A publication of
The Williamsburg BioProcessing Foundation

Fall 2006

BioProcessing™ JOURNAL

The Most Trusted Source of BioProcess Technology™

Vol. 5 No. 3

www.bioprocessingjournal.com

TIPS: Titerless Infected-Cells Preservation and Scale-Up

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Baculovirus, particularly AcMNPV (*Autographa californica* multiple nucleocapsids polyhedrosis virus), is widely used for heterologous protein expression.[2,3,6,8] There are several shortcomings in the current practice of preserving and scaling up baculovirus: 1) extracellular baculovirus stocks, routinely prepared in large volumes and stored at 4º C, are often unstable;[11,12] 2) laborious and time-consuming steps to amplify and titer the baculovirus stocks are often necessary, and generally recommended, for achieving consistent viral infection and protein expression;[4,5,7-10] 3) once prepared, the baculovirus is suspended and stored in conditioned medium. Given the complex, undefined, and unstable nature of the spent media components, including proteases and nucleases, protein expression tends to vary even when steps are taken to titer the virus stock and adjust the amount of stock used for infection.[1] Here, we will report a new method for preserving and scaling up baculoviruses that: 1) provides a new form of viral stock more stable than the traditional, extracellular stock; 2) eliminates the need for virus amplification and retitering; 3) drastically reduces the turn-around time and resources required for scale-up; and 4) improves yield and consistency in protein expression. While this article is specifically

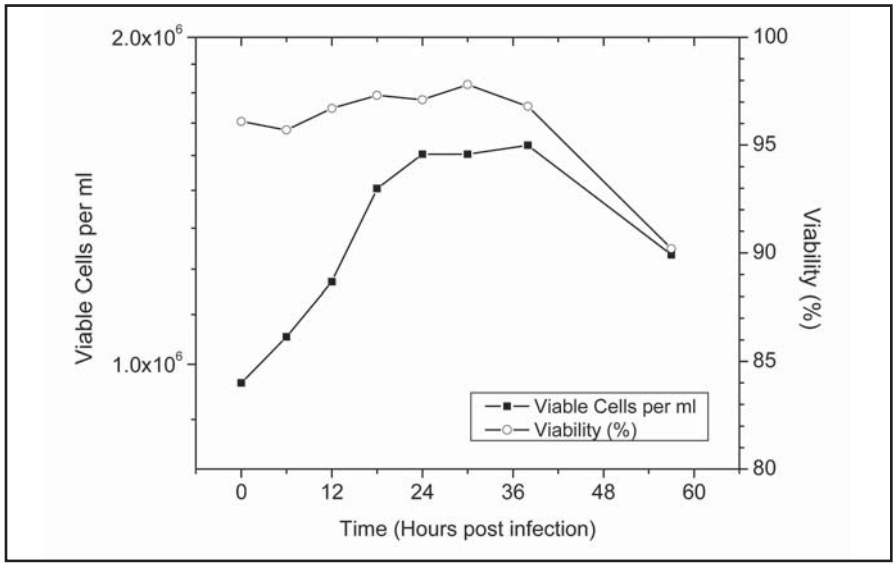

Figure 1. The viable cell density (cells ml-1) and viability (%) of an Sf9 insect cell culture after it was infected with an extracellular recombinant β-secretase virus stock.

