

# Combination of Baculovirus-Mediated Gene Delivery and Packed-Bed Reactor for Scalable Production of Adeno-Associated Virus

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## ABSTRACT

The production of recombinant adeno-associated virus (rAAV) commonly requires plasmid cotransfection, which hinders its mass production. Herein we describe the development of a novel process for rAAV production by combining the advantages of baculovirus-mediated gene delivery and BelloCell bioreactor (a novel packed-bed reactor for animal cell culture; CESCO Bioengineering, Hsinchu, Taiwan). We constructed three baculoviral vectors: Bac-LacZ carries the *lacZ* gene flanked by AAV inverted terminal repeats, Bac-RC harbors AAV *rep* and *cap* genes, and Bac-Helper carries helper genes derived from adenovirus. Cotransduction of HEK-293 cells with these three baculoviruses resulted in successful production of rAAV, and the protein and rAAV yield did not decrease with Bac-RC passage for up to four passages. By adjusting the dose ratio of Bac-LacZ to Bac-RC, adding sodium butyrate, and transferring the production process to the BelloCell-500-AP (500 ml), which allowed for high-density culture and effective baculovirus-mediated transduction of HEK-293 cells, the maximal specific rAAV yield reached  $\approx 3.8 \times 10^4$  vector genome (VG) or 247 infectious viral particles (IVP) per cell, which corresponded to  $\approx 1 \times 10^{14}$  VG or  $8.5 \times 10^{11}$  IVP per reactor run. The yield was comparable or superior to those obtained with other production systems. Baculoviral transduction is simple and cost-effective and the BelloCell-500-AP offers high-density culture of HEK-293 cells. Altogether, the combination of baculoviral transduction and BelloCell reactor culture provides a novel and economically viable approach for rAAV production.

## INTRODUCTION

ADENO-ASSOCIATED VIRUS (AAV) has been widely used as a gene therapy vector (Prockop *et al.*, 2003). The AAV genome, encompassing the *rep* and *cap* genes and the flanking left and right inverted terminal repeats (ITRs), is encapsidated in a nonenveloped icosahedral capsid. The ITRs serve as the primers and origins of replication for DNA replication, and are essential for packaging the viral genome into the virus capsid and for integration into and excision from the host chromosome. The Rep proteins, including Rep78, Rep68, Rep52, and Rep40, are expressed from the endogenous p5 and p19 promoters and are required for replication, site-specific integration, and rescue of the AAV genome from its integrated state. The Cap proteins, expressed from the *cap* gene, include VP1, VP2, and VP3 and

are essential for virus assembly. In addition to these gene products, productive AAV infection requires adenovirus (Ad) or herpes simplex virus (HSV) to provide helper functions (for review, see Flotte and Carter, 1999).

To date, the production of recombinant AAV (rAAV) usually involves the cotransfection of HEK-293 cells with plasmids that carry (1) the vector genome with the ITR-flanked target gene, (2) *rep* and *cap* genes, and (3) helper genes (e.g., Ad E2A, E4, and VA RNA genes) (Xiao *et al.*, 1998; Zolotukhin, 2005; Durocher *et al.*, 2007). However, cotransfection typically requires cell attachment to multiple tissue culture flasks or roller bottles (Okada *et al.*, 2005), which necessitates more than 5000 175-cm<sup>2</sup> flasks for a human clinical trial (Urabe *et al.*, 2002) or more than 20,000 roller bottles for the treatment of 50 patients (Farson *et al.*, 2004). These processes are cumbersome,

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time-consuming, and hinder the commercialization and wide-spread use of rAAV vectors. Although rAAV production can be adapted to suspension culture, the transfection efficiency is low at high cell density (Durocher *et al.*, 2007). A disposable reactor with large surface area for cell attachment (Nunc Cell Factory; Nalge Nunc International, Rochester, NY) has been tested for rAAV production, but the specific yield was 5- to 20-fold lower because of limitations in oxygenation (Okada *et al.*, 2005). The genes required for rAAV production may also be delivered into cells by Ad (Gao *et al.*, 1998) or HSV (Conway *et al.*, 1999), but the presence of Ad or HSV complicates rAAV purification. Alternatively, rAAV vectors can be produced in stable cell lines that require neither transfection nor helper virus infection, but the yield is low (Qiao *et al.*, 2002).

