Transient Expression of Recombinant Immunoglobulin in HEK-293 and CHO-S Cells Using BacMam Transduction

By CHRISTOPHER W. KEMP, ANITA GUGEL, and APRIL BIRCH

**Introduction**

In 1996, Boyce and Bucher\(^1\) reported that recombinant baculoviruses carrying foreign genes, under the control of mammalian promoters, could be used to transduce mammalian cell lines and achieve gene expression. Fifteen years later, this “BacMam” technology has evolved into a robust transduction system for a variety of mammalian cell lines including stem cells and cell lines of interest to the biopharmaceutical community.\(^2-5\) The introduction of the vesicular stomatitis virus G-protein (VSV-G) gene into the BacMam vector\(^6\) to create a second-generation virus resulted in a lowering of the multiplicity of infection (MOI) ratio required for efficient transduction to levels that are similar to those used for baculovirus transduction of insect cells. The expression of the VSV-G fusion protein also broadened the field of cell lines that may be successfully used as hosts for BacMam-mediated transient protein expression.\(^6\) The result of these improvements is the foundation upon which BacMam technology is being developed as a tool to express genes of interest in mammalian cell lines using large-scale bioprocess methodology.

The development of transient gene expression protocols in mammalian cell lines at scales above 1 L has largely relied on polyethyleneimine (PEI)-mediated transfection of plasmid DNA into a limited range of mammalian host cells (CHO and HEK-293).\(^7\) PEI-based transient transfection systems are readily scalable to the 100 L level and beyond, and may be performed with relatively low transfection reagent costs. The limitations of the system are associated with the quantity

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of highly purified plasmid DNA required for transfection (typically 1 mg/L culture), the limited number of cell lines that exhibit high transfection efficiencies (HEK-293 and CHO), and the limited number of commercially-available, serum-free media (SFM) formulations that support PEI transfection (e.g., Life Technologies Gibco® Freestyle™ for CHO, and Lonza Walkersville Pro293™ S-CDM for HEK-293). Of these limitations, the quantity of plasmid required to support larger scale transfections is the most costly and logistically-limiting factor. The packaging of a gene of interest into a viral vector eliminates the need for the amplification and purification of plasmid DNA as the gene is amplified through viral replication and packaged in a form that is readily introduced into the host cell. Viral transduction systems have been available for many years and include adenovirus, adeno-associated virus, lentivirus, vaccinia, and other vectors. The use of mammalian viruses as gene delivery vehicles for large-scale transduction is limited by the logistics of scale-up and biosafety issues. Most mammalian viruses require amplification and packaging in mammalian cell lines or through cotransfection of insect cells with viral structural genes and vector cassettes. A number of the mammalian viral vectors are BSL-2 agents and require specialized equipment and handling, especially at larger scales. Baculovirus and BacMam viral vectors are readily amplified to 10^8 virus particles per mL using simple and cost-efficient methods in a serum-free environment. Both baculovirus and BacMam vectors are listed as BSL-1 agents; therefore no specialized equipment or procedures are required for larger scale production.

The BacMam vectors utilized in this study are based on the Life Technologies Gateway® system. Separate vectors carrying genes for heavy chain (HC) and light chain (LC) humanized IgG were cloned into standard Gateway entry vectors and transferred by a transposition reaction into a BacMam destination vector. Bacmids and recombinant BacMam virus high-titer stocks (HTS) were generated using conventional methods for insect cell transfection and virus amplification. We found that the overall process of virus generation and amplification was streamlined by the ability to monitor virus particle concentrations in virtually real-time using the InDevR ViroCyt® 2100 Virus Counter®.

BacMam technology simplifies the essential elements of gene amplification and transfer necessary for successful transient protein expression in mammalian host cells. We have used the construction of recombinant immunoglobulin G (rIgG) vectors as a tool to demonstrate the feasibility of utilizing the BacMam system to generate significant quantities of recombinant protein in a cost and time-effective manner. We report our initial efforts involving vector construction, virus generation, and initial expression of rIgG. We are currently using the same constructs to expand our studies into process development of bioreactor-based expression protocols.

