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Normal Human Astrocyte Instructions for Initiation of Cultures from Cryopreserved Cells and Subculture

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14. ABSTRACT This technical report outlines procedures that have been developed to provide a cost effective way to produce large quantities of Normal Human Astrocyte cells (NHA) for studying the mechanism(s) of action of chemical warfare agents (CWAs) and medical counter measurements against CWAs. Five milliliters of a supplement provided by Clone Express was added to 467 mL of DMEM F12 media as well as 25 mL of fetal bovine serum plus 0.3 mL gentamicin and 2.5 mL of fungizone. Cell viability and cell proliferation were evaluated using the Coulter Z ₁ Particle Counter.					
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Introduction

Human neural cells forming synapses at the neural muscular junction are the organs attacked by many chemical warfare agents (CWAs) commonly used in military and terrorist attacks. It is believed that the organophosphates stimulate cytokines and generate neurodegeneration responses in the immune system (Allan et al., 2001). Our goal was to examine the cytokine response exhibited by astrocytes derived from the human brain. This response may be different for each of the nerve agents Sarin (GB) and Soman (GD) because of differences in their chemical structure. If this is true, measurements of cytokine activity could be used to generate a profile for diagnosis of exposure to specific nerve agents. This diagnosis would allow countermeasures for the specific nerve agent involved. Since many of these CWAs exhibit species- and tissue-specific metabolic changes, a human brain cell derived model would be more reliable for military research than the established animal models because of human gene expression (Boulter et al., 1990). The purpose of this technical report is to describe the processes of growing normal human astrocytes used for studying biochemical and biomolecular changes occurring in the presence of CWAs. The acquired knowledge will allow the development of profiles for CWA effects which should lead to very specific medical countermeasures against CWAs.

Methods

Normal Human Astrocytes and media

The NHA cell line (cell line designation: HAST040) derived from human fetal brain tissue was purchased from Clonexpress (Clonexpress, Inc., Gaithersburg, MD, USA). Two other cell lines were purchased from Clonetics a subsidiary of Cambrex (Cambrex Bio Science Walkersville, Inc. Walkersville, MD, USA). These were 3F0484 and 3F0710. 3F0484 was derived from a 21-year-old male and 3F0710 was derived from a 19-year-old female. NHA were shipped frozen in a cryoprotective cell medium to United States Army Medical Research Institute of Chemical Defense (USAMRICD). Upon arrival at the USAMRICD, the NHA were removed from the shipping container and immediately placed in liquid nitrogen storage at -154° C. The NHA were cultivated in a media consisting of 5 mL of a supplement provided by Clonexpress which was added to 467 mL of DMEM F12 media (Gibco, Invitrogen Corporation Carlsbad, CA, USA), as well as 25 mL of fetal bovine serum was added to the DMEM F12. An aliquot containing 0.3 mL gentamicin from Gibco (catalog # 15750-060) and 2.5 mL of fungizone (Gibco catalog # 15290-018) was also added to the DMEM F12 for a total volume of 500 mL. NHA received from Cambrex were grown in Astrocyte Growth Media (AGM) purchased from Cambrex (catalog # CC-3186). The frozen NHA ampoules were thawed by hand warming and seeded in four T-75 flasks then incubated at 37° C in a humidified 5 % CO_2 atmosphere. The NHA cells were cultivated for seven days, divided and seeded in 10 T-150 flasks at $\sim 700,000$ cells per flask for seven more days (Bressler et al., 1985; Chao et al., 1992). The cells at passage three (P_3) were exposed to CWAs. The cell media were removed from the flasks and assayed for cytokine production.

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 cm². 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 making it easy to keep them sterile. Twenty-four well and six-well plates were outfitted with coverslips (Thermanox, Nalge Nunc International International, Rochester, NY, USA). Thirteen mm diameter round coverslips were placed in the wells of a 24-well plate while twenty-two mm diameter coverslips were placed in the wells of a six-well plate. The wells of the 24-well plates were seeded with a minimum of 60,000 cells per well to a maximum of 80,000 per well. The six-well plates were seeded with 120,000 cells per well and grown for two or three days.

