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A high-yielding serum-free, suspension cell culture process to manufacture recombinant adenoviral vectors for gene therapy

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Abstract

We have developed an efficient, reproducible, and scaleable cell culture process for a recombinant adenoviral vector expressing therapeutic transgenes for clinical trials. HEK 293 cells – which support the propagation of E1 deficient adenovirus – were first adapted to serum free media and suspension growth. Subsequent studies focused on the infection, virus production and harvest from suspension culture bioreactors. Future studies are planned to address the kinetics of adenovirus production in HEK 293 as well as in other cell lines.

Abbreviations: Ad5 – adenovirus serotype 5; cGMP – current good manufacturing practice; CPE – cytopathic effect; DMEM – Dulbecco's modified Eagle's medium; EPD – end point dilution assay; FBS – fetal bovine serum; HEK 293 – human embryonic kidney cells transformed with Ad5 E1 DNA; HPLC – high pressure liquid chromatography; ip – number of infectious virus particles; MOI – multiplicity of infection; p – total number of virus particles (infectious and non-infectious); PBS – phosphate buffered saline; SFM – serum free medium

Introduction

The number of gene therapy protocols using adenoviral vectors has increased steadily and reached ten percent of the two hundred and seventy three worldwide protocols by the end of 1996 (Marcel and Grausz, 1997). These vectors are preferable to retroviral vectors when transient gene expression is required (Crystal, 1995). The adenoviral vectors for gene therapy programs are currently produced by infection of a suitable permissive cell line with a recombinant E1 deficient adenovirus containing a transgene insert. To meet commercial and regulatory requirements, this process must be high yielding, scaleable and reproducible. This process should also preferably avoid the use of animal-derived medium constituents, such as serum.

Some researchers have described the use of suspension adapted HEK 293 cells in serum-containing media for protein production via adenoviruses (Garnier et al., 1994; Nadeau et al., 1996). Other workers have discussed their use of attached or clumped HEK 293 cells in serum-containing media to produce adenovirus for gene therapy applications (Shabram et al., 1997; Hehir et al., 1998). There is no literature, however, on adenovirus production in serum-free mediumg with suspension adapted HEK 293 cells. In addition, little data is available on optimal multiplicity of infection or in-process virus stability (Graham and Prevec, 1994).

Our goal was to develop an efficient, reproducible, and scaleable cGMP manufacturing process for the production of an adenoviral vector expressing therapeutic transgenes using HEK 293 cells. These requirements were met by establishing a cell culture process employing stirred tank fermentors, suspension culture adapted HEK 293 cells, and serum free cell culture medium. All process steps, from preparation of cell and virus banks to collection of recombinant virus

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from the cell culture harvest, were studied. This article specifically addresses development work on the adaptation of cells to suspension culture, the selection of serum-free medium and adaptation of cells to that medium. The characterization of adapted cells, the production of virus in adapted cells, and the cryopreservation of cells in serum free medium are also discussed, as well as performance aspects of our long-term, large-scale cultures. This article further describes clonal purification of virus, studies to determine optimal multiplicity of infection; the infection of cells at large scale, and yields and stability of raw virus.

Materials and methods

Cell culture

HEK 293 cells were obtained from Magenta Corporation (Rockville, MD). These HEK 293 cells were maintained and passed in DMEM (Gibco BRL, Life Technologies, Grand Island, NY) with 10% FBS (Hyclone Laboratories Inc., Logan, UT). Serum free media were provided by JRH Scientific (Lenexa, KS), Irvine Scientific (Santa Ana, CA), and BioWhittaker (Walkersville, MD). Cell counts were done using standard hemocytometers and a Coulter counter (Coulter Electronics LTD, Luton, Beds., England). Stock cells were passed when at $>1 \times 10^6$ viable cells mL^{-1}, and reseeded at $>4 \times 10^5$ viable cells mL^{-1}. Cells were photographed using a Sony CCD-IRIS Color Video Camera (Sony Corporation, Japan).

Shake flasks

Small scale experiments were conducted in 125 to 2800 mL (total volume) shake flasks, that were either disposable flasks (Corning, Corning, NY), reusable plastic flasks (Nalgene, Rochester, NY) or reusable glass flasks (Bellco, Vineland, NJ). The reusable shake flasks were sterilized by autoclaving. The flasks were maintained in 5% CO_2 incubators with temperature control at 37 °C (VWR Scientific, So. Plainfield, NJ). Shaker tables were operated at 140 rpm (New Brunswick Scientific, Edison, NJ). The pH was checked by off-line measurements with a pH meter (Corning Inc., Corning, NY). Dedicated instruments (YSI Instruments, Yellow Springs, OH) measured glucose and lactate off-line.