Materials and Methods

Cell Lines and Media Formulations

The KB-HEK-293 cell line is a clone of HEK-293 adapted to serum-free suspension culture. The cells were maintained in a modified SFM formulation of Pro293S-CDM. The KB-CHO-S cell line is a clone of CHO-K1 and has also been adapted to serum-free suspension culture. The cells were maintained in Gibco CD-CHO (Life Technologies). The Sf9 cell line (Gibco) was cultivated in Gibco Sf-900™ III SFM.

Vector Construction

Plasmids containing genes encoding the KB008 heavy chain and light chains of humanized rIgG were amplified, purified, and analyzed to confirm the correct HC and LC sequences. The plasmids were digested at unique restriction sites and separated by agarose gel (E-Gel®, Life Technologies) electrophoresis (Gel Box, Bio-Rad Laboratories). The bands containing the genes of interest were excised from the gel and the isolated DNA fragments were cloned into the pENTR1A™ entry vector (Life Technologies). Plasmid DNA was isolated from at least four clones and characterized by restriction enzyme digestion. The KB008 genes in the entry vector were shuttled into the pDEST™ destination vector (Life Technologies) through the LR reaction using the Gateway LR Clonase™ II enzyme mix (Life Technologies) as per the manufacturer’s instructions. The resulting plasmids were amplified, purified, and transformed into MAX Efficiency® DH10Bac™ competent cells (Life Technologies) for bacmid formation. The final bacmid clones were subjected to multiple restriction enzyme mapping and sequenced to assure that the HC and LC sequences were correct.

Sf9 Cell Transfection and BacMam Generation

Purified bacmid DNA was prepared for each of eight clones (four heavy chain and four light chain) using a QIAprep Spin MiniPrep plasmid kit (Qiagen®). For the transfection, 2 × 10^6 Sf9 cells growing in serum-free media
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were plated into T-25 tissue culture flasks (Corning) and the volume was adjusted to 5 mL. The cells were allowed to attach for 60 min at 27°C before the medium was removed and replaced with 5 mL of Grace’s Insect Medium–Unsupplemented (Life Technologies) with 10% fetal bovine serum (FBS) (Mediatech, Inc./Corning). Bacmid DNA (5 µg) was mixed with 20 µL of Cellfectin® II reagent (Life Technologies) in a sterile 1 mL conical tube and incubated at room temperature for 30 min. The mixture was added dropwise to the T-25 flask, mixed gently, and incubated for 5 h at 27°C. The transfection mixture was then replaced with 5 mL of SFM and incubated for 72–96 h at 27°C or until there were visible signs of virus infection. The culture supernatant was harvested, filter-sterilized, and stored at 2–8°C as the “passage 0” (p0) virus stock.

**Virus Detection and Enumeration**

BacMam virus particles were rapidly quantitated using the InDevR ViroCyt® 2100 Virus Counter®, a compact flow cytometer specifically designed for this application. Intact virus particles were quantified using two-channel fluorescence by detecting colocalized proteins and nucleic acids. Samples were stained using a combination of two fluorescent dyes specific for the nucleic acid and protein content of the virus particles, and then the number of events occurring simultaneously on both detection channels were calculated using the analysis time and measured sample flow rate. The Virus Counter reported a total intact virus particle concentration in virus particles per mL.

**Virus Amplification**

The heavy and light chain BacMam viruses were amplified by infecting Sf9 cells using a multiplicity of infection of 0.02. Serum-free suspension cultures of Sf9 cells were infected at an initial cell density of 1 × 10^6 viable cells per mL. The cultures were incubated at 27°C for 96 h using a G2 Gyrotary shaker incubator (New Brunswick Scientific) with the agitation rate adjusted to 100 rpm. The culture supernatant was harvested, filter sterilized, and the virus particle count was determined using the Virus Counter. All high-titer virus stocks were stored at 2–8°C, protected from direct light.

**Transduction**

Suspension cultures of HEK-293 and CHO-S cells were cotransduced with HC and LC BacMam high-titer virus stocks in equal ratios. The virus stocks were added directly to the suspension cultures. The cell density at transduction was 1–2 × 10^6 viable cells per mL and the cultures were incubated with agitation (100 rpm) for up to five days post-transduction. A media exchange was not performed after the addition of the virus and the virus was added in its original insect cell media formulation. The MOI was between 10 and 20 (50% HC: 50% LC) and the volume of HTS added was kept below 10% of the total culture volume to avoid possible inhibitory effects due to metabolic waste products.

