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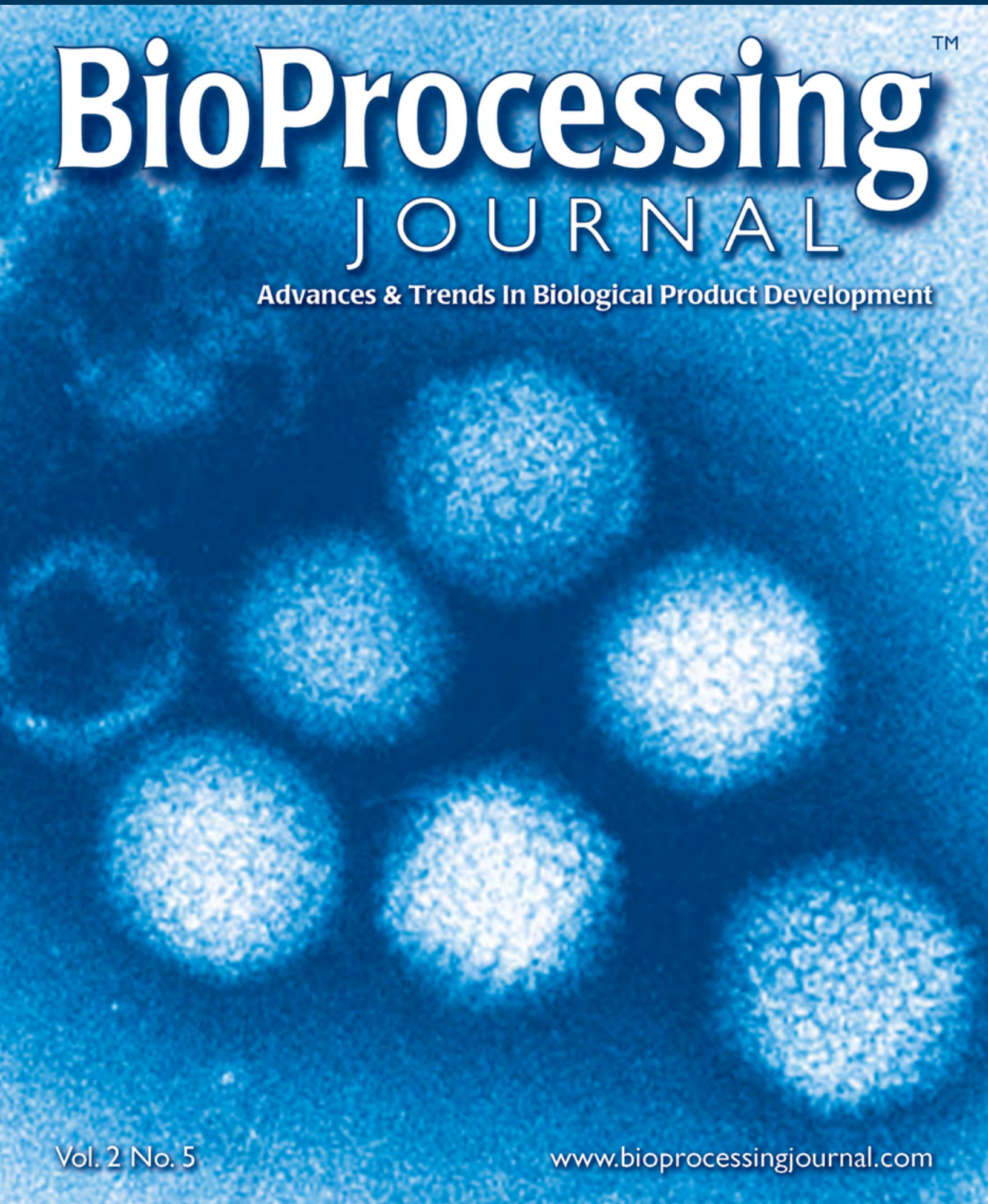

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Advanced Methods of Adenovirus Vector Production for Human Gene Therapy: Roller Bottles, Microcarriers, and Hollow Fibers

BY TATYANA ISAYEVA, OLGA KOTOVA, VICTOR KRASNYKH, and ALEXANDER KOTOV

Various types of viral vectors are being employed extensively as gene therapeutics to treat cancer and genetic diseases. Among the viruses that have been produced for human clinical trials (i.e. retrovirus, adenovirus, poxvirus, adeno-associated virus, and herpesvirus vectors) adenoviruses exhibit the lowest pathogenicity yet still infect an extensive range of cell types with high efficiency. These key characteristics make recombinant adenoviruses efficient gene-delivery vehicles and excellent research tools. However, the time-consuming and complex processes of generation, amplification, purification, and quality testing associated with production of recombinant adenoviruses make it difficult for many researchers to utilize these vectors. This is particularly true with respect to cell culture optimization and the virus propagation protocols employed in vector production. In this regard, the development of innovative cell culture techniques has become vital for optimizing vector production for gene therapy.

This article summarizes our testing of three different large-scale cell cultivation systems to produce two adenoviral vectors, with the goal of developing the most productive, reproducible, cost-effective, and scientifically sound manufacturing system.

