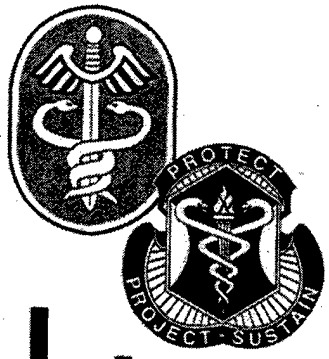

U.S. ARMY MEDICAL RESEARCH INSTITUTE OF CHEMICAL DEFENSE

USAMRICD-TR-02-01

Human Dermal Fibroblasts (Adult): Instructions for Initiation of Cultures from Cryopreserved Cells and Subculture

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April 2002

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
April 2002	Technical Report	

4. TITLE AND SUBTITLE
Human Dermal Fibroblasts (adult): Instructions for Initiation of Cultures from Cryopreserved Cells and Subculture

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
62384

6. AUTHOR(S)
Kahler, D.W. and Arroyo, C.M.

5d. PROJECT NUMBER
TC2

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER
USAMRICD-TR-02-01

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Chemical Defense
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Aberdeen Proving Ground, MD 21010-5400

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
This technical report outlines procedures that have been developed to provide a cost effective way to produce large quantities of normal human skin fibroblast cells (NHSFs) for studying the mechanism(s) of action of chemical warfare agents (CWAs) and medical counter-measurements against CWAs. Various growth factors were added to media recommended by the supplier, American Type Culture Collection (ATCC), Manassas, Virginia, USA. Cell viability, cell differentiation and cell proliferation were evaluated using different tissue culture assays in particular the CyQUANT® Cell Proliferation Assay kit obtained from Molecular Probes, Inc., Eugene, Oregon, USA. This particular assay measures cell proliferation and was used as indicated in the experimental protocol provided by the product brochure. We found cultured human skin cells to be a useful model for skin irritation testing.

15. SUBJECT TERMS
Human skin fibroblasts, cell viability, cell proliferation, chemical warfare agents, sulfur mustard, HD

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			David W. Kahler
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UNLIMITED	16	19b. TELEPHONE NUMBER *(include area code)* 410-436-5100

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Introduction

Skin is a major target organ for many chemical warfare agents (CWAs) commonly used in military and terrorist attacks (Reid and Walker, 1969; Walker and Thatcher, 1968; Eckert, 1989). Recently, it has been reported that significant changes in enzyme activation are caused by sulfur mustard (HD) in 3T3 fibroblasts (Detheux et al., 1997). Since many of these CWAs exhibit species- and tissue-specific metabolic changes, a human skin-derived model would be more reliable for military research than the established animal models because of gene differences in the animal models. In the past, we have documented the damage caused to normal human epidermal keratinocyte cells (NHEKs) caused by HD (Arroyo et al., 1997, 1999, 2000) (Kurt et al., 1997). However, keratinocytes are not the sole components of skin. Fibroblasts are also present and, therefore, constitute an important target for cellular damage caused by HD. Alkylation of DNA on NHSFs has been reported to be directly proportional to the concentration of HD (Niu et al., 1996). The purpose of this technical report is to describe the processes of growing NHSFs to be applied to studying biochemical/biomolecular changes occurring in the presence of CWAs and for evaluating medical countermeasures against CWAs.