Bioreactors

Long term perfusion cell cultures and batch cell infections were conducted in 3 and 10 L Applikon glass bioreactors (Applikon, Schiedam, the Netherlands). The reactors were all sterilized by autoclaving, and all sterile connections were made using a sterile tubing welder (Terumo Medical Corporation, Elkton, MD). Temperature was maintained at 37 °C by a single loop controller (Fenwall, Ashland, MA), using a platinum RTD to sense temperature, and a heating blanket (Applikon, Schiedam, the Netherlands) to heat the reactor. Dissolved oxygen was maintained at 50% of air saturation by single loop controller (B. Braun Biotech, Allentown PA), with a oxygen sensing probe (Mettler-Toledo Process Analytical Inc., Wilmington, MA) and pulsed sparging by time-proportioning on/off control of a solenoid valve. The pH was maintained at 7.1 by a single loop controller (B. Braun Biotech, Allentown PA) with a pH sensing probe (probe (Mettler-Toledo Process Analytical Inc., Wilmington, MA) and time-proportioning on/off control of either a pump feeding 0.2 N NaOH, or control of a solenoid valve adding CO_2 to the sparge system. The pH was checked by off-line measurements with a pH meter (Corning Inc., Corning, NY). Liquid level was controlled at 3 or 10 L working volume by a single loop controller (B. Braun Biotech, Allentown PA) using a conductance probe to sense level, and time-proportioning on/off control of a media feed pump (Cole-Parmer, Vernon Hills, IL). Cells were retained in perfusion culture using an external vortex flow filtration device (Membrex Inc., Fairfield, NJ) adapted for this purpose, as described earlier (Roth, et al., 1997). The rate of recirculation through and the rate of harvest from the vortex flow filtration device were set manually at the recirculation and harvest pumps (Cole-Parmer, Vernon Hills, IL). Typical recirculation rates were 50–200 mL min^{-1} and harvest rates were 1–2 culture volumes day^{-1}. A cell purge rate was set manually at the purge pump (Cole-Parmer, Vernon Hills, IL), and used as needed to maintain cell density in long term perfusion culture. Dedicated instruments (YSI Instruments, Yellow Springs, OH) measured glucose and lactate off-line.

Virus plaque purification

Plaque purification was done following an adaptation of a prior method (Green and Wold, 1979). Non-confluent HEK 293 cells were used to seed uncoated 60 mm plates (Corning Glass Works, Corning, NY) at 2.5×10^6 cells/plate in DMEM with 10% FBS. After

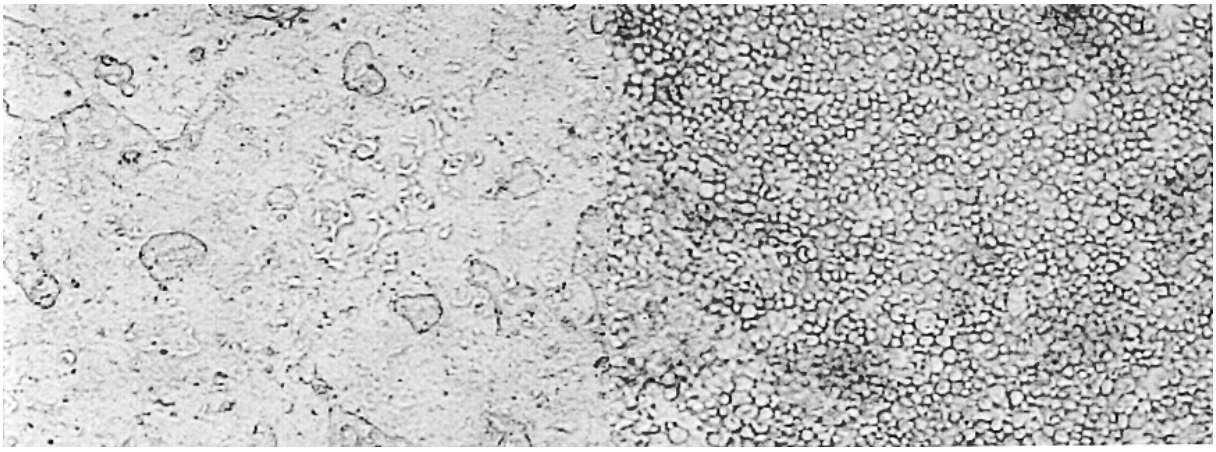


Figure 1. HEK 293 monolayers from a plaque purification dish, showing a non-plaque region on the left, and an early plaque formation (pre-lytic) region on the right. As described in the text, round pre-lytic regions such as this could be detected visually and confirmed microscopically prior to harvest from the dish, without requiring a second staining overlay.

