A New Medium for Adenovirus Production

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Introduction

The use of recombinant DNA technology to restore defective gene function (gene therapy) offers the potential for improving the lives of individuals afflicted with inborn errors of metabolism. Many of the vectors being investigated for use in gene therapy are based on viruses, with adenoviruses being one of the most commonly used delivery systems. Several helper cell lines are employed for the production of gene therapy vectors derived from adenoviruses. One of the inherent problems associated with many of the cell line-vector combinations used for gene therapy is the generation of wild-type replication competent adenoviruses (RCAs). The generation of RCAs results from recombination between the vectors and viral sequences in the helper cells used to grow the vector that can contaminate a vector preparation intended for therapeutic use. One approach to eliminating this problem is the construction of vector-helper cell combinations that do not contain overlapping viral sequences eliminating the potential for homologous recombination between the vector and viral sequences in the helper cells. The Per.C6® helper cell line is engineered to contain a specific sequence from the adenovirus serotype 5 (Ad5) E1A- and E1B-encoding regions. The use of this cell line in combination with vectors which have the E1 region deleted to remove overlapping sequences eliminates the concern of generating RCAs by recombination. Similarly, the possible presence of adventitious agents associated with animal-derived components frequently used in cell culture medium also represents a potential source of contamination of products produced using cell culture systems. These concerns have spurred the development of animal component-free protein-free media for use in manufacturing. With these objectives in mind, Sigma developed a medium optimized for the growth of Per.C6® cells and designed to meet the regulatory needs of manufacturers for the production of biotherapeutic agents.

Materials and Methods

All materials were supplied by Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise stated.

Per.C6® cells are engineered to contain a specific portion of the E1 region of adenovirus serotype 5 (Ad5) to enable this cell line to support the replication of adenovirus-derived vectors deleted for the E1 region. Per.C6® cells were cultured as attached cells in T-flasks in DMEM + 10% FBS (Product Code F 2442). Agitated small-scale suspension cultures were grown in 125 ml-1 L spinner flasks in protein-free medium on magnetic stirrer platforms at 50-60 rpm. Cells were seeded into medium at 2.5 x 10^5 viable cells/ml and incubated at 37 °C in a humidified atmosphere of 5% CO_2. Total cell counts were determined using an electronic particle counter. Cell viability was estimated by trypan blue dye exclusion.

Cells were grown in protein-free medium for multiple passages and did not require adaptation prior to evaluation of growth and virus production in batch culture. Cells grown in serum-supplemented medium were adapted to protein-free medium using the following regimen. When cells cultured in serum-supplemented medium reached approximately 80% confluence, the medium was removed and replaced with protein-free medium and the flask incubated overnight. The following day the flask was rapped sharply against the palm of the hand to dislodge cells into suspension and the cells were collected by centrifugation (200 x g for 5 minutes). Only cells that went into suspension readily were collected. Cells were never released by mechanical scrapping or enzymatic action. Cells were resuspended in fresh protein-free medium at 5-8 x 10^5 viable cells/ml and transferred to an appropriately sized T-flask. The cultures were counted daily and when the cell titer reached 1-1.2 x 10^6 cells/ml fresh medium was added to reduce the titer to 5-8 x 10^5 /ml. When the number of cells was sufficient to seed a minimum of 75 ml of medium at 3-4 x 10^5/ml the cultures were transferred to a spinner flask at 50 rpm. Suspension cultures were maintained by initiating new cultures at 2.5 x 10^5 viable cells/ml when the cell titer reached 1-1.5 x 10^6 cells/ml.