Table 1. Comparative yield of HEK 293 cells in different culture systems

	Total cell yield, x 10^6			
Experiment ## 1-8	Per T-flask (175 sq cm)	Per Triple Nunc flask (500 sq cm)	Per Roller Bottle (850 sq cm)	Per 3-Liter μ–carrier culture
Average ± st dev	52 ± 3	120 ± 10	214 ± 18	4,605 ± 364
Microcarrier yield equivalent (number of units)	90	39	21	1
Working volume	50 mL	100 mL	200 mL	3000 mL
Total volume	4500 mL	3900 mL	4200 mL	3000 mL

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Materials and Methods

Cells and Viruses

The human embryonic kidney 293 (HEK 293) cell line was used to propagate adenovirus type 5 (Ad5)-based recombinant vectors. Five vials of master cell bank (MCB) HEK 293 cells (Magenta Corporation, Rockville, MD) were used to generate the Working Cell Bank (WCB). Upon characterization, the cells were accepted by the Adenovirus Reference Material Working Group as a cell bank for the Adenovirus Reference Material (ARM) project.[1] All information regarding the development and characterization of the ARM is available at The Williamsburg BioProcessing Foundation website, www.wilbio.com. The cells were maintained in DMEM: Ham's F-12 (50:50 mix) medium supplemented with 10% fetal bovine serum (FBS) and 4 mM L-Glutamine. Confluent cultures of HEK 293 cells were infected with the virus at a multiplicity of infection (MOI) of 100 viral particles per cell, at which point the growth medium was replaced with the medium containing 2.5% FBS. Infected cells were incubated until a cytopathic effect (CPE) was observed (typically 48 to 72 hours post-infection). Two adenoviral vectors, Ad5.Luc and Ad5.GFP, encoding either luciferase or green fluorescent protein, respectively, were used in the virus production optimization experiments.

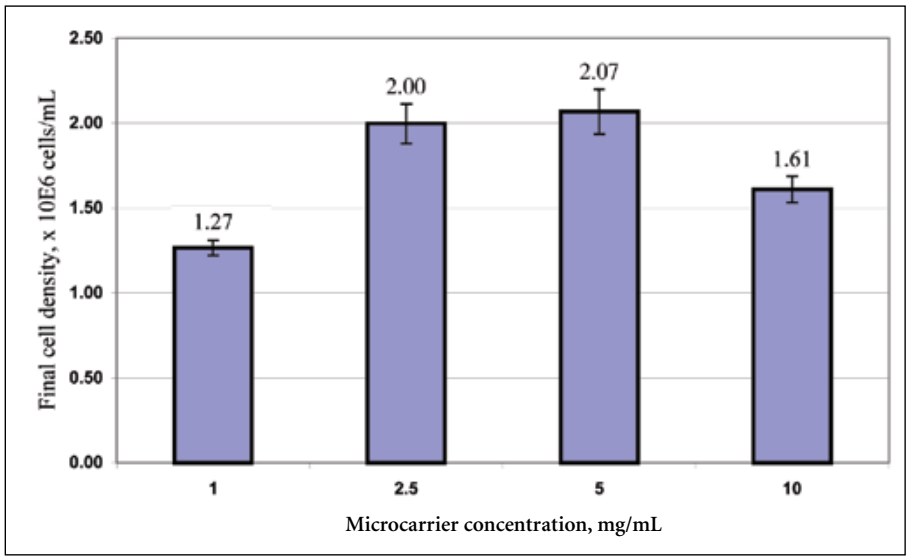

Figure 1. Effect of microcarrier concentration on final cell density

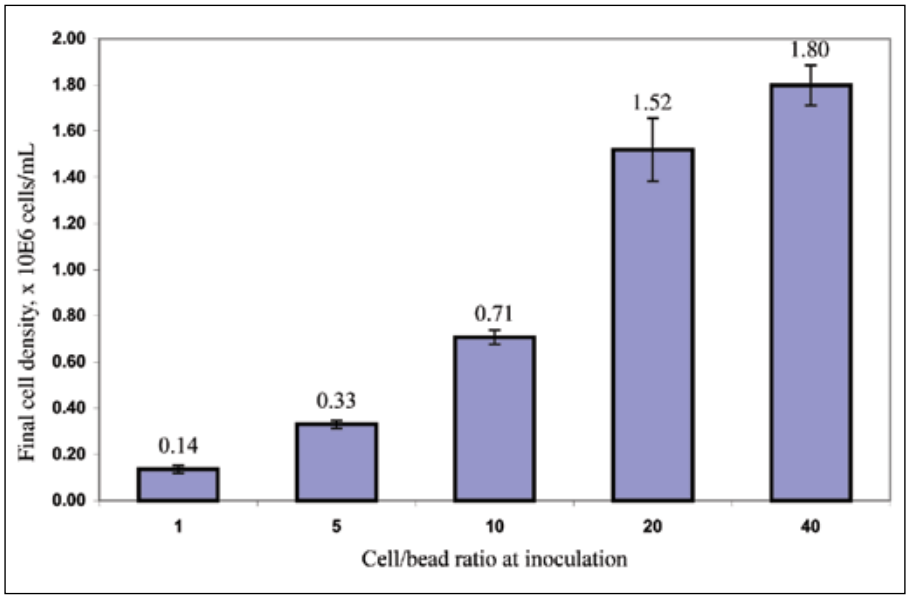

Figure 2. Effect of starting cell/bead ratio on final cell density of HEK 293 cells in microcarrier culture

Cell Counting

Infected cells were harvested from the culture vessel (such as a flask or roller bottle) and counted in a hemocytometer. Cell viability was determined by trypan blue exclusion. In the microcarrier experiments, a simpler way of monitoring cell growth was to count the released nuclei as originally described by Sanford *et al.* with modifications by van Wezel.[2,7] Specifically, cells grown on microcarriers were incubated in a hypotonic solution of citric acid and the nuclei released by lysis were stained with 0.1% w/v crystal violet, followed by enumeration in a hemocytometer. At least 100 cells (or nuclei) were counted for each time point, with these counts being performed in quadruplicate for each cell density data point.