48 hr, media was aspirated, and each plate was infected with 100–200 μL of virus diluted in DMEM without FBS. These plates were incubated at 37 °C in a humidified CO_2 incubator for 90 min, and then each plate was covered with 5 mL of a standard overlay containing 0.5% agarose and 6% FBS in DMEM without phenol red (the infection medium was not removed prior to adding the overlay). The plates were again incubated at 37 °C in a humidified CO_2 incubator. Plaques formed after five to seven days, and the associated areas of the agarose gel, were individually removed with a sterile wire loop or Pasteur pipette and diluted in 1 mL PBS with 2 mM $MgCl_2$. These collected plaques were stored at –70 °C.

Harvest

Adenovirus preparations were harvested from the infected HEK 293 cells by adaptation of a prior method (Shabram et al., 1997). Infected cultures were centrifuged at 700 x g for 5 min. Cell pellets were re-suspended in PBS containing 2 mM $MgCl_2$. The suspensions were frozen in dry ice and isopropanol, then thawed in a 37 °C water bath. These suspensions were freeze-thawed for two to three cycles, and then centrifuged at 700 x g for 5 min The pellets were discarded and the supernatants stored at –70 °C.

Assay for total virus particles

Determination of total viral particles was by analytical HPLC following the method of Shabram et al., 1997.

Assays for infectious virus particles

Infectious virus particles were determined using a variation of the end-point dilution assay (Nielsen et al., 1992), and by a simple cytopathic effect (CPE) assay, as follows.

In the end-point dilution (EPD) assay, confluent HEK 293 cells from a T-75 at >90% viability were used to seed a 96 well plate at 5×10^3 cells well^{-1}. The virus samples were first diluted in DMEM by an estimated three orders of magnitude, based on expected infectious titer, then were serially two-fold diluted in DMEM up to twelve times, and finally 25 μL of each viral dilution in DMEM was added to each of 8 wells. Negative controls (DMEM only) and positive controls (Virus standards diluted to known infectious virus particles mL^{-1}) were used on other plates run in parallel. The plates were incubated at 37 °C in a 5% CO_2 incubator, with media supplementation at day 1 (60 μL well^{-1}) and day 4 to 6 (100 μL well^{-1}). Wells were checked under a microscope for cytopathic effect on day 10 to 12, and scored either positive or negative for infection (infected cells are rounded and detached). A Microsoft Excel (Microsoft Corp., Redmond WA) spreadsheet was used to compute the infectious titer, based on the assumption that a dilution that produces 50% positive wells implies 0.5 infectious virus particles well^{-1} at that viral dilution.

In the CPE assay, HEK 293 cells were plated in 6 well plates at $\sim 2.5 \times 10^6$ cells well^{-1} and grown to confluence. The virus samples were initially diluted three-fold in PBS (Gibco BRL, Life Technologies, Grand Island, NY). Five wells were then infected with