Methods

NHSFs and Medium

The NHSF cell lines (cell line designation: CCD-32Sk; Catalog No. CRL-1489 and CRL-1501; Homo sapiens, normal, skin, upper chest, age: one month, gender: male) were purchased from American Type Culture Collection (ATCC), Manassas, VA, USA. NHSFs were shipped frozen in a cryoprotective cell medium. Upon arrival at the institute, the NHSFs were removed from the shipping container and immediately stored in liquid nitrogen storage at -154° C. The NHSFs were cultivated in 425 mL of minimum essential medium (MEM Eagle, Sigma® Chemical Corp., St. Louis, MO, USA; catalogue # M5650) supplemented with 5 mL of MEM vitamins 100× (Sigma®, catalogue # M6395), 5 mL of MEM nonessential amino acid 100× (Sigma®, catalogue # M7145), 10 mL of glutamine (Sigma®, catalogue # G7513), 50 mL of fetal bovine solution (Sigma®, catalogue # F2442) and 5 mL of 50× amino acids (Flow Laboratories, McLean, Virginia, USA; catalogue # 16-011-49) for a total of 500 mL. The pH of this fibroblast growth medium (FGM) was adjusted with sodium bicarbonate (Sigma®, catalogue # S8875) to pH = 7.4. The frozen NHSF ampoules were thawed and seeded in a T150 flask at ~ 400,000 cells per flask then incubated at 37° C in a humidified 5% CO_2 atmosphere. The NHSF cells were cultivated for seven days, divided and seeded in a T150 flask at ~ 400,000 per flask for seven more days.

Culture Vessel

The type of assay determined the style, size, and quantity of culture vessels required by investigators. Culture vessels were obtained from several sources, such as Corning® Corning Incorporated Life Sciences (Acton, MA USA) and Falcon™ (Becton Dickinson and Company, Bedford, MA, USA). The Corning® cell culture flasks have a cell growth area of 150 cm^2. The Falcon Corporation has developed a 96-well plate, which is very reliable for cell culture. These plates proved to be very suitable for CyQUANT® Cell Proliferation Assay kit analysis. The

plates arrive pre-sterilized by gamma radiation from the manufacturer and are individually wrapped. These safeguards make it easy to keep them sterile.

Thawing of Cryovial

The cryovials were removed from the liquid nitrogen freezer, then warmed in the sterile gloved hand of the technician and opened within the sterile field of a biological hood. All surfaces within the field were sprayed with a solution of 70% ethanol in water. The fibroblast growth media must be placed in a water bath and warmed to 37° C, prior to the thawing procedure. After reaching the appropriate temperature, the bottle of Fibroblast Growth Media (FGM) was removed from the water bath, dried with a paper towel, and sprayed with 70% ethanol before it was placed in the biosafety cabinet. The contents of the cryovials were placed in a test tube and cell growth media added until the total volume measured 10 mL. Two hundred fifty microliters (μL) of this solution was placed in a Coulter Z_1 Particle Counter (Coulter Corporation, Miami, FL, USA), and the number of cells per mL of growth media was calculated. This calculation was used to determine the amount of thawed cell solution needed to add to the Corning flasks to initiate growth of the passage two (P2) cells. After seven additional days of growth the cells were removed from the flasks using cell dissociation solution (Sigma®, catalogue # C5789) recounted and placed in Corning T-150 flasks to begin growth as passage three (P3) cells. Then after seven additional days of growth, the cells were removed from the flasks and placed in the final container to be used for the particular experimental assay.

All flasks were labeled using a permanent marker, and the information recorded included cell type, date, and passage number. Tissue culture flasks were placed in (Forma Scientific) CO_2 water-jacketed incubators (5% CO_2) equipped with a HEPA filter at 37° C. Cell culture flasks with vented flask caps, such as Corning catalog number 430825, were used to insure that the ventilation within the flask was sufficient. The vented flask caps insured that the pH of the growth media remained optimum within the flask to insure maximum cell growth. Furthermore, technician handling of flasks was kept to a minimum to prevent accidental introduction of contaminant organisms such as molds and mildews. Many of these microorganisms are airborne and very difficult to eliminate.

Subculture Technique

Using an Olympus IX Inverted Research Microscope, the flasks containing the cultured cells were examined, and a count of the individual cells comprising the monolayer was estimated (confluency). When the desired confluency was reached (approximately 80%), subculturing began. The type of vessels and concentration of cells per vessel were predetermined by the requirements of each research team. All subculturing took place within a sterile field inside the biosafety cabinet. The working area was wiped down with 70 % ethanol prior to placing all materials in the hood. The 500-mL bottle of fibroblast growth media was wiped down with 70% ethanol, after removal from a 37° C water bath, as were all reagents used in the subculture process even if they were not placed in the water bath.