Cell Lysis, Virus Purification, and Analysis

Once CPE developed, the cells were harvested and resuspended in the conditioned medium in a volume representing 5% to 10% of the volume of cultivation medium. The cells were then freeze/thawed three times in dry ice/ethanol bath, cell debris was removed by centrifugation, and the clarified lysate was treated with sterile Benzonase enzyme at 50 U/ml for 30 minutes at room temperature to digest cellular DNA. The supernatant was layered onto a preformed step gradient of CsCl with a density range of 1.4 to 1.25 g/ml and centrifuged at 23,000 rpm for 90 minutes at 4° C in a Beckman SW28 rotor (Beckman Coulter, Inc., Fullerton, CA). Banded virus was collected, diluted twice with 10mM Tris-HCl, pH 8.0, 2mM $MgCl_2$ and purified by equilibrium centrifugation in the same CsCl gradient once more. The virus was dialyzed against 10mM Tris-HCl, pH8.0, 2mM $MgCl_2$, 10% glycerol and stored at –80° C. The virus' physical and infectious titers were determined according to standard operating procedures (SOPs), established at the University of Alabama

at Birmingham (UAB) vector and vaccine production facility. The concentration of adenoviral particles in purified preparation of virus was measured by UV spectrophotometry at 260 nm. The infectious titer method used CPE that adenoviruses have on cells as the read-out to detect infection in HEK 293 cells 10 days post-infection with an adenovirus sample (tissue culture infectious dose, TCID-50 assay). Both optical density at 260 nm and the TCID-50 protocols were adapted from ARM SOPs.

Virus Propagation

The cells were propagated and adenovirus vectors were produced in 500-cm^2 triple flasks (Nalge Nunc International, Rochester, NY) that were placed in a regular CO_2 incubator, 850-cm^2 roller bottles (Corning Incorporated, Acton, MA) at 0.25 rpm, and in two condition-controlled systems. One of these was the 3-L Cell Optimizer System (Wheaton Science Products, Millville, NJ) that was employed with Cytodex 3 microcarriers (Amersham Biosciences, Piscataway, NJ). Cell growth and virus propagation in the Cell Optimizer System took place in a spinner flask with four side arms. The system's Control Tower and Support Unit automatically controlled pH via addition of sodium bicarbonate and CO_2, whereas the dissolved oxygen was adjusted with airflow. The volumetric airflow rate was from 4 to 10 liters per liquid liter per hour throughout the production cycle. Temperature was maintained with a heating pad, which eliminated the need for an incubator. The system's BIOPRO software provided the data acquisition capability for the controller and records storage.

The other system used was the FiberCell Hollow Fiber Cell Culture System (Bellco Glass, Inc., Vineland, NJ). Hollow fibers are relatively new tools in cell cultivation that allow designing compact systems.[3] Potted at both ends with medical-grade polyurethane, these hollow fiber filters create a semipermeable barrier between the extracapillary area where the cells grow and the intracapillary space where the medium flows. The naturally hydrophilic polymer provided a high filtration rate, thereby improving cell viability. This design allows the cells to grow on and around the fibers to extremely high densities or more than 10^8 cells/ml. A typical system consists of a microprocessor-controlled pump, a medium reservoir, and a cartridge. The microprocessor-controlled pump assembly allows control of pressure wave shape and provides flexible control options. The system's positive-pressure displacement pumping system ensures long cartridge life and facilitates nutrient and waste exchange across the fiber.

Figure 3. 3-liter Cell Optimizer System (Wheaton) with microcarriers

Results

Before proceeding to virus production, preliminary experiments to determine the yield of the HEK 293 cells in each propagation system were performed. The cells were maintained as described in the Materials and Methods section. Table 1 shows the average total yield in each system type (in million cells) after 72 hours of cultivation. The data was calculated based on eight independent measurements after harvesting cells from each cell propagation system. These results indicate that the same number of cells can be grown in one 3-liter microcarrier culture, twenty-one roller bottles, thirty-nine triple flasks, or ninety T-175 single flasks. In addition, the microcarrier culture seemed to be the most economical in terms of media costs.

At this point in the study, we employed roller bottles as a standard technique for vector production with good results. While scaling up the production process, our concerns were: improved control of culture parameters (pH and gas tensions, for example), reduced requirements for labor, and lower risk of contamination, all benefits of microcarrier culture. Although microcarrier culture is an advanced technique, it is based on prior knowledge of a cell type's growth characteristics. Information about cell morphology, plating efficiency, and other growth properties in traditional monolayer culture is invaluable when optimizing cell propagation on microcarriers. In this regard, the most efficient technique ensures maximum attachment of the cell inoculum to the microcarriers and results in a rapid, homogeneous growth of cells to the highest possible density.