Thawing of cryovial

The cryovials were removed from the liquid nitrogen freezer, 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 AGM was placed in a water bath and warmed to 37° C, prior to the thawing procedure. After reaching the appropriate temperature, the bottle of AGM was removed from the water bath, dried with a paper towel, and sprayed with 70 % ethanol before it was placed in the biological hood. 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 Z₁ 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 days of growth the cells were removed from the flasks, using trypsin (Cambrex Subculture Reagent Kit, catalog # CC-5034) 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, usually 96-, 24- or 6-well plates to be used for the 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) CO₂ water-jacketed incubators (5% CO₂) equipped with a HEPA filter at 37° C. Cell culture flasks with vented flask caps (Corning catalog # 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 insuring 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 (confluence). When the desired confluence was reached (approximately 90 %), 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 a biological hood. The working area was sprayed down with 70 % ethanol prior to placing all materials in the hood. The 500 mL bottle of AGM was sprayed 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 T-150 flasks were prepared for subculturing by removing the AGM and adding 7 mL of (HBSS) (Cambrex Subculture Reagent Kit, catalog # CC-5034) for five minutes. This media was then removed and 7 mL of trypsin/EDTA was placed in the flask for 5 minutes. The flasks were then scraped using a cell scraper (BD Falcon 353086) and 7 mL of Trypsin Neutralizing Solution was added. 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 AGM. Two hundred fifty (250) μ L of this cell suspension was added to 9.75 mL of Isoton II (Coulter Corporation, Miami, FL, USA) diluent and counted in the Coulter Z₁ 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 showed that the optimum growth for seven days in a T-150 flask required a seeding density of 700,000 NHA for Clonetics cell line 3F0710. This density resulted in 80-90% confluency in 7 days. Cell line 3F0484 seeded at the same density as 3F0710 grew at only half the rate of 3F0710 reaching 80-90% confluency in 14 days. The optimum seeding densities varied between cell lines because of genetic differences (different donors) between cell lines. After growing for seven days in the secondary flasks (P₃) the NHA were removed using the same procedure as that used for removal from primary (P₂) flasks. The cells were finally ready to be placed in 96-well plates as needed for an ELISA assay. If large numbers of cells were needed for an NMR or EPR experiment, the cells remained in the secondary flasks for treatment with CWA. The treated cells were then removed from the secondary flasks for spectroscopy analysis. NHA 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. Clumping of cells caused 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

NHA were counted using the Coulter Z₁ Particle Counter. A 10 mL suspension of cells to be counted was prepared. Because gravity pulls astrocytes to the bottom of containers, cells were briskly agitated before a 250 μ L 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 for subculture. Dissociation solution was attempted as in the case of keratinocytes, but cell clumping ruled out this process as a viable alternate to trypsin. Optimum subculturing occurs when individual cells are distributed evenly throughout the container. If NHA 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 NHA as clumps of cells can block the aperture of the Coulter particle counter interfering with the counting procedure.

The trypsinization process used for NHA contained in a 150-cm² cell culture flask required chemicals manufactured by Sigma-Aldrich. An initial five-minute incubation in 7 mL of a calcium free media, Hanks Balanced Salt Solution (HBSS) modified calcium free (Sigma[®], catalog # H-8394), was completed after the 30 mL of AGM had been removed from the flask. After incubation, the HBSS was removed and 7 mL of ½ X Trypsin EDTA Solution (Sigma[®], catalog # T-3824) a half X dilution was prepared by doubling the volume of X trypsin with a like amount of HBSS modified. The ½ X trypsin solution was added to the flask and incubated for five minutes. The flask was then scraped using a BD Falcon cell scraper and 7 mL of ½ X Trypsin Inhibitor Solution (Sigma[®], catalog # T-6414) was added to the flask. The ½ X Inhibitor solution was prepared the same way as the ½ X Trypsin solution combining equal volumes of Inhibitor and HBSS modified calcium free. 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 diluted with 10 mL of cell culture media and then counted.

Contamination

Incubator

The incubators used to grow NHA 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 biweekly, of ChlorhexiDerm (Nolvasan) were employed to reduce contamination problems. During humid summer months, airborne levels of contaminants became so high that the sheer number of airborne contaminants overwhelmed the best cleaning methods.

A major source of contamination was the incubators. The units used for NHA cell culture were difficult to disassemble and clean thoroughly. All shelves, side panels, water pans, gaskets, and fan covers of the incubator were removed to insure adequate cleaning. The inside

chamber was cleaned with a phenol reagent, rinsed and sprayed with 70 % ethanol. The removable pieces were cleaned in the same fashion, then autoclaved for 25 minutes at 120° C. The HEPA air filter that comes with the incubator is manufactured (Donaldson Co., Inc., Minneapolis, Minnesota, USA) 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 when airflow is inadequate within the incubator chamber.