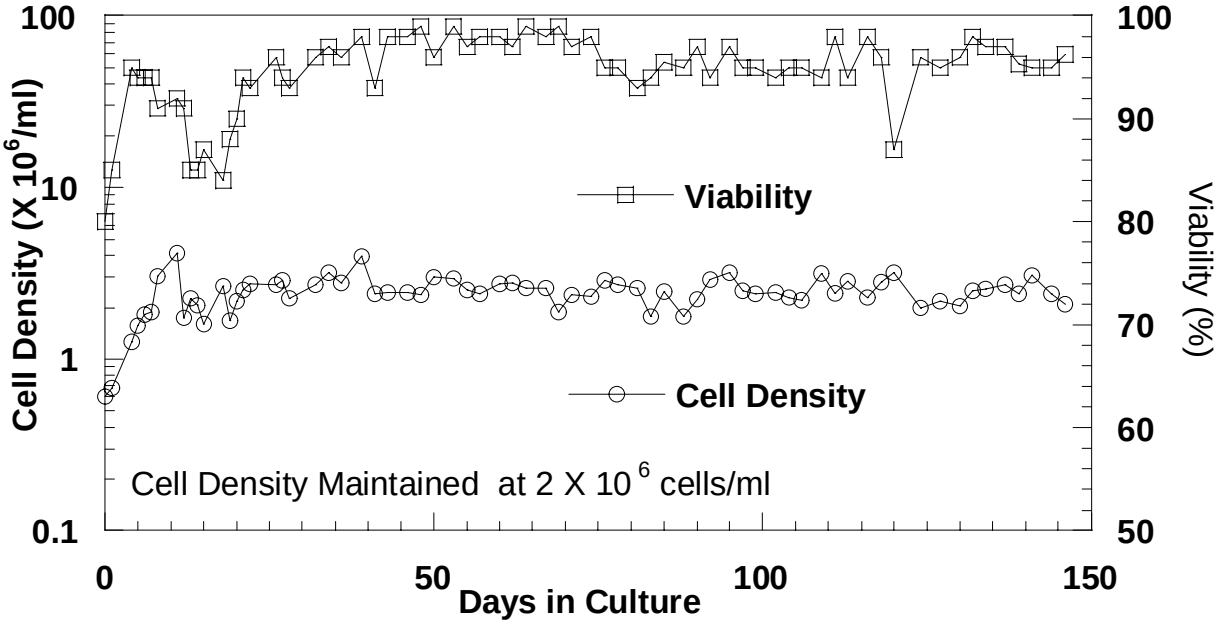


Figure 2. Long-term culture of HEK 293 cells in a 10 L perfused bioreactor using serum-containing medium. The reactor controls and conditions are detailed in the Section Materials and Methods. As described in the text, cells were removed from this reactor throughout the run and infected to produce virus. Over this time period, there were no detectable changes in the quality or amount of virus produced from these cells.

100 μL of serial 10-fold dilutions of the viral samples in PBS, and one well was left un-infected as a negative control. Infectivity was scored at 48 hr where the well that had approximately 50% total cell rounding and detachment (CPE) was scored as half-infected. For each well, it was assumed that there were 4×10^5 cells per cm^2 at confluence and 21 cm^2 per plate, giving approximately 8.4×10^6 cells/plate. The infectious titer per mL was estimated for each well assuming one infectious virus per infected cell. If half the cells were infected, there were 4.2×10^6 infectious virus particles well^{-1}, or 4.2×10^7 infectious virus particles mL^{-1} in the diluted sample. This rapid assay usually agrees with the EPD assay within an order of magnitude and allows for a rapid estimate of infectious titer.

Results

Plaque purification of clonal virus

A method was developed for plaque purification of virus, based on previously reported techniques (Green and Wold, 1979). In contrast to earlier methods, however, early plaque formation (pre-lytic) was identified by microscopy which is a faster approach than the second overlay of neutral red staining typically executed for Ad5 plaques (see Figure 1). These plaques could be detected visually at an early stage, and they grew radially as expected for slowly migrating plaques. Three sequential plaque purifications were executed using this technique to produce three or more clonal virus seeds for a master viral bank.

Long-term culture in bioreactors in serum-containing medium

Initially, HEK 293 cells were suspension adapted, and cultured in stirred tanks using serum-containing medium. These cultures were continuously perfused and cells were retained using a vortex flow filtration device. High-density growth at the 3 and 10 L scales were sustained for 99 and 148 days respectively (see Figure 2, data from a 10 L reactor). Cells were removed periodically for batch infection and proved to be capable of virus production over the life of these reactors. No loss in cell productivity or change in infectivity of the viral harvest was detected (data not shown). This study demonstrated that a long-term perfusion culture could provide cells for batch infections in reactors. Subsequently, we attempted to develop a similar process using cells adapted to SFM.