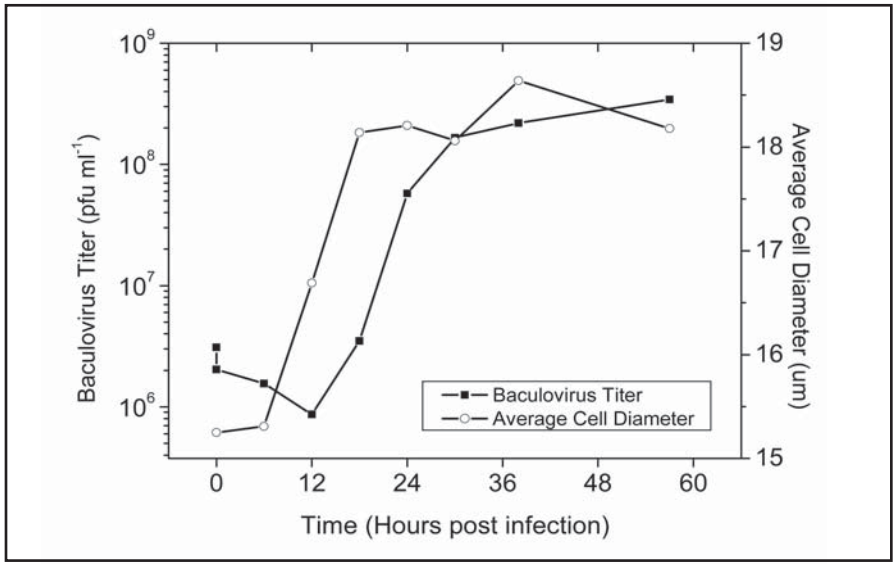

Figure 2. The extracellular baculovirus titer (pfu/ml-1) and average cell diameter (µm) during the infection (see Figure 1).

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directed to baculovirus, the method can be applied to other viruses. What we have developed is a very simple, fast, and versatile method for titerless infected-cells preservation and scale-up (TIPS).

Materials and Methods

Cell Culture

Spodoptera frugiperda Sf9 cells were obtained (Invitrogen Corp., Carlsbad, CA) and cultured in SF-900-II SFM (Invitrogen), supplemented with 5 mg l^{-1} gentamicin (Invitrogen) and 10% (v/v) of fetal bovine serum. Fetal bovine serum was omitted in the beta-secretase expression study. Sf9 cells were cultured (100 ml medium/500 ml flask) in disposable Erlenmeyer flasks (Corning Inc./Life Sciences, Acton, MA) and incubated at 27° C. The Sf9 cultures were continuously agitated at 130 rpm using an Innova 2150 platform shaker (New Brunswick Scientific, New Brunswick, NJ).

Baculovirus

The beta-secretase baculovirus was obtained from Matt Griffor (Pfizer Global Research and Development, Groton, CT). It was engineered to express his-tagged, beta-site amyloid precursor protein cleaving enzyme (BACE). The protein was expressed and secreted into the culture medium.

Analysis

Cell density, cell viability, and average cell size were determined using a Cedex cell analyzer (Innovatis AG, Bielefeld, Germany). Baculovirus titer was measured by the standard plaque assay.[7,10] Western blot was performed using cell-free, conditioned medium samples that were collected at various time points during the infection process and were stored at –20° C. Recombinant protein was detected using a tetra-his antibody (Qiagen, Inc., Valencia, CA).

Cryopreservation of Baculovirus-Infected Insect Cell (BIIC) Stocks

Sf9 cells were grown in SF-900-II SFM as described above. When the cell density was between 0.7-1.2x10^6 viable cells/ml, the Sf9 culture was infected with baculovirus at a multiplicity of infection (MOI) of three. At selected time points, infected Sf9 cells were removed from the culture and processed for cryopreservation. To pellet the cells, a portion of the culture was centrifuged at 90-100 relative centrifugal force (RCF) for ten minutes at room temperature. The supernatant was removed from the cell pellet and the infected cells were resuspended to 1x10^7 cells/ml in cryoprotective media containing 90% (v/v) SF-900-II SFM, supplemented with 5 mg l^{-1} gentamicin and 10 g l^{-1} (w/v) bovine serum albumin (Sigma-Aldrich Co., St. Louis, MO), and 10% (v/v) DMSO (Sigma-Aldrich). Aliquots (1 ml) of the cell suspension were placed into 1.8 ml cryovials (NalgeNunc International, Rochester, NY). The cryovials containing the infected cells were transferred to a Nalgene Cryo 1° C freezing container (NalgeNunc) that was then placed into a -75° C freezer for at least eight hours. For long-term storage, the frozen infected cells stock was transferred to a liquid nitrogen storage container.