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 removal of any potential contaminants. Frequent cleaning of the vacuum equipment was necessary because 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. All cleaning equipment 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 washed hands and put on gloves, lab coats and safety glasses 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 Clone Express was used, since these were the first astrocyte cultures commercially available. Clone Express recommended adding 5 mL of astrocyte growth supplement (shipped with the astrocyte cultures) to 467 mL of DMEM F12 media a product of Gibco (Invitrogen Corporation Carlsbad, CA, USA), as well as 25 mL of fetal bovine serum plus 0.3 mL gentamicin (Gibco catalog # 15750-060) and 2.5 mL of fungizone (Gibco catalog #15290-018). When Cambrex astrocytes became available, these cells were grown using Cambrex AGM (catalog # CC-3186). Growth rates for the Clone Express cell lines varied greatly when grown in the Gibco medium. The growth rate of Clonetics also varied greatly when grown in Clonetics astrocyte media. Typically two groups of flasks using the same cell line were prepared: one group was grown in Gibco cell culture media, the other group was grown in Cambrex AGM. After seven days, the technician evaluated the flasks to determine whether one group of flasks had outperformed the other group. It was determined that a 50/50 mixture of Gibco media and Cambrex AGM provided the optimum growth rate. The NHA 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 AGM were not prepared ahead of time, 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, T-150 flasks were easiest to move from the incubator to the sterile field and back again. The largest difficulty was caused by the slipperiness of surfaces treated with 70 % ethanol which resulted in spills within the sterile field. Large numbers of flasks placed within the biological hood interfere with airflow and allow little working area within the hood. However, frequently opening the incubator provides an access route for contamination organisms so the technician must develop a balance between a cluttered working space and the need to open and close the incubator.

Trouble Shooting

All container 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 NHA growth media would not be stored for more than 30 days since several of the components begin to deteriorate after 30 days. Water in water baths was changed biweekly because of contamination concerns. Incubators required periodic calibration to insure that temperatures remain at 37° C and that carbon dioxide (CO₂) levels remained constant at 5%, because CO₂ levels influence the pH of the cell growth media.

In an effort to cut down on costs on preparing media, Fibroblast Growth Media (FGM, CC-4134), which was purchased from Clonetics® (Bio Whittaker, Inc., Cambrex Company Walkersville, MD, USA) and available in the lab from previous fibroblast protocols and was examined as a substitute for AGM. KGM-2 was also available from previous fibroblast protocols and was examined as a substitute for AGM.

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 (CC4134). It contains, human recombinant Fibroblast Growth Factor Basic (hFGF-B) 1 µg/mL 0.5 mL, (CC4065), 50 mg/mL gentamicin, 50µg/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) 2mL; insulin (bovine), 0.5mL (CC-4321); hydrocortisone, 0.5mL (CC-4331); transferrin, 0.5mL (CC-4345); epinephrine, 0.5mL (CC-4346); gentamicin/amphotericin-B, 0.5mL (CC-4381).

Cell Line Variations- Considerations

Two cell lines were provided by Cambrex although the donors were of similar age, the growth rates were very different. Cell line 3F0484, which was derived from a 21-year-old male grew at only half the rate observed for cell line 3F0710, which was derived from a 19-year-old female. The seeding efficiency and the cell viability are 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, since slower growing cell lines require larger seeding densities to develop to 90% confluency in 7 days. Each cryovial contained a varying number of cells dependant on lot number. We were unable to use manufacturer recommended seeding densities because the plasticware, we used to grow the cells in the large quantities required for our assays were not recommended by Clonexpress. The technician evaluated several seeding densities to determine which one offered the optimum growth rate.