To determine such a technique, several optimization experiments were performed. Figure 1 shows the effect of microcarrier concentration on the final cell density. In most situations, microcarriers are used in stirred cultures at a concentration from 1 to 5 mg/ml. In our initial experiments, the optimal bead concentration was between 2.5 and 5 mg/ml and all further studies were

performed at 3 mg/ml. Optimization of this parameter ensured a balance between sufficient surface area for cell growth and minimized microcarrier costs.

The other parameter to optimize was the cell density at inoculation (Fig. 2). Cells' survival and growth rates depend on the inoculation density and conditioning effects. At low plating efficiency the cells tend to be very sensitive to culture under low-density conditions. The starting cell number, or inoculation density, affects both the proportion of beads bearing the cells at the plateau stage and the total cell yield. Figure 2 shows that 20 to 40 cells per bead are required for maximum utilization of these microcarriers. All the experiments for the production of adenovirus vectors were subsequently accomplished at starting cell densities of 300,000 to 500,000 cells/ml, which were calculated based on the finding above and the known number of beads per mg of microcarriers.

The microcarrier bioreactor system we used for cell culture process optimization and viral production studies was the 3-L Cell Optimizer System, a complete, small-volume cell culture spinner. This system provides quick, accurate determination of optimal parameters for cell growth. The Cell Optimizer System's vessels are consistent and similar in their design (1 to 45 L volumes), which, along with its modular construction, makes the system convenient, versatile, and easy to scale up.

Using this cultivation system in combination with microcarrier-based culture allows easy aseptic sampling (Fig. 3) at any time, making it possible to monitor both cell expansion and virus production. Because the system is in a biosafety cabinet, the culture was under required containment conditions, thereby allowing easy removal of the samples for microscopic examination, cell counting, titration, and other assays.

Figure 4 shows a typical growth curve of HEK 293 cells in the microcarrier culture system, demonstrating that there was no drop in cell density below the starting cell number during the lag phase (first 24 hours in culture), which often occurs when culture initiation conditions are not optimal. In addition, the final cell density was generally at least 1 x 10^6 cells/ml after 72 hours in culture (infection point). The virus titer accumulation curve is shown in Figure 5. Vector harvesting was typically between 48 and 72 hours post-infection.

Microcarrier culture facilitates microscopic observation of the virus-producing cells due to transparency of the beads. Examining cells by microscopy is a vital part of the technique, in order to assess the state of the culture, confluency, cell counting, and CPE development (Fig. 6A, B, and C). Counting cells after trypsinization can be used to quantify cells, but more a rapid method is to count the released nuclei. In this method, cells grown on microcarriers are incubated in a hypotonic solution and nuclei released by lysis are stained then counted with a hemocytometer (Fig. 6D).

Overall, in contrast to roller bottles, the microcarrier-based system introduced new options and required less hands-on time. This production system facilitated the high yield production of HEK 293 cells and vectors. Although the results we obtained with microcarri-

