup shelf in a separate incubator. Discard only when you make the backup for the next week. At each transfer, add an aliquot (5 ml) of the complete medium to a 15 ml centrifuge tube and store at 37°C to check for contamination; discard when the study flask is used.

Calculation of the Cumulative Population Doubling Levels (CPDL). To monitor the growth of the cells requires calculation of the Cumulative Population Doubling Level (CPDL). The presentation of passage number (i.e., the number of "splits") does not convey enough information about the biological age of the cells in culture. Population doubling levels (PDL) are calculated using the formula:

$$N_t \div N_i = 2^{\text{PDL}} \text{ where } N_t = \text{number of cells harvested, } N_i = \text{number of cells inoculated}$$

$$\text{Therefore, PDL} = \frac{\log(N_t/N_i)}{\log 2}$$

For example, if one week after seeding a T75 flask with the standard inoculum of 75 X 10⁴ cells, 60 x 10⁵ cells were obtained, the calculation would be as follows:

$$\text{PDL} = \frac{\log(60 \times 10^5 / 75 \times 10^4)}{\log 2} = 3$$

Addition of the PDL for each passage to the already accumulated population doubling level gives the Cumulative Population Doubling Level (CPDL) at seeding.

Freezing and thawing fibroblast lines.

Freezing cells. Harvest and count the cells as described above. Remove the media by centrifugation and resuspend the cells in freeze media (see above) to a concentration of 1.5 X 10⁶ cells/ml. Triturate well and aliquot 1.5 ml cell suspension (2.25 x10⁶ cells) into cryotubes. Each cryotube has a capacity of 2 ml. Cells should be frozen slowly by leaving them in a -80°C Revco freezer in a Nalgene cryo freezing container (Nalgene product number 5100-0001) for 24 hours. This cools the cells at a rate of -1°C/min. Cells are then transferred to a liquid nitrogen container for longer storage. It is critical that a responsible person ensure that the level of liquid N₂ in the container is maintained.

Thawing frozen cells. Cells should be thawed quickly. Remove the vial from the liquid nitrogen container and place it immediately in a 37° water bath. Once thawed, transfer the cells into a T25 flask containing 10 ml of complete medium and incubate. To remove all traces of DMSO from the culture, remove the medium from the flask after 8-12 hours of incubation, and replace with a fresh 10 ml complete medium.

Receiving and shipping cell lines to other laboratories. To ship cells, fill confluent flasks with normal growth media and replace the lids with non-vented caps. Wrap

the flasks very carefully with shock resistant packing material and ship them the quickest way possible. Ensure that the cells are not frozen (e.g., in an unheated baggage compartment of an airplane) or left in the sun on a loading dock for hours or days. Call the people receiving the cells when they are sent and give them the weigh bill number, carrier and estimated time of arrival.

Upon arrival of the cells, first check them under the microscope. Then wash the flask with alcohol and place it in the incubator for 24 hours. If the cells are not confluent after the 24 hour equilibration period, the media should be changed and the cap replaced with a vented lid. If cells are confluent after equilibration and the day that they are to be routinely harvested is more than three days away, change the media and the cap replaced with a vented lid. Once the cells are confluent they are harvested by standard methods.

Precautions to avoid mycoplasma contamination. Mycoplasma can easily contaminate cell lines. Assurance from the donating laboratory that the cells are "mycoplasma free" requires confirmation in the accepting laboratory. Two simple procedures prevent mycoplasma contamination. If cells are brought into the laboratory, they are maintained in a separate culture room until they have tested negative for mycoplasma contamination. Secondly, by omitting antibiotics from the media, inadvertent contamination with mycoplasma also leads to more easily detected bacterial or fungal contamination. Testing for mycoplasma can be done in the laboratory by a variety of published methods, but having it done commercially is reliable and time efficient. Cell lines are pooled for these tests and if a positive result is obtained, individual cell lines are tested. The vendor that we have used is Bionique Laboratories, Bloomingdale Road, Saranac Lake NY 12983. The procedure that they use combines an indirect DNA staining approach to detect non-cultivable mycoplasmas with a direct culture methodology. The indirect approach involves the inoculation of the sample into a mycoplasma free VERG indicator cell line and performing a DNA fluorochrome assay after 72-96 hours of incubation. The direct culture aspect of the test utilizes three different mycoplasma media including both broth and agar formulations. The sample is inoculated into each of the three broth formulations and also onto plates for each of the three agar formulations. Subcultures from broth to fresh agar plates is carried out after 7 days incubation. Agar plates are incubated aerobically and anaerobically in order to detect any colony forming units morphologically indicative of mycoplasma contamination. The tests meet the requirements of the Food and Drug Administration and the United States Department of Agriculture. Bionique will provide complete protocols for all of the tests.