Fibroblast Growth Media

Because of the similarity in appearance of astrocytes to fibroblasts, the technician considered the possibility that a growth media that increased cell proliferation of fibroblasts might also increase proliferation of astrocytes. This theory was the basis for the development of the Kahler media. In an effort to cut down on costs on preparing media, Fibroblast Growth Media CC-4134 which was purchased from Clonetics® (a subsidiary of Bio Whittaker, Inc A CAMBREX Company Walkersville, MD, USA) and available in the lab from previous fibroblast protocols, was examined as a substitute for AGM. KGM-2 was also available from previous fibroblast protocols and was also examined as a substitute for AGM because it had improved fibroblast proliferation. As a result various combinations of all four cell culture medias were tested and the Kahler Media was developed. 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 (CC4134). It contains Human recombinant Fibroblast Growth Factor Basic (hFGF-B) 1 µg/mL 0.5 mL, - CC4065, 50 mg/mL Gentamicin, 50µg/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) 2mL; Insulin (bovine), 0.5mL - CC-4321; Hydrocortisone, 0.5mL - CC-4331; Transferrin, 0.5mL - CC-4345; Epinephrine, 0.5mL - CC-4346; Gentamicin/Amphotericin-B, 0.5mL - CC-4381. Many of these bullet kits were used well beyond their expiration date, since they had remained frozen until formulated. They continued to improve cell proliferation even though they were used as late as one year after the expiration date.

Conclusion

Normal human astrocytes were cultured for use in assays designed to track cytokine response to challenge by nerve agents Sarin (GB) and Soman (GB). This report details the step by step process used to culture those cell lines.

Astrocyte cell line 3F0710 Passage 16 grown curve in Clone Express media.