Aside from the aforementioned systems, baculovirus is a DNA virus that infects insects and has been widely used for recombinant protein production. Thanks to their efficient gene delivery into a wide variety of mammalian cells and ease of production, baculoviral vectors have been used for *in vitro* and *in vivo* gene therapy (for review, see Hu, 2005, 2006; Kost *et al.*, 2005), development of cell-based assays (Condreay *et al.*, 2006), vaccine candidates (Yang *et al.*, 2007), cancer therapy (Wang *et al.*, 2006), genetic modification of stem cells (Ho *et al.*, 2006; Zeng *et al.*, 2007), and tissue engineering (Chen *et al.*, 2007; Chuang *et al.*, 2007; Sung *et al.*, 2007). In addition, we have demonstrated that cotransduction of baby hamster kidney (BHK) cells with two baculoviruses expressing essential proteins for the assembly of hepatitis delta virus-like particles (HDV VLPs) results in efficient synthesis of HDV VLPs (Wang *et al.*, 2005). The production process is further transferred to the BelloCell-500 bioreactor (CESCO Bioengineering, Hsinchu, Taiwan), which consists of a packed bed in the upper chamber and a compressible “bellow” in the lower chamber. The oscillating compression and relaxation of the bellow enables the cells immobilized in the packed bed to be sequentially submerged into medium for nutrient supply and exposed to the ambient air for gas exchange. Baculovirus-mediated transduction of BHK cells in the BelloCell-500 is highly efficient (efficiency,  $\approx 90\%$ ) and the specific HDV VLP yield is superior to that obtained by the commonly employed transfection method (Chen *et al.*, 2005).

In light of the limitations in current rAAV production methods, this study aimed primarily at developing a novel scalable rAAV production process by exploiting the advantages of baculovirus-mediated transduction and the BelloCell reactor. We constructed three recombinant baculoviruses: Bac-LacZ harboring the reporter gene (*lacZ*) flanked by AAV serotype 2 (AAV-2) ITRs; Bac-RC harboring AAV-2 *rep* and *cap* genes; and Bac-Helper carrying Ad E2A, E4, and VA RNA genes. Cotransduction of HEK-293 cells with these three viruses resulted in successful rAAV production and the process was successfully transferred to a new BelloCell-500-AP reactor.

## MATERIALS AND METHODS

### *Cells and recombinant baculoviruses*

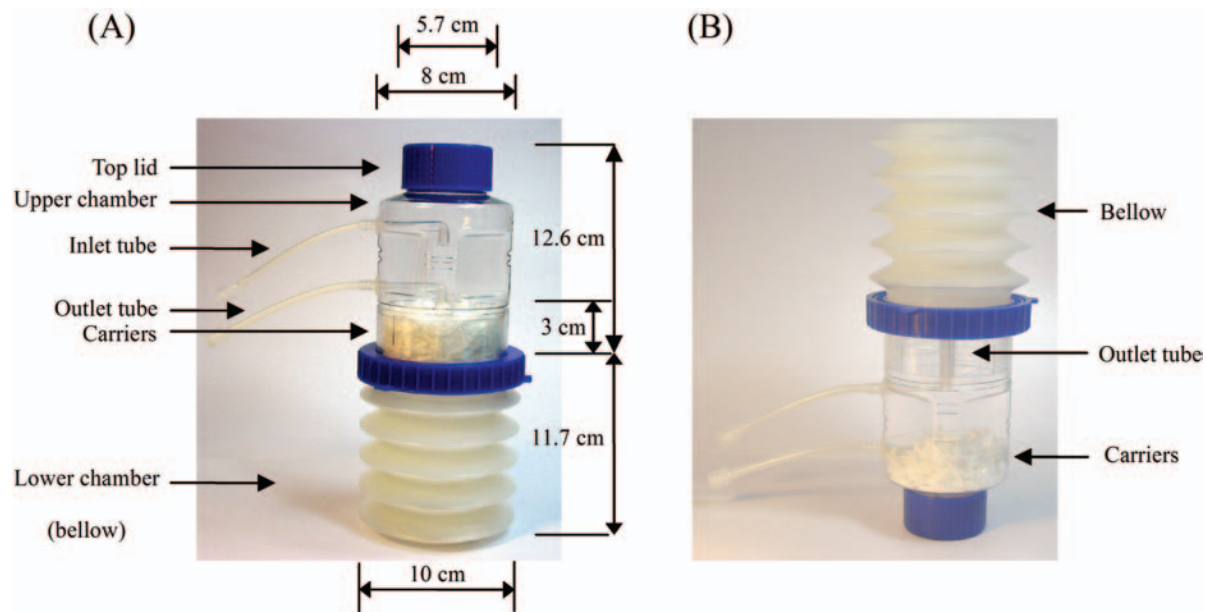
Insect cells (Sf-9) for baculovirus generation and propagation were cultured in TNM-FH medium containing 10% fetal