The “HEK-Direct” cotransduction was performed using high-density cryovials of HEK-293 cells. For the transduction, vials containing 1 × 10^6 cells each were thawed and diluted to 100 mL using serum-free medium. The BacMam viruses were added and the cultures were incubated at 37°C with an agitation rate of 100 rpm for 66 h. Samples of culture supernatant were aseptically removed at 48 and 66 h post-cotransduction and analyzed by SDS-PAGE for rIgG secretion.

**SDS-PAGE and Western Blot**

Recombinant IgG was analyzed using a combination of SDS-PAGE and Western blot techniques. SDS-PAGE gels, 4–20% Tris-glycine (Life Technologies) were run according to the manufacturer’s instructions. Western blots were obtained by wet-transfer to PVDF membranes (Life Technologies) according to the manufacturer’s instructions. Western blot and Dot-blot preparations were probed with mouse anti-human IgG (whole molecule) alkaline phosphatase conjugate, (Sigma-Aldrich, Inc.) and color was developed using BCIP/NBT (Sigma-Aldrich, Inc.) according to the manufacturer’s instructions.

**Quantitation of Immunoglobulin**

The quantitation of rIgG in samples collected from the various expression experiments was accomplished using affinity chromatography. The assay was performed using 1 mL HiTrap™ Protein G columns (GE Biosciences). The columns were attached to a Bio-Rad BioLogic LP chromatography system and equilibrated using 10 column volumes (CV) of PBS (Mediatech, Inc./Corning) at a flow rate of 1 mL/min. Samples of filter-sterilized culture supernatant (10–20 mL) were loaded onto the column at a flow rate of 0.5 mL/min and the column was washed with 10 CV using PBS. The rIgG bound to the column was eluted with 0.1 M glycine buffer (pH 3.0) at a flow rate of 1 mL/min into tubes containing 40 µL of 1M Tris, pH 9.0. The eluate samples were analyzed by BCA protein assay (Bio-Rad) and for purity by SDS-PAGE. The total quantity of rIgG purified was calculated and expressed as mg/L culture volume.
**Results**

**rIgG Vector Construction**

The two rIgG BacMam vectors were constructed from existing heavy and light chain vectors designed for PEI-mediated transient expression in HEK-293 cells. The procedure for cloning (as presented in the Materials and Methods section) is outlined in Figure 1. Each original plasmid was sequenced and restriction sites were selected that could be used to excise the genes and transfer them to the pENTR1A vector for entry into the cloning system. The rIgG genes contained in the pENTR1A-KB008-HC and pENTR1A-KB008-LC vectors were transferred to the pDEST-BacMam vector through Clonase® catalyzed transposition (Life Technologies).

**Transfection of Sf9 Cells and Amplification of BacMam Viruses**

Our standard incubation time for p0 virus stock production in Sf9 cells is approximately 96 h or until the cell monolayer exhibits visible signs of virus infection (increase in cell diameter, pleomorphic cellular morphology, and cell death). The monolayers for the eight BacMam clones transfected for this study (denoted LC-1 through LC-4 and HC-1 through HC-4) showed no signs of viral infection at 72 h post-infection when compared to the untransfected control. When the same samples were analyzed using the Virus Counter, we found that the titer estimates ranged from $7.5 \times 10^6$ to $2.2 \times 10^7$ virus particles per mL for the eight virus stocks (Figure 2). These titers were of a sufficient level to

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**FIGURE 1.** Schematic diagram of the cloning procedure for obtaining heavy chain and light chain BacMam constructs.

**FIGURE 2.** Viral particle counts for eight Sf9 cell supernatant samples collected 72 hours after transfection with BacMam constructs. The titer estimates were obtained and then used to set up 50 mL p1 virus amplifications at an MOI of 0.02.
initiate 50 mL p1 virus amplifications using an MOI of 0.02. The data presented in Figure 3 demonstrates the utility of using the Virus Counter to monitor virus particle accumulation in timed samples of virus amplifications. We found that for the three p1 virus stocks measured, there was an increase in titer at each time point and a 0.5–1.0 log increase in titer between the 96 and 120 h samples. Table 1 summarizes the titer data generated for the two KB008 BacMam constructs selected for use in the development of the rIgG expression process (HC-3 and LC-3). Both constructs produced high-titer virus stocks exceeding $3 \times 10^8$ VP/mL and support transduction MOI ratios in excess of 10 for each BacMam without the need for concentration or diafiltration of the virus stock. These two constructs were selected based on amplification titers and Dot-blot expression data (Figure 4).