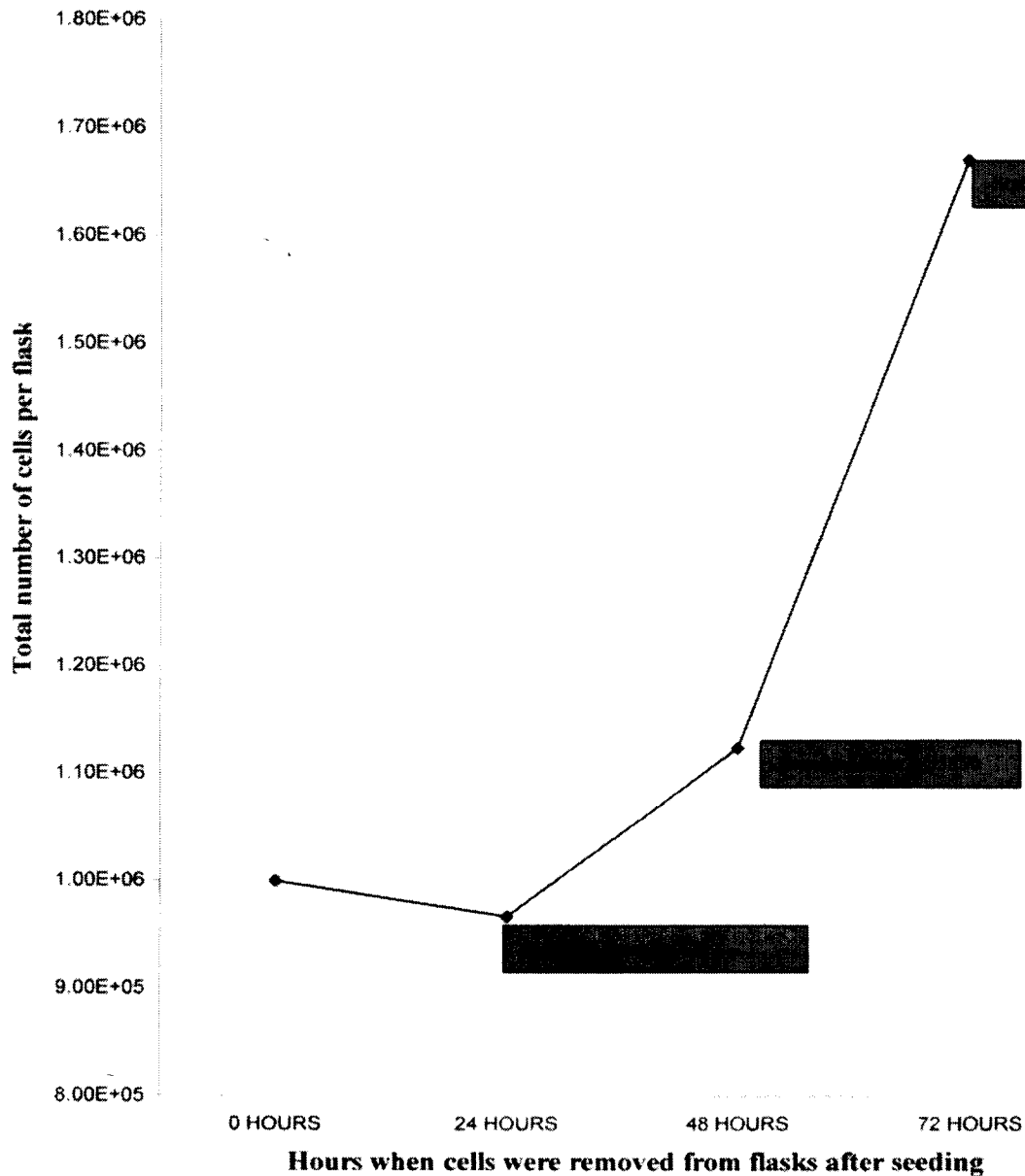


Figure 1. Astrocyte cells growing in Clonexpress Media. The total number of cells per flask were counted every 24 hours for a 3-day period. The amount of cells was measured in exponential phase growth (1×10^6 cells/flask) after the seeding process. This graphic represents the decrease in number of cells growing after 24 hours and the significant increase in number of cells growing after 48 hours.

Astrocyte Cell Line 3F0710 grown in Clonetics AGM

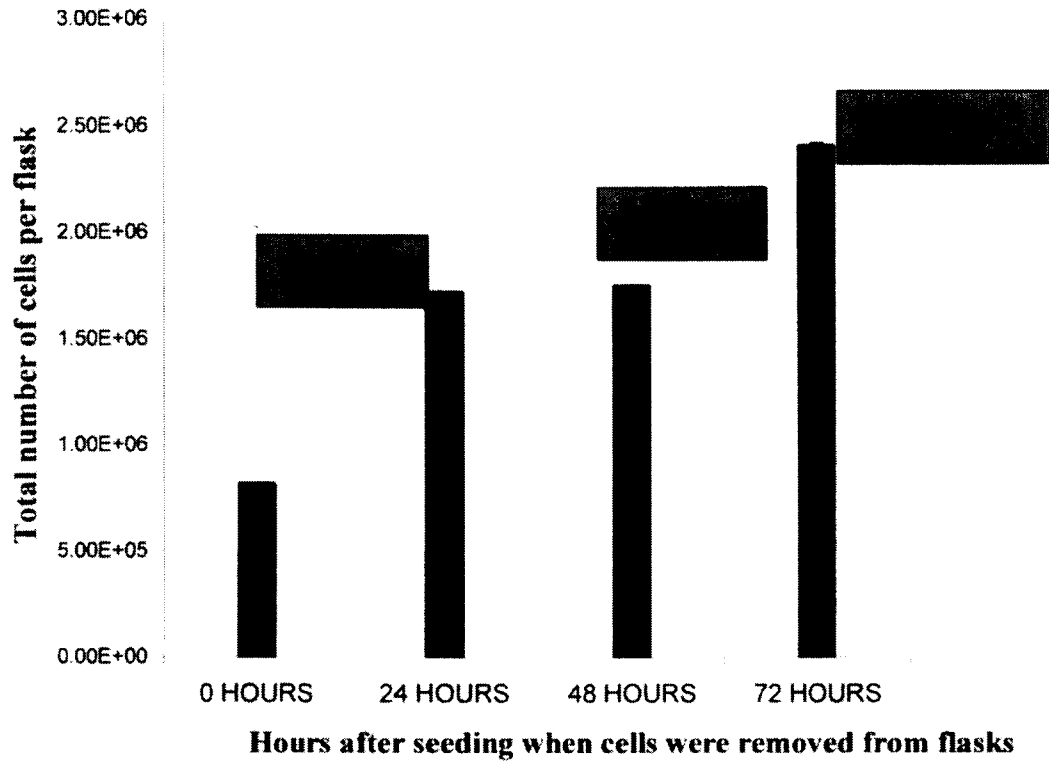


Figure 2. Astrocyte cells growing in Clonetic's Astrocytes Growth Media (AGM). The total number of cells per flask were counted every 24 hours for a 3-day period. The amount of cells was measured in exponential growth phase (1×10^6 cells/flask) after the seeding process. This graphic denotes that between periods of 24 hours to 48 hours cell growth is constant, then after 72 hours cell growth increases.

Astrocyte cell line 3F0710 Passage 16 grown in Clone Express media.