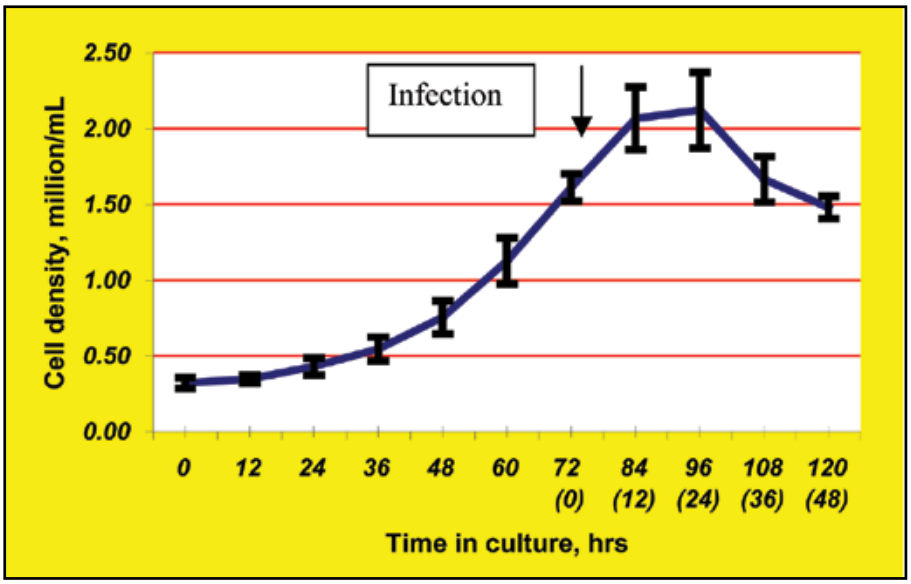

Figure 4. Growth Curve of HEK 293 Cells in Microcarrier Culture

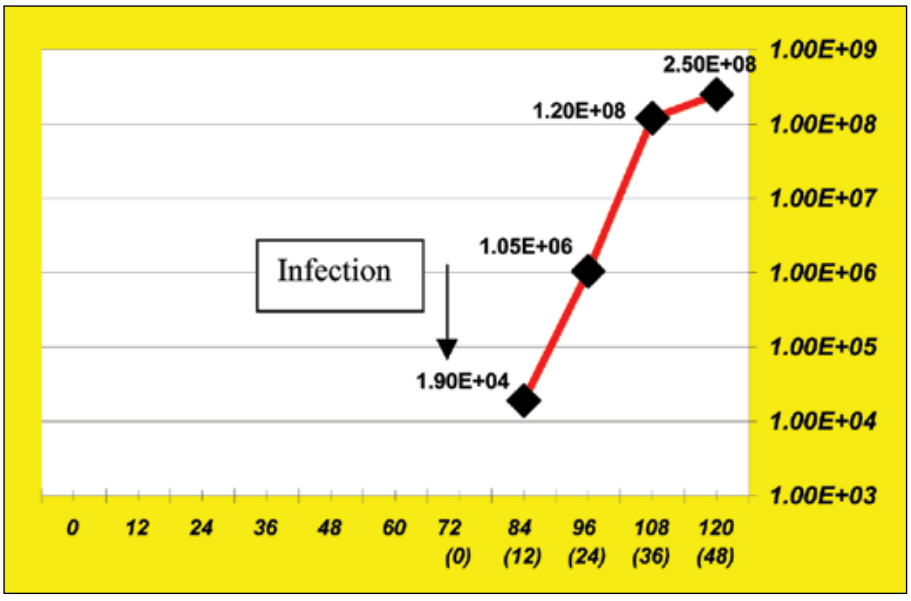

Figure 5. Ad5.Luc Production in Microcarrier Culture, TCID-50/ml

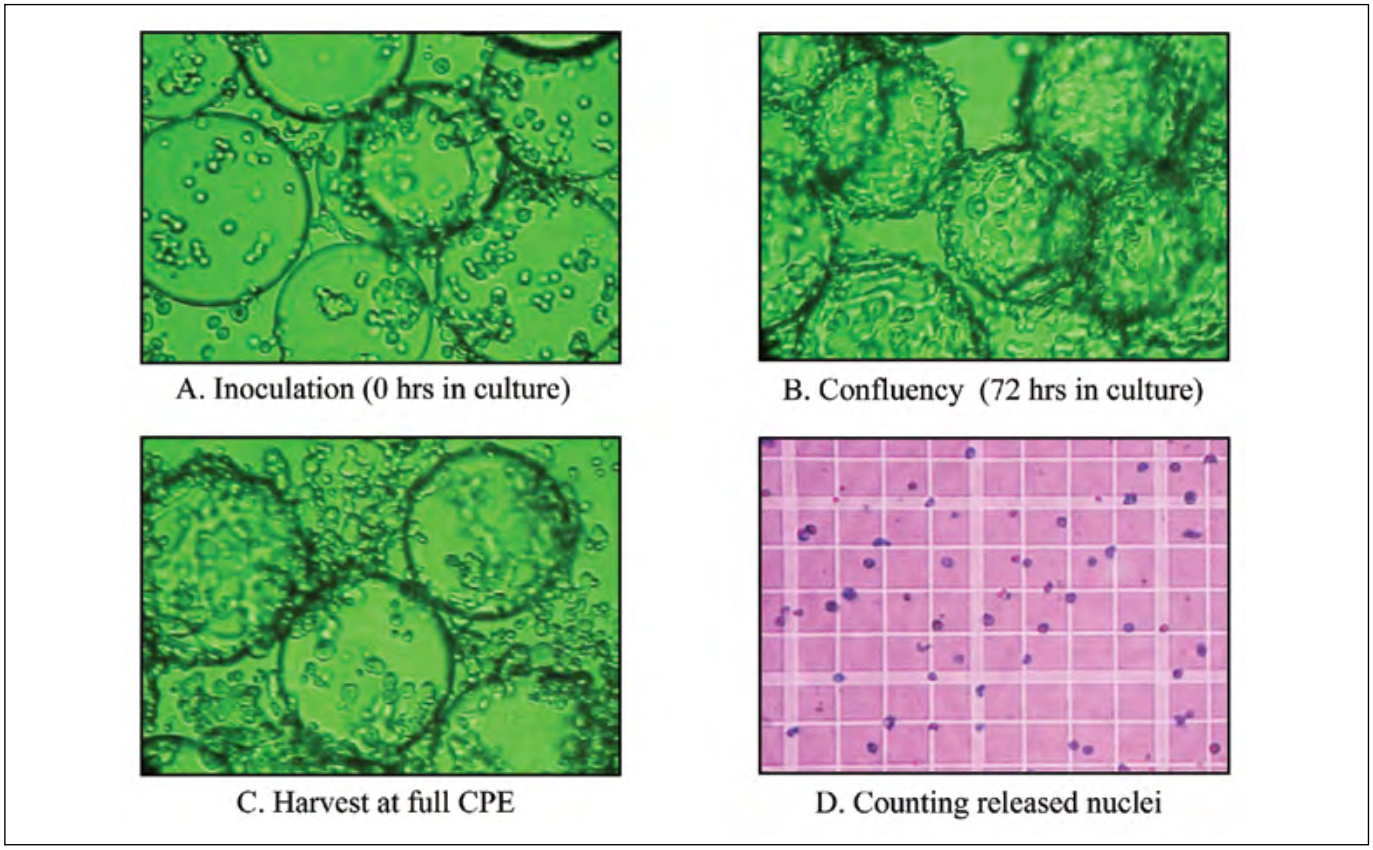

Figure 6. Monitoring of microcarrier culture

er culture were very promising, cGMP regulations and the need to validate cleaning, sterilization, and preparation of reusable equipment (for example, spinner flasks) encouraged us to test the recently introduced FiberCell system, which comes presterilized, certified, and ready to use.[8]