**Expression of rIgG-KB008 in HEK-293 Cells**

Figure 4 summarizes the initial expression screen data for the KB008 rIgG obtained using Dot-blot assays. Immunospecific staining was observed for all combinations of HC and LC BacMam constructs tested with the exception of HC-4 (data not shown). We observed a range of signal strengths from the different expression pairs and found that the pairing of HC-3 and LC-3 resulted in a robust signal. This observation and the p1 titer data were used as selection criteria for the HC-3 and LC-3 pair as the combination for further amplification and process development.

The development of expression protocols for the rIgG BacMam constructs was initiated with shake-flask experiments. For this study we generated expression data at the 50 mL scale using 250 mL vented Corning disposable shake-flasks. Two types of HEK-293 cell cultures were tested for their ability to express the rIgG KB008 from BacMam cotransductions. First cultured were the HEK-293 cells maintained using a routine

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**TABLE 1.** Virus titer estimates obtained using the Virus Counter for amplifications of the p0 virus stock of the LC-3 and HC-3 BacMam constructs used in the expression experiments. The amplifications were performed using Sf9 cells infected with an MOI of 0.02. The p1 virus stocks were harvested at 120 h post-infection and the p2 virus stocks were harvested at 96 h post-infection.

<table>
<thead>
<tr>
<th>Plasmid ID</th>
<th>p0 Titer (VP/mL)</th>
<th>p1 Titer (VP/mL)</th>
<th>p2 Titer (VP/mL)</th>
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</thead>
<tbody>
<tr>
<td>LC-3</td>
<td>7.5 x 10^7</td>
<td>4.0 x 10^8</td>
<td>3.5 x 10^8</td>
</tr>
<tr>
<td>HC-3</td>
<td>1.3 x 10^8</td>
<td>5.6 x 10^8</td>
<td>3.4 x 10^8</td>
</tr>
</tbody>
</table>
split schedule in SFM. The cell density was maintained between 5\times10^5 and 3\times10^6 cells per mL and cultures were incubated at 37°C with an atmosphere of 95% air and 5% CO2. (These cultures are typically replaced with freshly thawed and expanded cells on a monthly basis.) The suspension cultures were agitated at a rate of 100 rpm. The second type of HEK-293 cultures tested (HEK-Direct) were cells frozen at high-density (1\times10^8 cells per vial) and used directly after thawing and dilution in serum-free medium. In both cases, the cells were transduced with BacMam viruses at an initial cell density of 1\times10^6 cells per mL. The MOI for transduction for the HEK-Direct cells was 20 (10 HC-3 and 10 LC-3) and the conventionally cultured HEK-293 cells were cotransduced with a total MOI of either 10 or 20. The Coomassie-stained SDS-PAGE gel results for the shake-flask experiments of HEK-293 and HEK-Direct cells are shown in Figure 5. The yield data was obtained using Protein G for purification and a Micro BCA™ assay (Pierce Biotechnology/Thermo Scientific) for protein quantitation. The average yield for rIgG-KB008 in HEK-293 cells cultivated under standard conditions was 4.4 mg/L for a total MOI of 10, and 8.0 mg/L for the cultures cotransduced with a total MOI of 20. The purified yield for the HEK-Direct cells was 18.9 mg/L, and that sample utilized a total MOI of 20.

**Expression of rIgG-KB008 in CHO-S Cells**

Shake-flask cultures of CHO-S cells cultivated in CD-CHO medium under serum-free conditions were used to test the ability of this cell line to express rIgG after cotransduction with BacMam constructs. Figure 6 illustrates the successful cotransduction and protein expression of rIgG-KB008 in CHO-S cells cotransduced with BacMam constructs HC-3 and LC-3 at a total MOI of either 10 or 20.

**FIGURE 5.** SDS-PAGE gel analysis demonstrating rIgG expression in HEK-293 or HEK-Direct cells cotransduced with KB008 BacMam constructs HC-3 and LC-3. Samples of culture supernatant collected at 48 and 66 hours post-cotransduction were analyzed under nonreducing conditions and visualized with Coomassie stain. Purified yield estimations, (mg/L) were established using a Protein G purification protocol and A_{280} measurements.