Virus production

Viral production experiments were set up in singlet by inoculating Per.C6® cells in 100-ml spinner flasks at a density of 2.5 x 10^5 cells/ml. After three days of growth at 37 °C and 5% CO_2, the spinners were infected with 3 x 10^9 virus particles (moi≈3) of the rAd5 containing the β-galactosidase (β-gal) reporter gene (Crucell, Leiden, The Netherlands). The infected cells grew for an additional three days before collecting the virus for quantification. The collection process consisted of centrifuging each infected culture sample at 200 x g for five minutes. The supernatant was removed and the pellet was resuspended in 10 ml of DPBS + 10% glycerol. Both supernatants and cell lysates were stored at –70 °C. To quantify the virus concentrations, a freeze/thaw process was performed three times to lyse the virus from the cells. The freeze/thaw process consisted of freezing the samples at –70 °C and immediately thawing at 37 °C. The samples were centrifuged at 200 x g for five minutes and the supernatant was drawn off for virus titration. For virus titrations, 96-well plates were set up by inoculating each well with 0.100 ml of attached Per.C6® cells (2.0 x 10^5 cells/ml) in DMEM + 4 mM glutamine + 10% FBS and incubated for three days at 37 °C and 5% CO_2. When setting up the virus titrations, six ten-fold serial dilutions were prepared from each cell lysate (10^{-5} to 10^{-10}) in DMEM + 4 mM glutamine + 10% FBS. Each dilution infected one column (eight wells) of a 96-well plate by adding 0.100 ml/well. A control column of wells was set up with 0.100 ml of DMEM + 4 mM glutamine + 10% FBS. The infected plates were incubated for six days at 37 °C and 5% CO_2. This 96-well plate design was set up for high-throughput analysis for use with β-galactosidase assay.

β-galactosidase assay

β-gal activity was assayed using a colorimetric β-galactosidase Reporter Gene Activity Detection Kit (Product Code GAL-A). After incubation of 96-well plates, the medium was removed from the infected wells and 100 µl of cell lysis buffer (1X) was added to each well. After a 20-minute incubation at 37 °C, 100 µl of β-gal assay buffer (2X) was added to each well. The plates incubated for 30 minutes at 37 °C. Next, a plate reader was used to determine absorbency values at a wavelength of 405 nm. The absorbency readings were applied to the tissue culture infectious dose ($TCID_{50}$) equation to obtain quantitative data.

Quantitation of virus production

The tissue culture infectious dose is defined as the dilution of sample at which 50% of the replicate cell culture inoculated with the sample becomes infected. The method for infectious virus quantitation as described by Karber[4] was used in calculating the $TCID_{50}$ value: -m = $\log_{10}$ starting dilution –[p-0.5] x d. The equation is defined where m is the $\log_{10}$ $TCID_{50}$ (per unit volume inoculated per replicate culture), d is the $\log_{10}$ dilution factor, and p is the proportion of wells positive for viral infection. The number of positive wells came from the data obtained by the β-gal assay. Positive wells are defined as having optical densities greater than 0.500.

Results

Frozen cell stocks of Per.C6® cells were recovered by culture in serum-supplemented medium and then adapted to growth in suspension culture in serum-free medium by the methods described. The cell growth characteristics of cultures established from multiple vials of frozen cell stocks and those adapted to suspension culture were compared to assess variability introduced due to selection during the recovery and adaptation processes. As seen in Table 1, the cells adapted quickly to suspension culture without significant changes in the population doubling time (PDT) observed with attached cultures in serum-supplemented medium (approximately 26 hours) and behaved consistently over multiple passages.

Initial studies indicated that cell populations grew to a maximum titer of 2-3 x 10^6 cells/ml with high viability and then exhibited very rapid decreases in viability. Studies were undertaken to understand the effects of key components on cell growth and viability. Based on these results, the original formulation was modified to produce a medium capable of supporting high cell titers and allowing the cultures to maintain high viability several days after reaching peak titer. Studies were then undertaken to compare the performance of the new formulation Gene Therapy Medium (GTM-3; Product Code G 9916), with other commercially available media. The results of cell growth studies of Per.C6® cells seeded at 2.5 x 10^6 cells/ml in 100 ml spinner cultures are shown in Figure 1. These indicate that cells grown in our modified medium (GTM-3) reached higher cell density (> 4.0 x 10^6 viable cells/ml) than cells grown in any of the other media tested. Similar results were also observed in cultures grown in roller bottles (Figure 2).