Testing of the FiberCell system began with adapting cells to serum-free conditions using the original HyClone protocol.[4] Briefly, the procedure was to subculture cells in decreasing concentrations of serum until a concentration of 2.5% serum was reached. Once cells were in 2.5% serum, and a passage was completed as normal, cells were incubated to form a monolayer; the serum-containing medium was then replaced with HyQ protein-free (PF) 293 medium (HyClone, Logan, UT). Thereafter, the cells were passaged twice a week in PF-293. Full adaptation was accomplished within three passages from first exposure to PF-293. Trypan-blue viability counts from an established HEK 293 culture growing in PF-293 were almost always above 95%. When HEK 293 cells are adapted to suspension culture, large quantities of adenovirus can be produced in a single FiberCell hollow fiber cartridge. Under these conditions, large numbers of cells (up to 3 x 10^9) were grown in a small volume (15-ml extracapillary space). Efficient viral infection occurs due to the high cell densities.

Because direct control over cell density in the FiberCell system is somewhat difficult, two alternative ways to monitor cell growth were used, based on either glucose consumption or lactate production (or both). In our Ad-vector production experiments we established the correlation of the lactate production rate with the cell number by taking small samples from the extracapillary space and counting the cells. Figure 7 demonstrates very good correlation between these two parameters. Based on these data, we established a conversion factor that allowed us to calculate the cell density in culture using the measured level of lactate concentration. This provided the necessary control over the infection time, MOI, and harvesting time. The infection process was contained inside the closed environment of the hollow fiber cartridge extracapillary space. Adenovirus harvesting took only a few minutes and resulted in highly concentrated samples of the virus. At least 10^{13} virus particles could be produced per preparation in

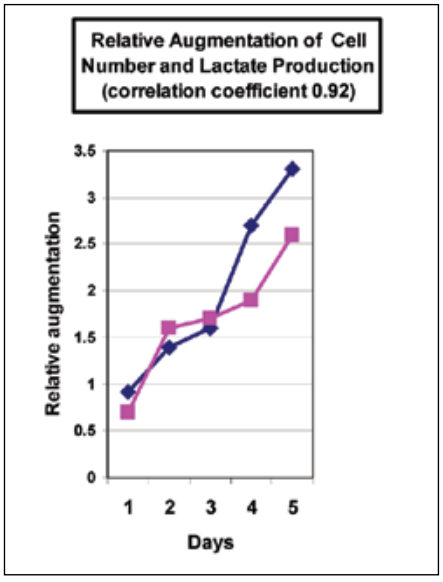

Figure 7. Relation between cell density and lactate level in the FiberCell system

this compact, biosafe system.

The data presented in Table 2 shows the final results. Using three tested systems for middle-scale adenovirus production we were able to obtain 0.9 to 3.6 x 10^{13} viral particles (VP) per production cycle (n = 4 for each system). Figure 8 shows a typical result of CsCl gradient banding (i.e. the purified vector after the second centrifugation). The scale of production is sufficient to support Phase I/II clinical trials at reasonable manufacturing costs.

Discussion

Biotechnologists are constantly challenged to produce more cells and cell products. Therefore, it is critical to develop and establish the most productive, cost-effective, time- and labor-efficient cell propagation and virus production process. The most important characteristic of any anchorage-dependent cell culture is not absolute surface area, but the area to volume ratio. In traditional monolayer techniques this ratio is rather low, typically approximately 4 cm^2/ml. In contrast, hollow fiber-, and microcarrier-based systems have been shown to be effective production tools, providing maximum area for cell growth per unit of media.[5] However, among routine, conventional methods, roller culture has three major advantages compared with static cultures such as dishes, flasks, or triple flasks:

- increase in surface area
- constant but gentle agitation
- improved gas exchange

For repeated harvesting of large number of cells, the roller bottle system seems to be the most economical, although labor intensive and requiring investment in a roller rack and a roll-in incubator. In addition, this manufacturing system requires accurate testing of plastic quality. For instance, for our particular cell line, the bottles from Corning were found the most suitable. Both bottle types from Greiner (soft and hard plastic), as well as the bottles from Nunc and Sarstedt were inappropriate for our laboratory's HEK 293 cells.

A microcarrier system allows propagation of large quantities of virus in compact culture units and provides an improved system for producing many vaccines.[6] Vaccines produced in microcarrier systems include polio, rubella, rabies, influenza, and foot-and-mouth disease (FMD). The advantages of using microcarrier culture for virus production include increased productivity, reduced cost, and reduced risk of contamination compared to other conventional methods. For example, with microcarrier culture, one technician can handle vaccine production equivalent to 900 roller bottles per week.[9] In our applications, the microcarrier system has shown to be an effective time- and labor-saving technique allowing the elimination of hundreds of flasks or dozens of roller bottles while demonstrating high virus yield.