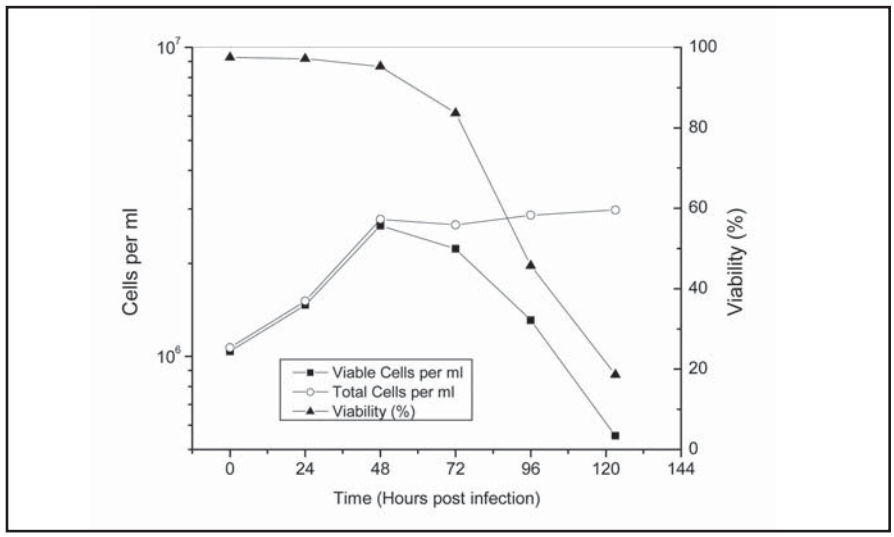

Figure 3. The viable cell density (cells ml-1), total cell density (cells ml-1), and viability (%) of an Sf9 insect cell culture after it was infected with a recombinant β-secretase BIIC stock.

Results and Discussion

Baculovirus has been widely used to express recombinant proteins. Unfortunately, baculovirus stocks are

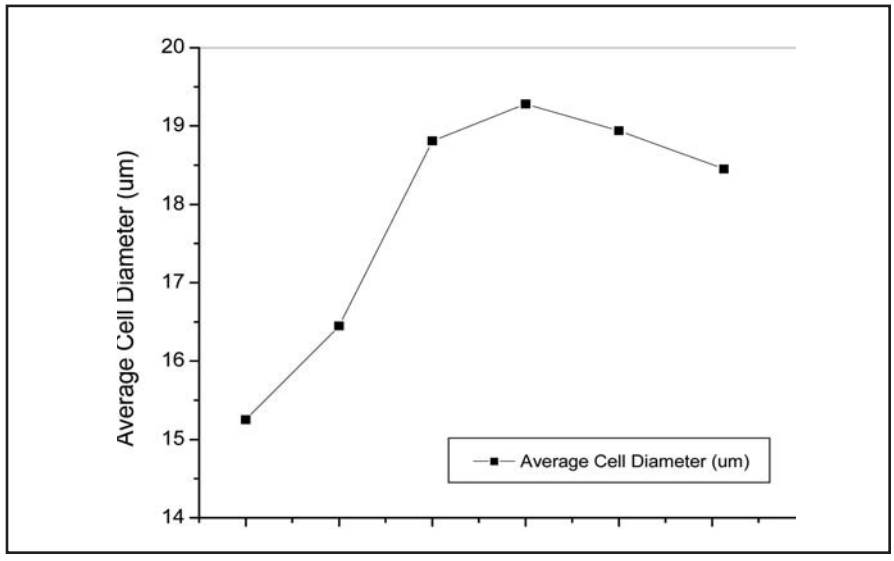

Figure 4. The average cell diameter (µm) during the infection (see Figure 3).