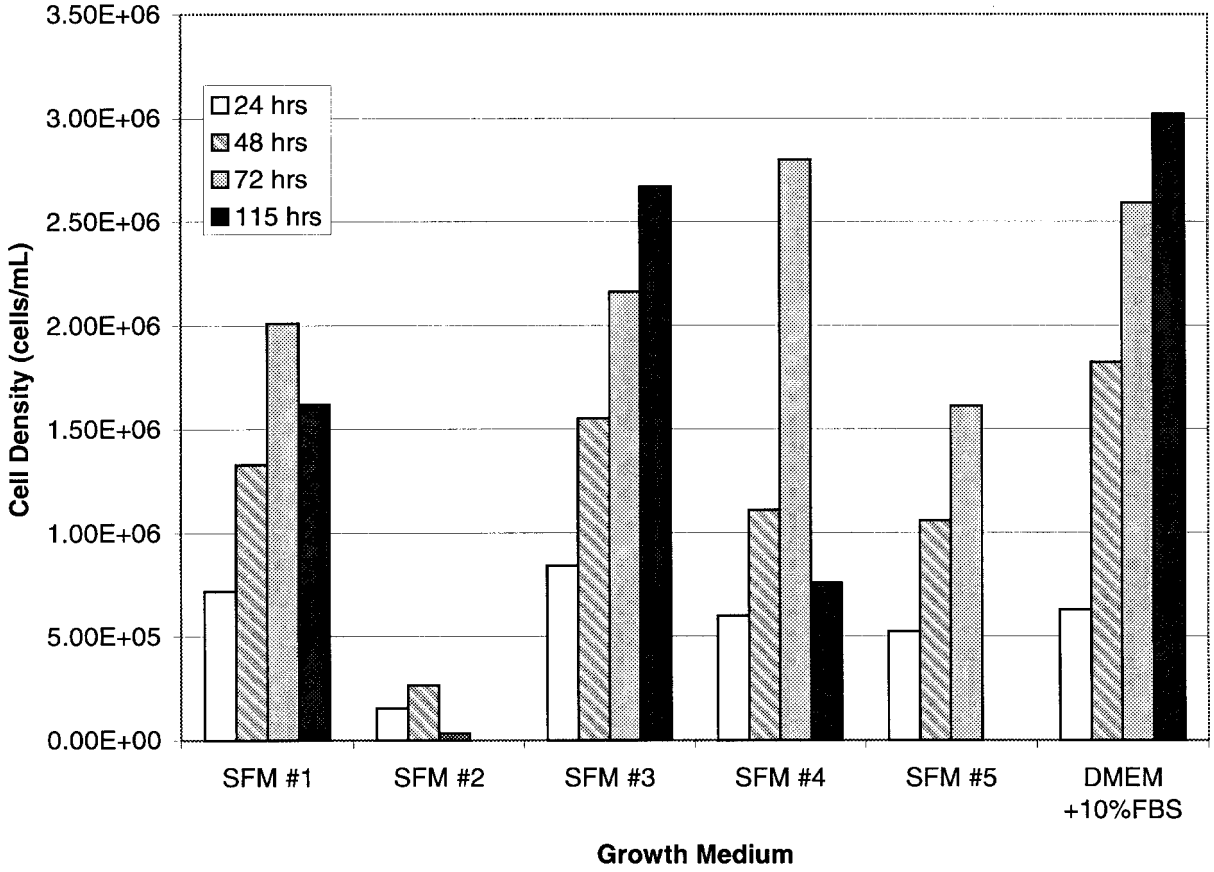


Figure 3. Evaluation of commercial serum-free media with HEK 293 cells using non-weaned cells. The graph shows cells densities for five SFM over 115 hr, compared to DMEM with 10% FBS. Attachment dependent cells were seeded at 5×10^5 cells mL^{-1} in 6-well plates, and a plate was harvested and counted at each time point. The DMEM w 10% FBS culture and the SFM#3 culture had viabilities >90% over the time of the experiment. All other cultures were less viable.

Serum-free medium

Five serum free media (SFM) from three different vendors, Irvine Scientific, BioWhittaker, and JRH Scientific, were screened for use with the HEK 293 cells. These media were first evaluated in 6-well culture plates with adherent cells (see Figure 3). One culture medium (SFM#3) supported growth and viability comparable to serum-containing medium. This medium (SFM#3) also yielded the best growth characteristics in suspension culture (shake flasks). Growth rate and viability of cells in this medium were comparable to that in serum-supplemented medium (see Table 1). Adenovirus production and infectivity of the product were found to be equivalent in both SFM#3 and the serum-supplemented medium, with yields of $\sim$2–5 $\times$ 10^4 viral particles per cell.

Table 1. Growth and viability in serum-containing vs serum-free medium. HEK 293 growth rates and viabilities in a serum-containing formulation compared with a commercial serum free media. Cells were seeded in multiple shake flasks at $\sim 5 \times 10^5$ cells mL^{-1}, and cultured for $\sim$72 hr. Specific growth rates and viabilities were calculated from the first cell doubling

		WEC 5.2.1 + 2% calf serum	SFM#3
Mean specific growth rate	($1\ day^{-1}$)	0.47	0.42
Mean viability	(%)	96	91