Bottle washing. Rinse media bottle and cap thoroughly with tap water when you have finished using the media. Soak for a minimum of two hours in a 1% solution of ICN Biomedical/Flow Linbro 7X Cleaning Solution. Make

sure the bottle is totally submerged. Use bottle brushes set aside for this purpose. Rinse with tap water at least three times to remove any traces of soap. Fill each bottle with distilled water and empty it. Repeat this process three times. Rinse each cap three times with distilled water. Place bottles and caps in the drying oven upside down and dry thoroughly. Put caps on the bottles loosely and place each bottle in an autoclave bag. Autoclave for 30 minutes.

RESULTS AND DISCUSSION

The method of subcultivation described here provides meticulously reproducible cultures of human skin fibroblasts. This procedure allows each cell line to be characterized including cumulative population doubling levels to phase out (i.e., senescence). The methods for doing this are described under "General Procedures for Transferring the Cells". The CPDL to phase out varies considerably between different cell lines (Table 2). Note in Table 2, Trial 2, that the fastest growing of the seven lines tested grew more than twice as fast as the slowest growing line.

Table 2. Comparison of multiple tests of CPDL to phase-out in several cell lines.

	CPDL to Phase Out			Coefficient	
	Trial 1	Trial 2	Trial 3	Mean	STDEV of variation
GM04260	45.4	45.2		45.3	0.1
GM03651	45.7	43.8		44.8	1.3
GM04560	46.5	41.2		43.9	3.7
AG08170	46.2	40.7		43.5	3.9
AG07661	28.6	28.1		28.4	0.4
AG06842	25.7	28.2		27.0	1.8
AG07637	25.4	21.4	18.7	21.8	3.4

CPDL were determined multiple times for each of the indicated cell lines. Flasks (3-5 T175) were taken to phase out and the average number is on the table. The cells from all of the flasks were pooled at each passage. The cell line was then re-ordered and the process was repeated. Since the repeat runs were so similar, phase outs were run three times on only one cell line.

The CPDL to phase out did not vary between the fibroblasts from the Alzheimer patients and controls (Table 3). The average CPDL for the phase out for all of the controls was 45.0 ± 10.6 while that for all of the Alzheimer lines was 46.7 ± 11 . In the Nova Scotian family, fibroblasts from the Alzheimer subjects appear to have a greater CPDL to phase-out, but the difference was not statistically significant.

Cells that were grown by this protocol have been used to reveal differences in several aspects of cell function in fibroblasts from patients with Alzheimer's disease. These include β -adrenergic stimulated formation of cyclic AMP (Huang and Gibson 1993, Vestling et al., 1997), bradykinin stimulated formation of cyclic IP₃ (Huang et al., 1991, 1993, 1995), several aspects of calcium homeostasis (Gibson et al., 1996a,b; Ito et al., 1994; Fohr et al., 1994; Etcheberrigaray et al., 1993; Huang et al., 1991) and α -ketoglutarate dehydrogenase activities (Sheu et al., 1994). The more standardized that the tissue culture methods are throughout the

world, the better the chances that cultures can be used to clarify the molecular and cellular mechanisms in aging and disease of aging including Alzheimer's disease.

Table 3. CPDL to phase out in 25 cell lines

YOUNG CONTROLS	CPDL	
GM04390	60.9	
GM02987	44.8	
GM-3377	50.1	
GM03652	62.6	
GM03651	44.8	
Mean $\pm$ STDEV	52.6	8.6
AGE-MATCHED CONTROLS		
GM04260	45.3	
GM04560	43.9	
GM04560	43.9	
GM0610	50.6	
AG03524	32.7	
AG08044	51.7	
AG06241	48.4	
AG09553	49.7	
Mean $\pm$ STDEV	46.0	6.5
CANADIAN AGE-MATCHED CONTROLS		
AG06842	27.0	
AG07661	28.4	
AG07603	34.2	
Mean $\pm$ STDEV	29.8	3.8
SPORADIC ALZHEIMER'S DISEASE		
AG04400	54.3	
AG05809	51.4	
AG05810	39.9	
AG06264	45.7	
AG06265	61.5	
AG07377	45.9	
Mean $\pm$ STDEV	49.8	7.6
CANADIAN ALZHEIMER'S DISEASE		
AG06848	56.9	
AG06840	45.9	
AG08170	43.5	
AG07637	21.8	
Mean $\pm$ STDEV	42.0	14.7

Each cell line was taken to phase out. Measurement of CPDL in some cell lines was repeated (see Table 2). Flasks (3-5 T175) were started and maintained until phase out was achieved. The cells from all of the flasks were pooled at each passage. Analysis of variance showed that the Canadian Age Matched Controls differ from all groups except the Canadian Alzheimer Disease lines.

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ABBREVIATIONS

CPDL. Cumulative population doubling level.

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