The flasks were prepared for subculturing by removing the FGM and adding 10 mL of Hank's Balanced Salt Solution (HBSS) modified (Sigma®, catalogue # H9394, calcium free) for five minutes. This media was then removed and 10 mL of Cell Dissociation Solution (CDS) (Sigma®, catalogue # C5789 or #C5914) was placed in the flasks for 15 minutes. The flasks were then scraped using a cell scraper (BD Falcon 353086). Contents of all flasks were placed in 50 mL centrifuge tubes and centrifuged at 1000 rpm for 5 minutes; we recommend the centrifuge from International Equipment Company (model MP4). All media was removed from the sample, and the cell pellet was resuspended in 10 mL of Fibroblast Growth Media. Two hundred fifty (250) μL of this cell suspension was added to 9.75 mL of Isoton II (Coulter Corporation, Miami, FL, USA) diluent and counted in the Coulter Z_1 Particle Counter (Coulter Corporation, Miami, FL, USA). The number of cells per mL was calculated to determine the volume of the cell suspension to place in each of the secondary culture flasks.

Previous seeding attempts had shown that the optimum growth for seven days in a T-150 flask required a seeding density of 400,000 CRL-1489 NHSFs per flask and a seeding density of 600,000 per flask for CRL-1501 NHSFs. Optimum seeding densities vary because of genetic differences between cell lines. After growing for seven days in the secondary flasks (P3) the NHSFs were removed using the same procedure used for removal from primary (P2) flasks. The cells were then ready to be placed in 96-well plates if needed for an ELISA assay. If large numbers of cells were needed for a nuclear magnetic resonance (NMR) or electro paramagnetic resonance (EPR) spectroscopy experiment, the cells remained in the secondary flasks for treatment with HD. The treated cells were then removed from the secondary flasks for spectroscopy analysis. NHSFs used in ELISA assays were seeded into 96-well plates at densities ranging from 40,000 to 60,000 cells per well. When subculturing cells, crowding of containers was avoided since this leads to clumping of cells. For example, the clumping of cells may cause zones of uneven growth in secondary containers. Prevention of clumping was given high priority to insure optimum growth rates in secondary containers. Agitation by pipetting of media repetitively in containers insured optimum dispersal of cells within the containers, preventing dead zones where cells do not grow well because they are too far apart. Uniform dispersal of individual cells within the secondary container proved to be a key factor for effective subculturing.

Cell Counting

NHSFs were counted using the Coulter Z_1 Particle Counter. A 10-mL suspension of cells to be counted was prepared. Because gravity pulls fibroblasts to the bottom of containers, cells were briskly agitated before a 250-μL aliquot was removed and added to 9.75 mL of Isoton II diluent. The mixed solution was placed in the particle counter, which had been programmed for a 1 to 40 dilution and counted three times. The final count was the average of all three counts.

Trypsinization Process

The trypsinization process was used when preparing cells used as standards. Since trypsinization is a faster process than cell dissociation and since standards usually involve large volumes of cells, technician-handling time was reduced. If cells were allowed to overpopulate

flasks, they were difficult to remove, since cells tend to clump when crowded. Optimum subculturing occurs when individual cells are distributed evenly throughout the container. If NHSFs are too crowded in the secondary container (P3), larger amounts of trypsin are required to remove the cells from the container. If the correct volume of trypsin is not used, cells will detach from the primary container in clumps. Clumping was the largest problem encountered working with NHSFs because it interferes with the counting procedure, since clumps of cells can block the aperture of the Coulter particle counter.

The trypsination process used for NHSFs, contained in a 150-cm 2-cell culture flask, required chemicals manufactured by Sigma-Aldrich. An initial five-minute incubation in 10 mL of a calcium free media, Hanks Balanced Salt Solution (BSS) modified (Sigma®, catalogue # H-8394), was completed after the 30 mL of fibroblast growth media had been removed from the flask. After incubation, the Hanks BSS was removed, and 5 mL of Trypsin EDTA Solution (1X) (Sigma®, catalogue # T-3824) was added to the flask for an additional five-minute incubation. The flask was then scraped using a BD Falcon cell scraper, and 5 mL of Trypsin Inhibitor Solution (Sigma®, catalogue # T-6414) was added to the flask. The contents of each flask were added to a 50-mL centrifuge tube and centrifuged at 1,000 rpm for 5 minutes. The supernatant was removed and the cell pellet frozen.