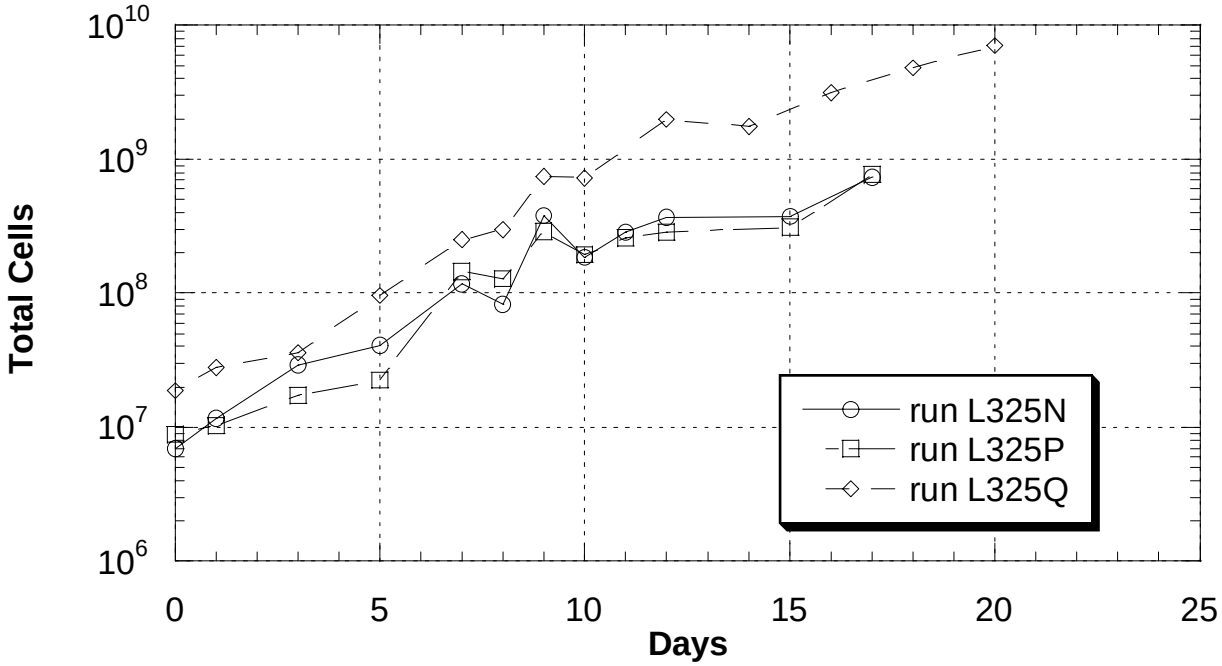


Figure 4. Cell growth qualification for serum-free medium and suspension adapted HEK 293 cells in shake flasks. All three cell cultures graphed on the figure were passaged into new flasks approximately every other day (as described in the materials and methods section). For that reason, the total cells are calculated to provide a clearer depiction of the cell growth. Viability was greater than 90% for all of these cultures.

Adaptation of HEK 293 cells to suspension and serum-free medium for cGMP

After selecting a SFM, HEK 293 cells were adapted to suspension and SFM under cGMP conditions. Suspension adaptation in shake flasks in DMEM + 10% FBS took 9 passages over 20 days. These adapted cells were used for long-term perfusion bioreactor runs using serum-containing medium.

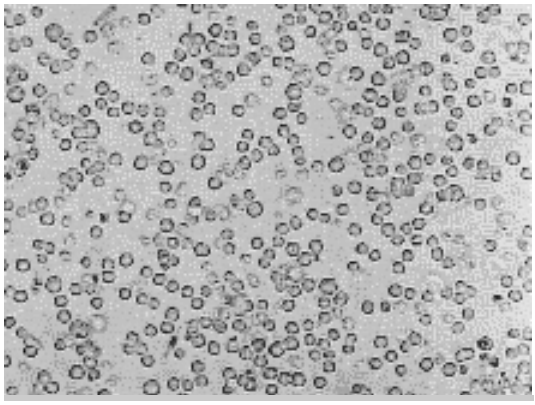
Once the cells were fully adapted to suspension, with similar doubling times and viabilities as achieved in serum-containing cultures, adaptation to SFM#3 was begun. The cells were first passaged multiple times in SFM#3 + 5% FBS until doubling times of no more than 2 days and viability above 90% were achieved. At that point, the cells were assumed to be fully adapted to the reduced level of serum and were transferred into and passaged in fresh medium containing further sequential step-reductions in serum concentration. The cells were finally passaged in SFM#3 without added serum after 30 days and 15 passages starting from the initial suspension adapted, cultures. The final suspension and serum-free medium adapted cells were frozen in medium with 10% DMSO. They were subsequently thawed and expanded in shake flasks. The cultures exhibited doubling times from 1.8 to 2.4 days (see Figure 4). In separate studies, samples of the adapted cells were infected in suspension, and virus production averaged 7×10^4 particles per cell and $1–3 \times 10^3$ infectious particles per cell, which was comparable to production in serum-containing medium.

Small scale cultures

Viral production requires thawing of cells from the cell bank and expansion in small shake flasks. Our best conditions for freezing HEK 293 cells in SFM was 5% DMSO and freezing at –70 °C, with later transfer to liquid nitrogen. These cells were best thawed by direct recovery into SFM, without centrifugation to remove DMSO.