**FIGURE 6.** Western blot demonstrating expression of rIgG-KB008 in 50 mL shake-flask cultures of CHO-S cells cotransduced with BacMam constructs HC-3 and LC-3 at a total MOI of either 10 or 20.
expression of rIgG-KB008 in 50 mL cultures of CHO-S cells cotransduced at a total MOI of either 10 or 20 using HC-3 and LC-3 BacMam constructs. While bands are detectable on Coomassie-stained SDS-PAGE gels (data not shown), the Western blot data shown in Figure 6 clearly demonstrates expression at both MOI levels tested.

Discussion

BacMam technology has established itself as a proven method for gene transfer and expression in the fields of cellular-based assays and the visualization of molecular events and structures at the subcellular level.[3,9,10] The application of this technology to the production of milligram and gram quantities of recombinant proteins is relatively new, but the data published to date and the data shown here suggest that BacMam-mediated transduction of HEK-293 and CHO-S cells can be developed as an alternative to PEI-based transient transfection.[11,12] As stated in the introduction, the use of BacMam vectors as transduction agents eliminates the need for plasmid amplification and purification. Using baculovirus as a gene amplification and cell transduction tool simplifies the logistics of the protein expression process and potentially lowers the overall production cost. The elimination of E. coli-based plasmid amplification also eliminates a potential source of endotoxin contamination. There are many aspects of the expression protocol that require optimization. However, the preliminary data that we have generated indicates that expression levels are above that which we were expecting from a nonoptimized system. Optimization will involve all aspects of the process from vector construction to bioreactor conditions and media formulation/supplementation. We are confident that optimization will yield a process that is superior to PEI-mediated transient transfection in both cost and performance.

By using the Virus Counter to obtain virus titer estimates, we have changed our approach to the amplification of our virus stocks from transfection supernatant through two passages of the BacMam viruses. It has been shown previously that Virus Counter measurements on dilutions of a high-titer baculovirus stock are linearly correlated to infectivity results (pfu/mL) obtained from traditional viral plaque assay.[13] The ability to measure virus particle concentrations known to be correlated to infectivity allowed us to rapidly and strategically select the best transfections for amplification and also improved our success rate with early expressions. For example, the failure of cotransductions performed using LC BacMam constructs paired with HC-4 was predicted by the Virus Counter data obtained for the p1 amplification. We routinely set up amplifications from transfection supernatants (p0) and expressions from p1 virus stocks on standard volumetric parameters. Having an estimation of titer for the p0 stocks within a few hours of harvest allowed us to set up our initial amplifications with a more consistent MOI and resulted in higher titers than we normally obtain from volumetric-based protocols.

We were intrigued by the successful cotransduction
of the cryopreserved HEK-Direct cells, developed to provide a degree of standardization for PEI-mediated transient transfection. This approach could be useful for quality control or for reproduction of results either longitudinally or between separate worksites. High-density cryopreserved cells eliminate the need to maintain cells in culture, simplifying the procedure for testing new constructs for protein expression characteristics. This could potentially serve as a platform for the expression of biosensors selected from a “toolbox” of BacMam constructs. We have also prepared banks of high-density cryopreserved CHO-S cells and they will be tested to determine if the results are similar to those observed with the HEK-Direct cells.

The expression levels of the rlgG-KB008 observed with the CHO-S cells were lower than that observed using the HEK-293 cells, but this was not unexpected. It has been our experience that CHO cells are more difficult to transfect or transduce than HEK cells. The fact that we were able to observe gene expression using a total MOI of 10 is promising, and the development of CHO as a viable host for BacMam expression is critical for utilizing this technology successfully in expressing proteins of interest for biopharmaceutical use. Transducing at higher cell densities or using genetically-modified CHO cells could possibly increase the productivity of this cell line with the BacMam system. Our laboratory will be exploring this in the near future.

We have demonstrated that BacMam vectors may be used to successfully express humanized rlgG in both HEK-293 and CHO-S cells. While there remains a great deal of optimization work to be performed, the initial results shown here illustrate that this technology has the potential to help advance the field of large-scale transient molecular expression for biopharmaceutical consideration.

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REFERENCES


NOTE

The pDEST-BacMam vector was obtained from Life Technologies under a sublicense agreement to Kempbio, Inc.