For immunoassays, the pellets were thawed and 10 mL of media was added for counting. NHSF cells were counted and placed in empty wells in a 96-well plate, immediately before the assay to insure cell counts were accurate.

Contamination

Incubator

The incubators used to grow NHSFs employed water pans to control the amount of humidity within the growing chamber. Since many contaminant particles are airborne, opening and closing the incubator door provided a route of entry to the water pans. Regular treatments, usually once every two weeks, of ChlorhexiDerm (Nolvasan) were employed to reduce contamination problems. During humid summer months airborne levels of contaminants became so high that the best cleaning methods were overwhelmed by the sheer number of airborne contaminants.

A major source of contamination was the incubators; the units used for NHSF cell culture were difficult to disassemble and clean thoroughly. All shelves, side panels, water pans, gaskets and fan covers of the incubator must be removed to insure adequate cleaning. The inside chamber was cleaned with a phenol reagent, rinsed, then sprayed with 70% ethanol. The removable pieces were cleaned in the same fashion, then autoclaved for 20 minutes at 120° C. The HEPA air filter that comes with the incubator is manufactured by Donaldson Co., Inc., Minneapolis, MN, USA, and was replaced each time an incubator was cleaned.

Large workloads lead to frequent opening and closing of incubator doors and increased contamination problems. Large numbers of containers within the growth chamber interfered

with normal airflow, which can lead to cool spots and result in inefficient cell growth. The stainless steel shelves may act as warm spots affecting the temperature of the media within the flasks directly above the shelves.

Water Bath

The water bath was cleaned on a routine (biweekly) basis using a non-abrasive cleanser and rinsed thoroughly. After air drying the water bath was wiped down with 70% ethanol, then refilled with autoclaved distilled water. Water baths should be turned on and left on because of the long time required for the temperature to equilibrate.

Vacuum Line and Waste Container

The cell culture growth media was changed every 2 or 3 days during periods of maximum growth. This procedure can be accomplished by pouring the growth media out of the flask into a waste container; this is a very slow process. A vacuum apparatus was used to remove the growth media from the flask, increasing technician efficiency. Before beginning any cell culture procedures, the vacuum line was rinsed with bleach to insure that any potential contaminants had been removed. Frequent cleaning of the vacuum equipment was necessary since waste media encourages growth of contaminants and can lead to high levels of airborne contaminants within the laboratory.

Used Plastic Ware

Used pipettes were rinsed with 10% bleach, water solution, and then placed in a broken glass container box, which was incinerated. All used flasks or plates were rinsed with 10% bleach and water or autoclaved for 20 minutes. These procedures are necessary to insure that airborne contamination is kept to a very minimum within the laboratory.

Waste (Plastic and Paper)

All waste containers were conveniently located within reach of the hood to insure that the technician was not forced to move in and out of the sterile field increasing the likelihood of contamination. Because of their proximity to a sterile field, all solid waste containers have a lining, which is changed frequently to prevent growth of potential contaminants.

Laboratory

The laboratory had to be cleaned regularly. All exposed surfaces, such as bench tops, centrifuges, and other laboratory equipment, were wiped down with a 10% bleach solution. The floor was mopped with a disinfectant. Most equipment used for cleaning was disposable; if not disposable it was stored in another room, because damp mops and clothes are breeding grounds for microorganism contaminants.