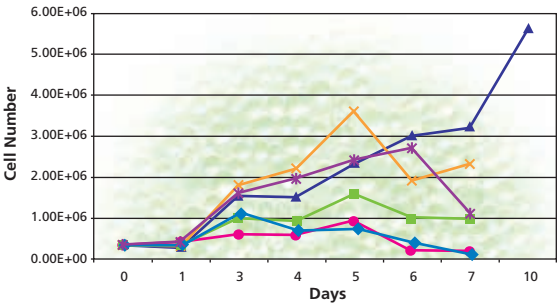

Figure 1. Comparison of cell growth of Per.C6® cells in GTM-1 and GTM-3. *Per.C6® cells were seeded at 2.5 x 10^5 cells/ml in spinner flasks with GTM-1 and GTM-3 and several other commercially available media. Samples were taken on a daily basis in order to monitor cell growth and viability. Each data point represents the average viable cell number from duplicate spinner flasks from multiple experiments. Per.C6® cells showed the best cell growth in the GTM-3 medium with 5.60 x 10^6 at day nine of the culture. The Per.C6® cells growing in GTM-1 reached a maximum density of 3.60 x 10^6 at day five of the culture.*

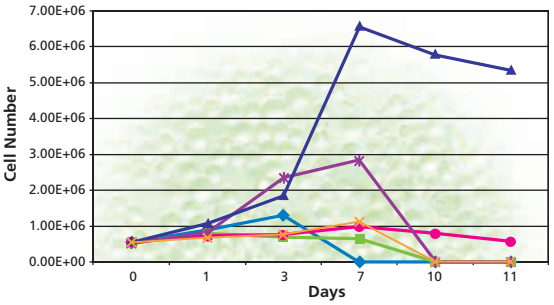

Figure 2. Comparison of cell growth of Per.C6® cells in GTM-1 and GTM-3. *Per.C6® cells were seeded at 2.5 x 10^5 cells/ml in roller bottles with GTM-1, GTM-3, and several other commercially available media. Samples were taken on a daily basis in order to monitor cell growth and viability. Per.C6® cells showed the best cell growth in the GTM-3 with 6.56 x 10^5 cells/ml at day seven of the culture.*

Table 1. Population Doubling Time (Hours)

	Passage 1	2	3	4	5	6	7	8	Average
Stock 1	27.5	29.7	55.8	25.4	27.5	32.3			33.0
2	24.9	23.9	20.8	21. 8		24.6	24.5	23.1	23.4
3		27.2	30.2	25.6	22.6	25.4	22.5	23.4	25.3
4	28.2	28.3	33.5						30.0
5	25.9	19.1	22.7	31.1		47.9	23.8	32.0	28.9
6		30.7	26.6	25.7	22.7	25.3	22.0	23.0	25.2

Table 2. Comparison of virus production from Per.C6® cells in GTM-1 and GTM-3.

Experiment	Medium	Fold Difference
Roller Bottle Assay	GTM-3	3.2
	GTM-1	1
Spinner Flask Assay	GTM-3	3
	GTM-1	1
Spinner Flask Assay		
2 days post infection	GTM-3	2.9
	GTM-1	1
3 days post infection	GTM-3	2.8
	GTM-1	1
4 days post infection	GTM-3	3
	GTM-1	1

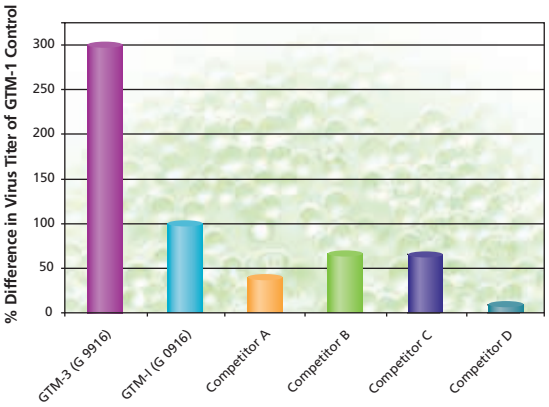

Figure 3. Comparison of virus production from Per.C6® cells in GTM-1 and GTM-3. *Per.C6® cells were seeded at 2.5 x 10^5 cells/ml in spinner flasks with GTM-1, GTM-3, and several other commercially available media. After three days of growth the cultures were infected with 3x10^9 virus particles (moi≈3) of the rAd5 containing the β-galactosidase reporter gene (Crucell, Leiden, The Netherlands). Samples were taken three days post infection and assayed for virus production by using Sigma's β-gal Reporter Gene Activity Detection Kit and Sigma's β-gal Reporter Gene Staining Kit. Quantitation of virus production was determined by $TCID_{50}$ equation (Karber). Evaluation of virus productivity qualitatively was obtained by a virus titer calculation. Per.C6® cells grown in GTM-3 supported a three-fold increase compared to virus produced from Per.C6® cells grown in GTM-1.*