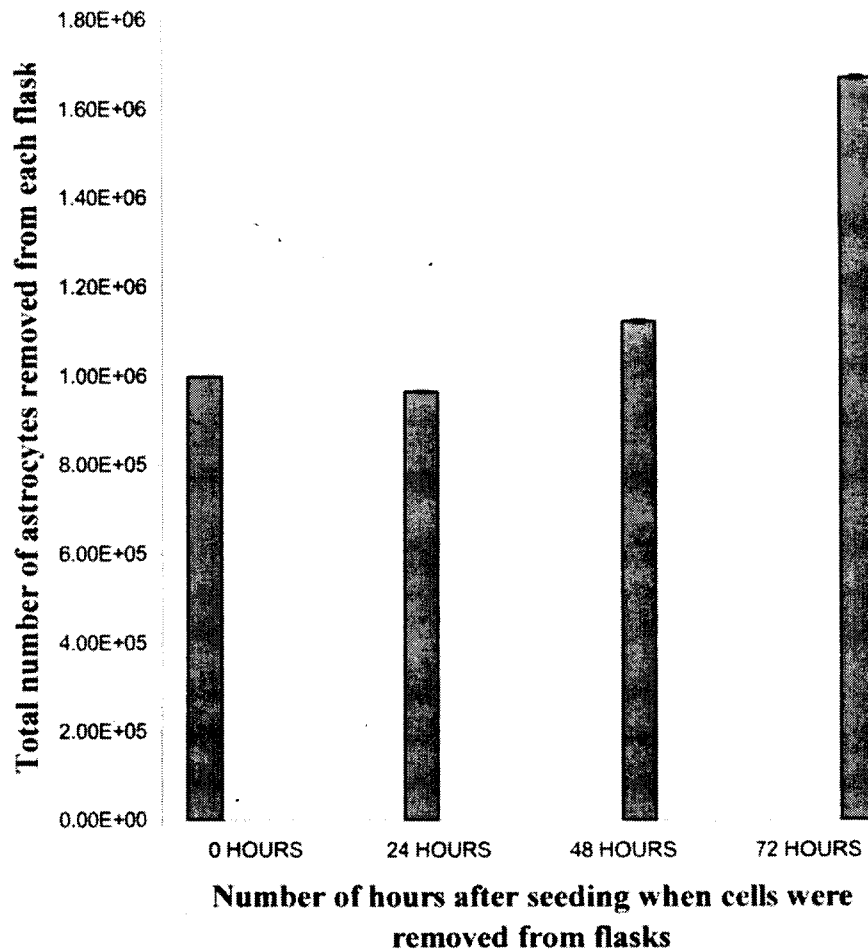


Figure 3. Astrocyte cells growing in Clonexpress Media. The total number of cells per flask was counted every 24 hours for a 3-day period. The amount of cells was measured in exponential phase growth (1×10^6 cells/flask) after the seeding process. This graphic represents the decrease in number of cells growing after 24 hours and the significant increase in the number of cells growing after 48 hours.

Cell Line 3F0710 Passage 16 grown in Clone Express astrocyte media (blue) and Kahler's astrocyte media (burgundy)