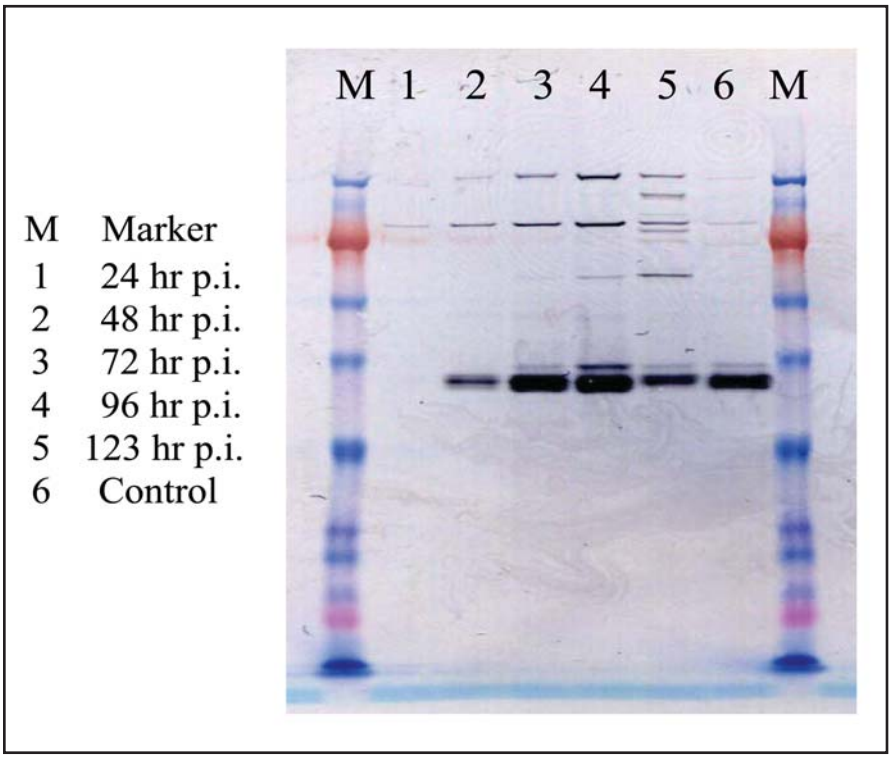

Figure 5. A Western blot for assessing the expression of the β–secretase extracellular domain. Lanes M are molecular weight markers. Lanes 1 through 5 are samples taken 24, 48, 72, 96, and 123 hours during the BIIC infection (see Figure 3). Lane 6, a "control," represents an optimized and best expression obtained from a prior study in which a conventional, extracellular stock of the same virus was used for infection. The dominating band is that of the β-secretase extracellular domain.

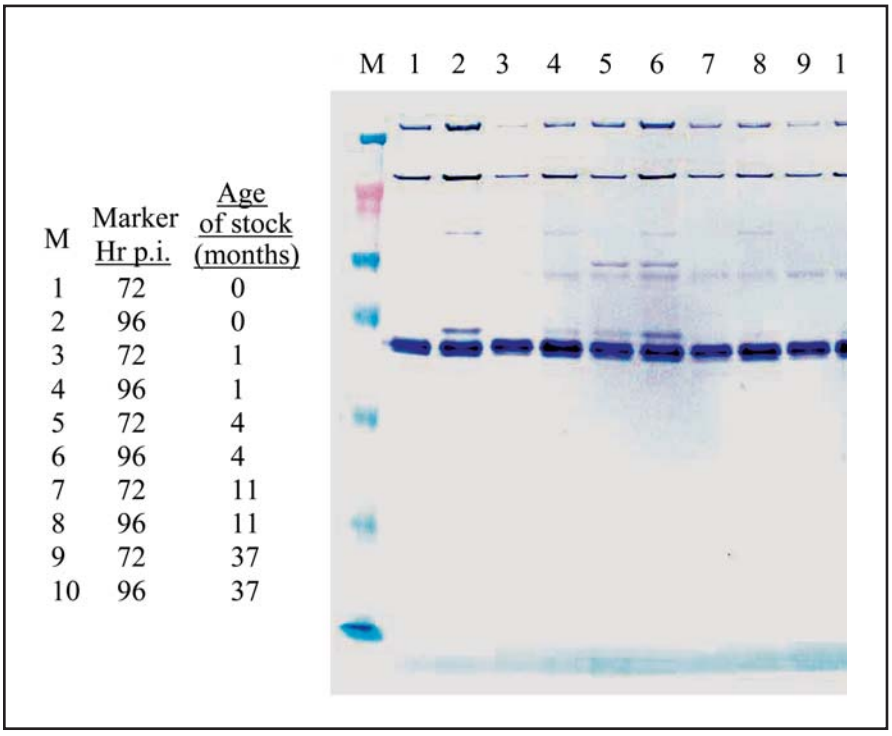

Figure 6. A Western blot of samples taken 72 and 96 hours post-infection initiated with a BIIC stock that had been stored frozen for up to 37 months. The dominating band is that of the β-secretase extracellular domain.

often unstable and show a reduction in virus titer after long-term storage at 4° C. It is therefore highly desirable to have a stable baculovirus stock that can be used repeatedly to produce recombinant protein in a consistent manner, without having to periodically retiter or amplify the baculovirus stock. Our approach for obtaining such a stock was to store the baculovirus inside the cell in the form of BIIC, as opposed to the traditional, extracellular virus stock.