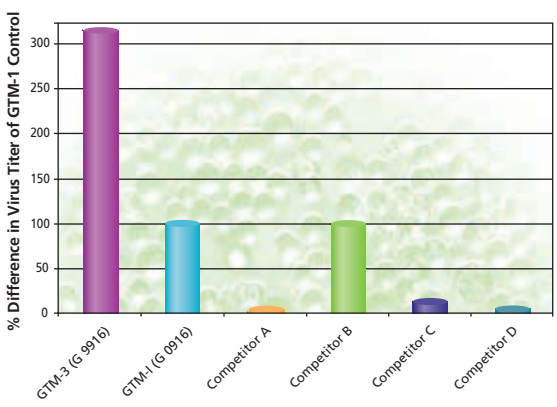

Figure 4. Comparison of virus production from Per.C6® cells in GTM-1 and GTM-3. *Per.C6® cells were seeded at 2.5 x 10^5 cells/ml in poly-HEMA coated 250cm^2 roller bottles rotating at 4 RPM with GTM-1, GTM-3, and several other commercially available media. After three days of growth the cultures were infected with 3x10^9 virus particles (moi≈3) of the rAd5 containing the β-galactosidase reporter gene (Crucell, Leiden, The Netherlands). Samples were taken three days post infection and assayed for virus production by using Sigma's β-gal Reporter Gene Activity Detection Kit and Sigma's β-gal Reporter Gene Staining Kit. Quantitation of virus production was determined by $TCID_{50}$ equation (Karber). Evaluation of virus productivity qualitatively was obtained by a virus titer calculation. Per.C6® cells grown in GTM-3 supported a three-fold increase compared to virus produced from Per.C6® cells grown in GTM-1.*

To confirm that the modified medium formulation also supports adenovirus production, a virus production was tested in the same group of media used in the cell growth test. Virus production was tested as described in Materials and Methods using a β-gal colorimetric assay. Three days post infection, GTM-3 resulted in a virus titer of 1.5 x 10^9 particles/ml and Gene Therapy Medium – 1 (GTM-1) resulted in a virus titer of 5 x 10^8 particles/ml, which is a three-fold increase in virus production (Figure 3). The same three-fold increase in virus production was also replicated in roller bottles (Figure 4). GTM-3 supported a virus titer of 2.37 x 10^8 particles/ml compared to the GTM-1 virus titer of 7.50 x 10^7 particles/ml (data not shown). These data suggest that Per.C6® cells produce more virus when growing in the GTM-3 than in GTM-1 or any of the competitor media (Table 2).

Conclusions

As biotechnology and pharmaceutical manufacturers strive to optimize downstream processing and meet increasingly stringent regulatory guidelines, development of new media to meet their needs must focus on two distinct and sometimes divergent goals. First, animal-derived components must be eliminated due to current and future regulatory concerns regarding raw materials used during the manufacturing of injectable biotherapeutic agents. Second, without serum, cell growth and productivity over a prolonged duration must be similar or exceed that of serum-containing medium. Additionally, it is often desirable to adapt adherent cell lines to suspension culture to facilitate the manufacturing process. Meeting these goals, we have developed a medium for Per.C6® cells, GTM-3, that is optimized for cell growth in suspension culture and maximizes virus production. The medium meets the need of biotechnology and pharmaceutical manufacturers for production of adenoviral vectors using the paired system of rAd5 vector and Per.C6® cells and has the following characteristics:

- Free of proteins and other animal-derived components
- Cells require little or no adaptation.
- Cultures consistently grow to a density of four to five million cells/ml versus two to three million cells/ml in other commercially available media.
- The medium facilitates a metabolic shift in the cells to allow the cells to maintain high viability for several days after reaching maximum cell density.
- Consistent growth of cultures for greater than two weeks.
- The medium supports viral production three times greater than cells grown in other media.

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ORDERING INFORMATION

Product	Product Description	Unit
G 9916	Gene Therapy Medium-3 for Adenovirus Production	500 ml
		1 liter
		6 x 500 ml
		6 x 1 liter

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