Laboratory Personnel

The largest single source of contamination in a cell culture laboratory is the technician. The amount of contamination is directly proportional to the number of technicians sharing a work area. Minimizing the number of technicians working in an area reduces contamination. Technicians were taught aseptic techniques and closely supervised by senior technicians. Technicians wore gloves and sleeve covers to insure that no contaminants present on the technician's skin would be introduced into the sterile field. Before performing any cell culture procedure (including preparing the sterile field within the hood), technicians put on gloves, lab coats and safety glasses with side shields and then sprayed the gloves with 70% ethanol. All reagent containers were sprayed with 70% ethanol before being placed in the sterile field. All containers warmed in the water bath were wiped dry and then sprayed with 70% ethanol before they were placed in the sterile field.

Cell Growth Media

Initially the cell media recommended by ATCC was used, since large amounts of cells were required for EPR spectroscopy experiments or NMR spectroscopy studies. Different growth factors were examined to try to speed the growth rate. Typically two groups of flasks were prepared; one group was treated the other group was not. After seven days the technician evaluated the flasks to determine whether the treated group had outperformed the untreated group. The NHSF growth media was refrigerated because components such as L-glutamine have a very short shelf life and must be kept frozen until use. Because of the large numbers of cells required, media such as Clonetics® Fibroblast Growth Media (FGM) and Keratinocyte Growth Media 2 (KGM^2) were evaluated and incorporated into the prepared formulation. Large quantities of FGM were not prepared, because single opening of small containers helped prevent contamination problems. Frequently reopening a larger container of media provides a route of entry for contaminating microorganisms.

Equipment

Plastic Ware

Various containers were evaluated for growth rate and ease of moving in and out of the incubators. Typically, T150 flasks were easiest to move from the incubator to the sterile field and back again. The greatest difficulty encountered was the slipperiness of surfaces treated with 70 % ethanol, which resulted in spills within the sterile field. Large numbers of flasks placed within the biosafety cabinet interfere with airflow and allow little working area within the hood. However, frequently opening the incubator provides an access route for contamination organisms. Therefore, the technician must develop a balance between a cluttered working space and the need to open and close the incubator.

Trouble Shooting

All containers, flasks and plates were examined daily under the microscope to evaluate growth and observed for signs of contamination. Small batches of media, usually one liter, were prepared to insure that the NHSF growth media would not be stored for more than 30 days. Water in water baths was changed every other week, because of contamination concerns. Incubators require periodic calibration to insure temperatures remain at 37° C and carbon dioxide (CO_2) levels remain constant at 5%, because CO_2 levels influence the pH of the cell growth media.

Clonetics Fibroblast Growth Media

In an effort to save time spent on preparing media, Fibroblast Growth Media CC-4134 was purchased from Clonetics® (a subsidiary of Bio Whittaker, Inc., A CAMBREX Company, Walkersville, MD, USA). This media was compared with the recommended ATCC Fibroblast Growth Media and found to be inferior to the ATCC media. Clonetics® KGM-2 BULLET KIT CC-4152 was also evaluated and found to be superior to Clonetics Fibroblast Growth Media but not superior to the ATCC Fibroblast Growth Media.

All bullet kit formulations have an extended shelf life of eight months while frozen. The frozen components (SingleQuots) of the bullet kit must be added immediately before use. After the SingleQuots are added to the bullet kits, the shelf life is two weeks. Each batch of cell growth media is slightly different, thus affecting the growth rate. The FGM bullet kit is based on Clonetics Corp. Media Development Laboratories (CC-4134). It contains, human recombined. Fibroblast Growth Factor Basic (hFGF-B) 1 µg/mL 0.5 mL, CC-4065; 50 mg/mL gentamicin; 50 µg/mL amphotericin-B, 0.5 mL ,CC4081; and insulin 5mg/mL 0.5 mL. KGM-2 BULLET KIT (CC-4152) SingleQuots contain bovine pituitary extract (BPE) 2 mL; insulin (bovine), 0.5 mL, CC-4321; hydrocortisone, 0.5 mL, CC-4331; transferrin, 0.5mL, CC-4345; epinephrine, 0.5 mL, CC-4346; and gentamicin/amphotericin-B, 0.5 mL, CC-4381.