The FiberCell System utilized serum-free cell culture medium and significantly simplified the production process by excluding the trypsin and the serum additives, thus avoiding additional safety testing. In the medium-sized hollow fiber cartridges, HEK 293 cells grew to a final density of 200 x 10^6 cells/ml. After purification using double CsCl gradient ultracentrifugation, the total yield of the

Table 2. Comparison of Timing and Production Expenses*

Production system	Roller Bottles	Microcarriers	Hollow Fibers
Cell Culture Units	20 x 850 cm^2	1 x 3L Spinner	1 x Cartridge (medium)
Person-hours			
Preparation of HEK 293 cells	4 h	1.5 h	1 h
Seeding cells for production	6 h	2.5 h	1 h
Media change and virus inoculation	2.5 h	1.5 h	1 h
Harvest of cells	3.5 h	2.5 h	1 h
Total hours	16 h	8 h	4 h
Supplies ($)			
Plastic-ware	$216	$74	$74
Microcarriers	$0	$62	$0
Cell-Fiber cartridge	$0	$0	$300
Media	$120 (8L)	$90 (6L)	$165 (5L/run)
Serum	$178	$136	$0
Trypsin	$12	$5	$0
Totals ($)	$526	$367	$539
Adenovirus produced after CsC1 purification (Virus Particles)	1.8-3.5x10^{13} VP	0.9-3.6x10^{13} VP	1.0-2.6x10^{13} VP

*Table does not include raw material or QC release/stability test costs nor QA labor.

Table 3. Characterization of Ad vector Manufacturing Systems

Production System	Advantage	Disadvantage
Roller Bottles	Simple and reliable.	Space-, and labor-consuming. Requires FBS/Trypsin. High contamination risk at scale-up. Little scale-up control over culture conditions. Sampling risky.
Microcarrier Systems	Effective and productive. Allows sampling, direct cell count and observation, control over production process. Easy to scale-up. Contamination risk reduced.	Requires FBS/Trypsin. Harvest is time-consuming. Overall relatively complicated.
Hollow Fibers	Effective and productive. Convenient and simple to operate. Space and labor saving. Environmentally sound (biohazard waste reduced). No FBS/Trypsin.	Sampling and cell count is difficult. Indirect control over culture conditions. Scale-up potential unclear.

adenoviral vector in microcarrier culture and hollow fibers was approximately 1.0 to 2.6 x 10^{13} viral particles per production run. This technique also reduced the risk of contamination and can be scaled up. The protocols used were reproducible, convenient, and biologically safe for the production of adenovirus vectors for laboratory use and have the potential to be scaled up for clinical applications. Table 3 reflects the major characteristics of each tested system.

Figure 8. Purification of Adenovirus vectors in CsCl gradient

In this paper, we described a few emerging technologies that could maximize production efficiency, while still meeting FDA regulatory expectations, and offer solutions for future scale up. From the "stone age" of petri dishes for large-scale virus production, the biotech industry needs to step up toward more safe, efficient, and scientifically sound manufacturing systems. However, to obtain more information and details about the advantages and limitations of each contemporary manufacturing method, further experiments and data analysis are needed.

ACKNOWLEDGEMENTS

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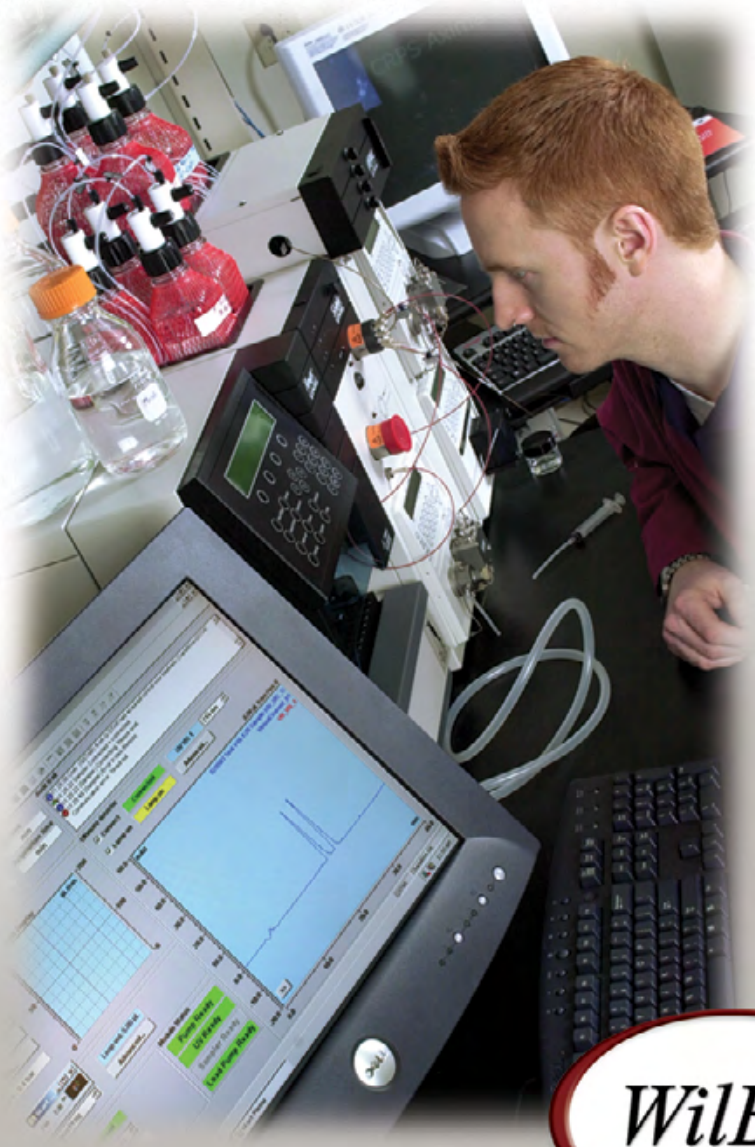
Characterization and Comparability for Complex Biological Products