To prepare the BIIC stock, we infected Sf9 insect cells with a baculovirus that was engineered to express a non-glycosylated form of the extracellular domain of human secretase. After infection, cell density, viability, and average cell diameter were monitored. Time point samples were collected for protein analysis and baculovirus titer.

Figure 1 depicts the viable cell density and viability changes of the Sf9 insect cell culture after it has been infected with the recombinant baculovirus. Figure 2 shows the extracellular baculovirus titer and the change in average cell diameter during the infection. Since the increase in cell size is directly related to the baculovirus infection, the change in cell diameter can be used to monitor the infection process in real time. Approximately 24 hours post-infection, when the cell viability was still in the 90th percentile and the average cell diameter had increased by more than 2 µm, a portion of the infected culture was removed for BIIC preparation. The infected cells were pelleted by centrifugation, and resuspended to a cell density of $1x10^7$ cells ml^{-1} in cryoprotective medium. Aliquots (1 ml) of the cell suspension were then frozen and kept in either a -75° C freezer, or in liquid nitrogen.

After cryopreservation, the BIIC stock was used to express the desired recombinant protein in Sf9 cells. To initiate the infection and protein expression, one frozen BIIC vial was removed from cryopreservation and quickly thawed in a 37° C water bath for approximately two minutes. When nearly thawed, 1 ml of BIIC suspension was diluted 1:100 in cell-free medium. Once diluted and adequately mixed, 1 ml of the diluted stock (approximately $1x10^5$ BIIC cells) was

transferred to a flask containing 100 ml Sf9 cells at a density of $1x10^6$ cells ml^{-1}. The newly infected culture was incubated as described. Cell density, viability, and diameter were monitored. Figure 3 illustrates the change in cell density and viability while Figure 4 shows the change in average cell diameter during the infection. Cell growth stopped and cell viability began to decline soon after the cell diameter plateau was reached at 48 hours post-infection. The decline in cell viability with the corresponding increase in cell diameter is sign of an active infection process.

Expression of his-tagged secretase extracellular domain in conditioned medium samples was monitored by Western blot using an anti-his antibody. In the Western blot shown in Figure 5, lanes M are the molecular weight markers, and lanes 1 through 5 are samples collected 24, 48, 72, 96, and 123 hours post-infection. Lane 6 is a control sample of the same volume. This sample represents an optimized and best expression that was obtained from a prior study in which a traditional, extracellular stock of the same virus was used for infection. These results indicate that the new form of infected cell stock can be used to express recombinant proteins, and in this case, produced infected beta-secretase extracellular domain at concentrations equal to, or greater than, the traditional extracellular stock under its optimal expression condition.

To evaluate the stability of the new BIIC stock, the expression experiment was repeated using the frozen BIIC stock after 1-37 months of storage. Figure 6 is a Western blot that summarizes secretase extracellular domain expression during this 37 month period. Time point samples collected at 72 and 96 hours post-infection from four different experiments indicated that protein expression was essentially unchanged for up to 37 months.

In the protein expression procedure described above, the overall BIIC dilution (10^{-4}) in the 100 ml shake flask expression experiment resulted in approximately $1x10^3$ BIIC per ml in the expression culture. Using this dilution scheme, one BIIC stock can be used to initiate the infection of a 10 liter culture (data not shown).

To date, many other proteins have been produced using this TIPS method in scales varying from small shake flask volumes to large bioreactor production volumes (data not shown). In certain cases, protein expression using cryopreserved infected cell stocks was actually better than the traditional, refrigerated extracellular virus stock. We believe the stability and uniformity of the new form of infected-cell stock contributed to the consistency and improvement in recombinant protein expression that we have seen. The TIPS method has had a significant impact on reducing resources required and time allotments for baculovirus scale-up and protein production.

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