HEK 293 cells are prone to aggregation in SFM. The aggregation of HEK 293 cells in shake flasks was found to be dependent on the flask size. Cell clumping was prevalent in 250 mL shake flasks containing 50 mL of culture (agitation set at 140 RPM), while mono-disperse cultures could be obtained in larger flasks (e.g. Fernbach's). Since clumping of cells was reversed by transfer of cells to a larger flask, the seed cell process included transfer of cells as soon as they

5(A) Day 0



5(B) Day 9

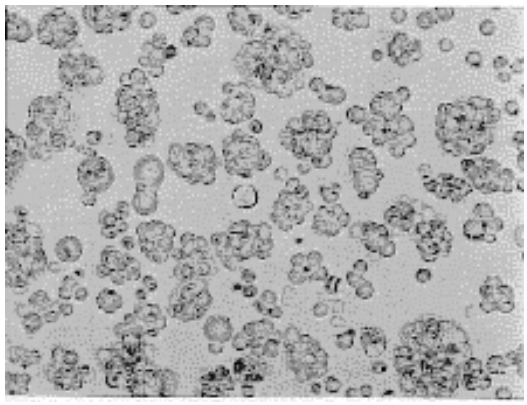


Figure 5. (A) HEK 293 cells in serum-free medium sampled from a bioreactor run at day zero, and (B) the same bioreactor sampled on day nine. The photographs indicate the progression of clumping of the cells over this time frame.

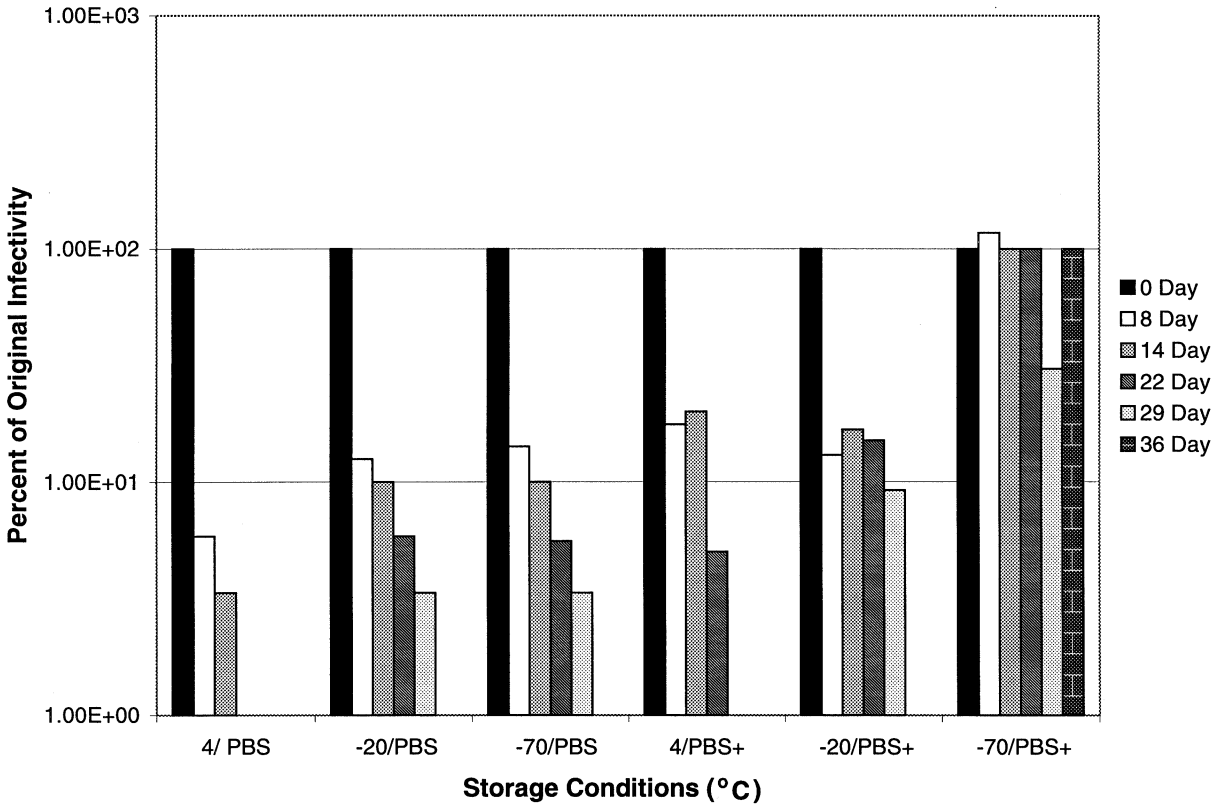


Figure 6. The stability of adenovirus, measured in percent infectivity of the original samples as assayed by CPE (see the Section Materials and Methods), when stored at various temperatures and in various diluents (PBS + is PBS with sucrose and $MgCl_2$). This data clearly shows that under the conditions of PBS+ at –70 °C, the raw virus is stable for at least one month.

Table 2. Determination of optimal MOI. Yields of total and infectious particles from 100 mL culture volume shake flask cultures at various MOI. Particles were measured by HPLC, and infectious particles were measured by EPD

MOI	Particles/cell	Infectious particles/cell
125	6.4×10^4	2.8×10^3
25	8.9×10^4	3.9×10^3
5	5.9×10^4	3.0×10^3
1	4.1×10^4	1.9×10^3
0	0	0

can seed the next largest flask (i.e., at $>4 \times 10^5$ cells mL^{-1} in 20% of the total volume of the flask).