Cell Line Variations - Considerations

CRL-1489 and CRL-1501 are produced from different donors. The seeding efficiency and the cell viability is different for each cell line. The amount of time required for the cells to double is dependent upon factors specific to the individual donor, such as age and sex. These factors must be taken into consideration when determining seeding densities. Each cryovial contained a varying number of cells, which was dependent on lot number. The CRL-1501 cell line grew much slower than the CRL-1489 cell line as compared in ***Figure 1*** and ***Figure 2***. As a result, flasks were seeded with twice as many CRL-1501 cells to accomplish 80-90% confluency in 7 days.

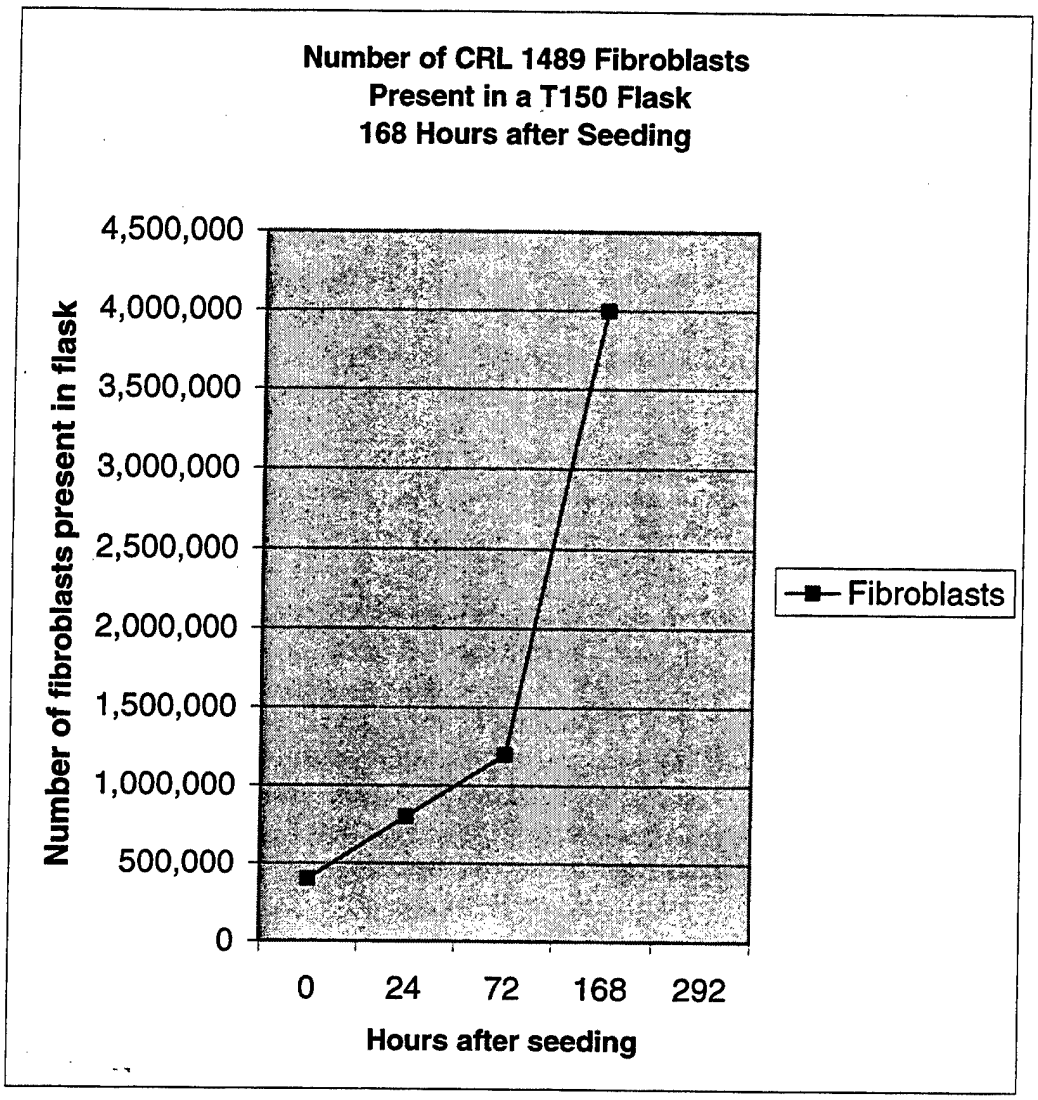

Figure 1. NHSFs CRL # 1489 growth curve as a function of hours after seeding ampoule.