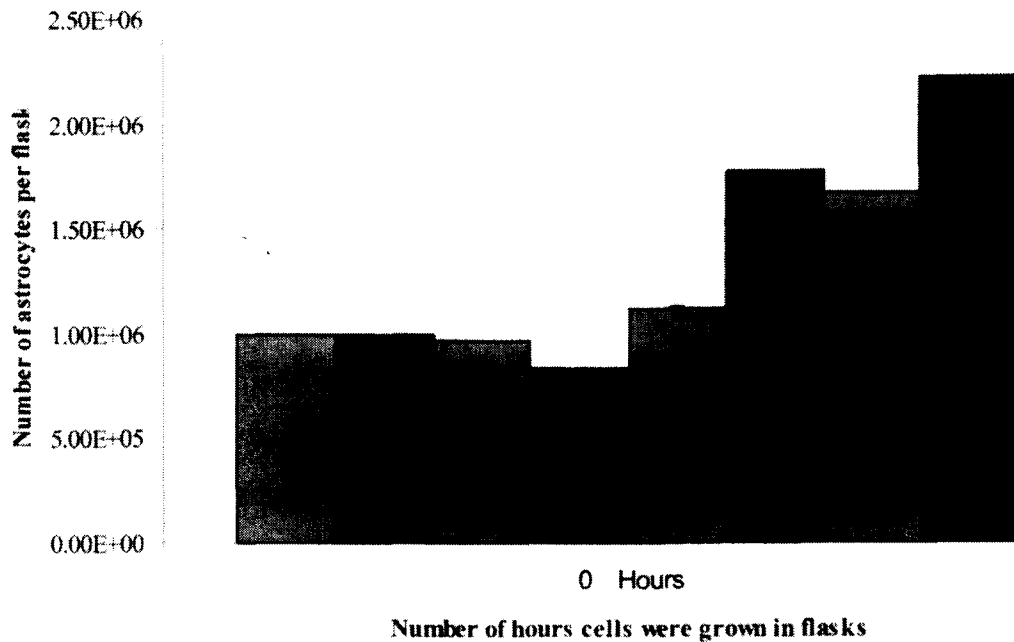


Figure 4. Astrocyte cells growing in Clonexpress Astrocyte Media and Kahler's Astrocyte Media. The total number of cells per flask was counted every 24 hours for a 3-day period. The amount of cells was measured in exponential phase growth (1×10^6 cells/flask) after the seeding process. This comparative graphic represents the decrease in the number of cells growing in Kahler's Astrocyte Media over Clonexpress Astrocyte Media in the first period of 24 hours. On the other hand, after 48 hours the number of cells increased substantially in Kahler's Astrocyte Media in contrast with the cells grown in Clonexpress Astrocyte Media.

**Astrocyte cell line 3F0710 grown
in two different media**

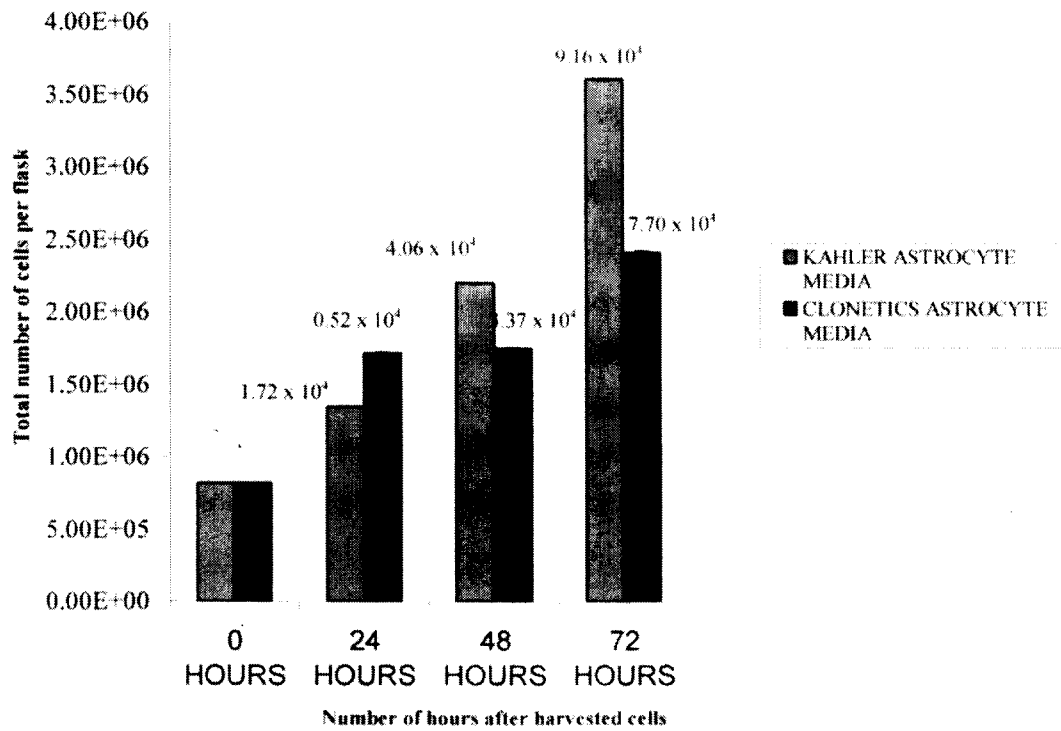


Figure 5. Astrocyte cells grown in Clonetics' Astrocyte Media and Kahler's Astrocyte Media. The total number of cells per flask was counted every 24 for a 3-day period. The amount of cells was measured in exponential phase growth (1×10^6 cells/flask) after the seeding process. This graphic indicates the linear growth of the numbers of cells in Kahler's Astrocyte Media. During the first 24 hours, the cells grown in Clonetics' Astrocyte Media were higher in number than Kahler's Astrocyte Media. After 48 hours the number of cells growing in Kahler's media was considerably larger than the number of cells grown in Clonetics' Astrocyte Media.

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