Growth in shake flask cultures in SFM was shown to be dependent on gassing conditions. The SFM used with these cells does not require CO_2 buffering, thus we have traditionally used airtight flasks rather than vented flasks in our 5% CO_2 incubators. Doubling times were lower (1.8 days) in vented flasks exposed to the incubator's atmosphere, compared to sealed flasks with doubling times of 2.4 days. This effect was shown not to be due to pH, but maybe due to either oxygen limitations or accumulation of CO_2 in the sealed flasks (we have observed that both occur, data not shown). This issue was not studied using serum-containing media. To maintain higher growth rates we now routinely culture cells in shake flasks with vented caps.

Long-term bioreactor cultures in serum-free medium

To develop long-term perfusion cultures, ten bioreactor runs were initiated in serum free medium. In all cases, the cells formed large aggregates (see Figure 5) and stopped growing after several days. A variety of conditions were tested, including increased agitation and decreased calcium to reduce the aggregation, and increased surfactants and decreased temperatures to reduce cell shear sensitivity. None of these conditions, however, resulted in a successful culture. While it might be possible to prevent aggregation with additives that inhibit cell/cell interaction, that is probably not a satisfactory approach due to difficulties in removal of such additives.

Effect of multiplicity of infection (MOI)

Table 2 lists viral yields from a wide range of MOIs. Cells were grown in small scale shake flask cultures up to densities of $\sim 1 \times 10^6$ cells mL^{-1} prior to infection. The flasks were harvested at 48 hr post infection, and assayed for total and infectious viral particles. The results indicate that the best MOI for these conditions was greater than or equal to one. A similar experiment in spinner flasks indicated that an MOI of 0.1 significantly reduced the yield of virus. There was no significant increase or loss of per cell productivity with MOIs from 1 to 125.

Adenovirus production in bioreactors in serum-free medium

Characterization of adenovirus production in bioreactors was done in nineteen 3 L runs using serum-free medium. HEK 293 cells were expanded in large shake flasks, introduced into a batch reactor, and infected at MOIs of $\sim$1 to 100. At approximately 48 hr post infection, cells were harvested by centrifugation, resuspended, and lysed by repeated freeze-thaws. Lysates were assayed for total and infectious viral particles. Data from these nineteen runs is presented in Table 3. The data indicates process reproducibility, with reasonably consistent viral yields (both total and infectious).

Storage conditions for stability of the raw virus

Conditions for long-term storage of the raw, unpurified adenovirus, liberated from cell lysates were studied to determine maximum hold times prior to further purification. Two diluents and three storage temperatures were examined. Storage at –70 °C in PBS with sucrose and $MgCl_2$ provided the greatest viral stability (see Figure 6), and was chosen as the standard condition for storage of raw virus.

Conclusions

We have developed an efficient, reproducible, and scaleable manufacturing process for an adenoviral vector expressing therapeutic transgenes. HEK 293 cells were adapted to suspension cultures and serum-free medium. These adapted cells exhibited similar growth, viability, and virus production to serum dependent cells. Although perfusion cultures of suspension adapted cells were maintained for up to 148 days

Table 3. Summary of adenoviral production runs in 3 L bioreactors (Suspension, SFM). Results from fourteen 3 L batch bioreactor runs producing adenovirus, infected at an MOI of 1 to 100, and harvested at ~48 hr. Viral particles were measured by HPLC and infectious particles were measured by EPD

Run result		Mean (n=14)	Standard deviation
Cell density at harvest	($\times 10^{-6}$)	0.82	0.38
Viral particles per run	($\log_{10}$)	14.2	0.22
Infectious particles per run	($\log_{10}$)	12.5	0.24
Viral particles per cell	($\times 10^{-4}$)	8.6	4.6
Infectious particles per cell	($\times 10^{-3}$)	1.8	1.0
Infectivity	(%)	2.2	1.0

in serum, these long-term cultures could not be established for SFM. The long-term SFM cultures appeared to fail due to cell clumping, and attempts to reduce this clumping were so far unsuccessful. It appears that in shake flasks, clumping is less pronounced in larger flasks.

Plaque purification steps for isolating clonal virus were simplified and accelerated by replacing the staining overlay with microscopic inspection. The best multiplicity of infection (MOI) in shake flasks was found to be in the range of 1 to 125, as confirmed by viral production in serum-free suspension bioreactors. The raw virus was shown to have good long-term stability when stored in a PBS buffer at –70 °C.

Development work continues, to establish long-term SFM suspension cultures, and to better characterize the kinetics of virus production in serum-free, suspension cultures.

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