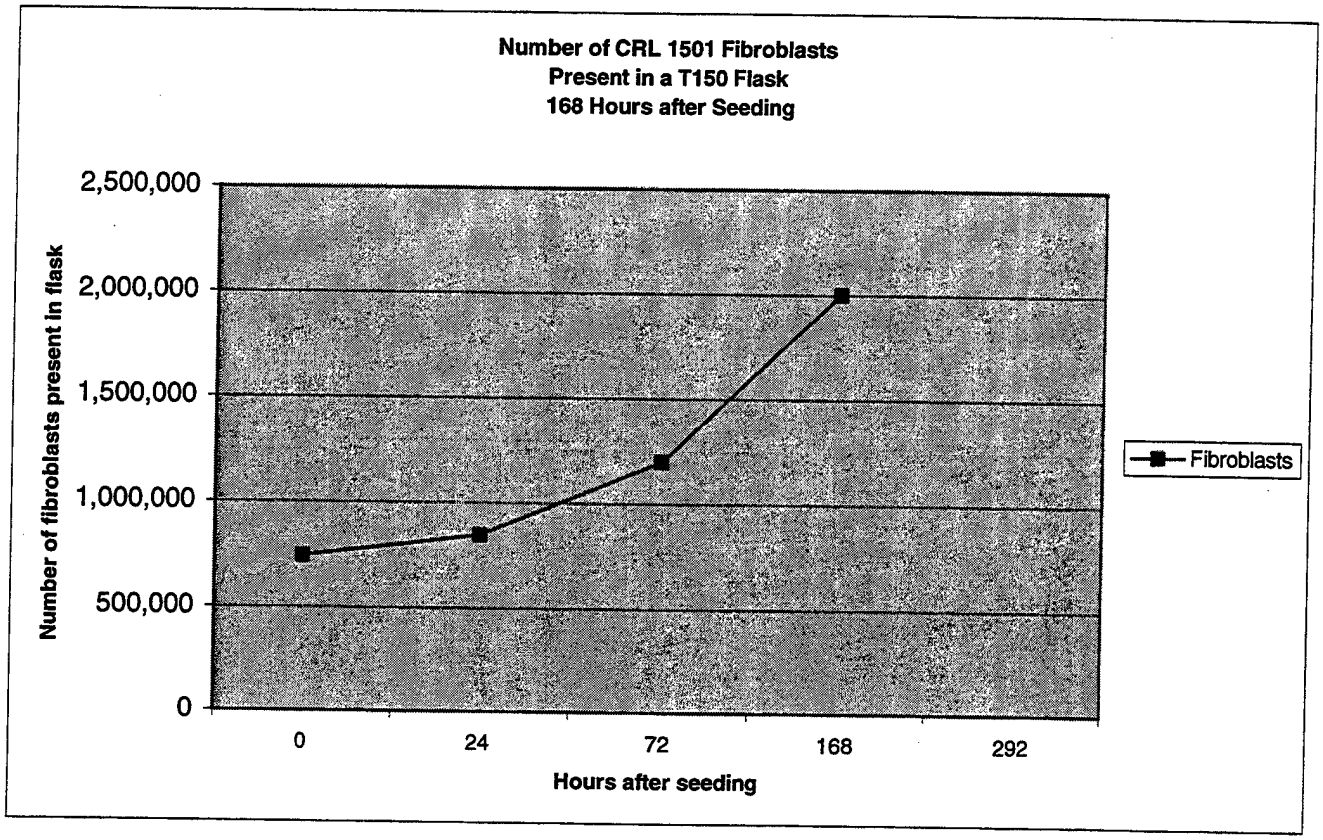

Figure 2. NHSFs # 1501 growth curve as a function of time (hours after seeding ampoule).

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