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Of particular interest will be the developments in rapid analytical methods and inprocess monitoring. We'll also evaluate the progress that is being made in validating product comparability for a number of product types, including the clinical methodologies that are providing the most meaningful results. To support the actual data, we will cover the more effective documentation and regulatory submission approaches that have been used to show comparability for established products. Finally, we will have exhibits and workshops from the industry's leading technology suppliers so that the latest analytical tools and capabilities can be evaluated.

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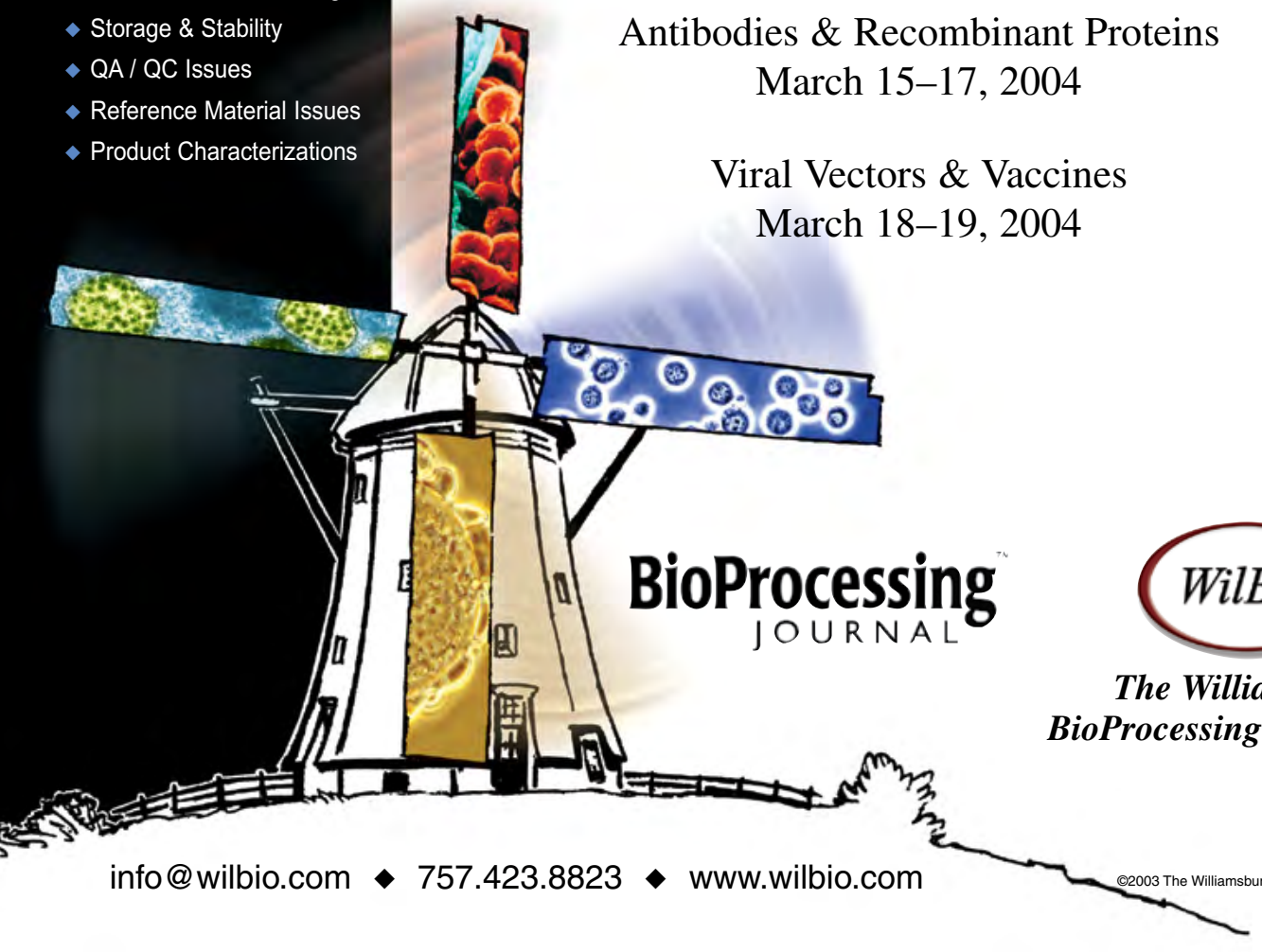

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