bovine serum (FBS; Invitrogen GIBCO, Grand Island, NY). HEK-293 and HT-1080 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS. Construction of recombinant baculovirus started with deletion of the polyhedrin promoter in the donor plasmid pFastBac Dual (Invitrogen, Carlsbad, CA). To this end, the polymerase chain reaction (PCR) was performed with pFastBac Dual as the template with two synthetic primers (5’-TAATGGGCCCGAG-TATACGGACCTTTAAT-3’ and 5’-AAATGGGCCCGAT-TATTCATACCGTCCC-3’). The amplicon (5.0 kb) was treated with *Apa*I and ligated to form the plasmid pFastBac $\Delta$ polh, which was identical to pFastBac Dual except that the polyhedrin promoter upstream of multiple cloning site I (MCS I) was removed. The recombinant donor plasmids, pBac-LacZ, pBac-RC, and pBac-Helper, were constructed by separately subcloning the genes in pAAV-LacZ, pAAV-RC, and pHelper plasmids (Stratagene, La Jolla, CA) into pFastBac $\Delta$ polh.

Specifically, pAAV-LacZ contained the cytomegalovirus (CMV) promoter-driven *lacZ* flanked by AAV-2 left and right ITRs. The complete 4.7-kb cassette was digested with *Pst*I, inserted into pBluescript II KS+ (Stratagene), and then subcloned into MCS I of pFastBac $\Delta$ polh by treatment with *Pst*I and *Hind*III. The recombinant plasmid was designated pBac-LacZ. To construct pBac-RC, the AAV-2 *rep* and *cap* genes ( $\approx 4.3$  kb) harbored by pAAV-RC were cleaved with *Eco*RV and *Sma*I and subcloned into MCS I of pFastBac $\Delta$ polh by *Stu*I treatment. pBac-Helper was constructed in two stages. The gene fragment encoding nucleotides 1–1692 of the Ad E2A gene was cleaved from pHelper with *Kpn*I and *Xho*I and subcloned into MCS I of pFastBac $\Delta$ polh to form pBac-E2A. The gene fragment encompassing the rest of the E2A gene and the downstream E4 and VA RNA genes carried by pHelper was then cleaved with *Xho*I and *Sal*I, treated with calf intestine alkaline phosphatase, and subcloned into pBac-E2A downstream of E2A. The resultant plasmid harboring the Ad E2A/E4/VA RNA genes (9.3 kb) was designated pBac-Helper. The recombinant baculoviruses Bac-LacZ, Bac-RC, and Bac-Helper were generated with the respective plasmids, according to the instructions for the Bacto-Bac baculovirus expression system (Invitrogen). The baculoviruses were passaged by infecting insect cells at a multiplicity of infection (MOI) of 0.1, harvested 4 days postinfection, and titrated by the end-point dilution method (O’Reilly *et al.*, 1992). The viruses were not concentrated by ultracentrifugation.

### *BelloCell reactor configuration and operation*

The BelloCell-500 reactor has been employed successfully for BHK cell culture and HDV VLP production (Chen *et al.*, 2005). To produce rAAV vectors using HEK-293 cells, we tested a modified version of the BelloCell-500: the BelloCell-500-AP (CESCO Bioengineering). Similar to the BelloCell-500, the BelloCell-500-AP (500-ml working volume; Fig. 1A) has a top lid equipped with a 0.22- $\mu$ m pore size filter for ventilation and is prepacked with BioNOC II carriers (CESCO Bioengineering) for cell immobilization (Chen *et al.*, 2005). The compressible lower chamber (i.e., the “bellow”) can be alternately compressed and released by the BelloStage (CESCO Bioengineering), with a linear moving rate ranging between 0.5 and 2 mm/sec (Lu *et al.*, 2005). The compression raises the medium



**FIG. 1.** Photographs of BelloCell-500-AP reactors. (A) BelloCell-500-AP. (B) Inverted BelloCell-500-AP. BelloCell-500-AP reactor has a top lid equipped with a 0.22- $\mu\text{m}$  (pore size) filter for ventilation and is prepacked with 71000 pieces (bed volume, 7100  $\text{cm}^3$ ) of BioNOC II carriers for cell immobilization. The compressible “bellow” can be alternately compressed and released by BelloStage. BelloCell-500-AP has inlet and outlet tubes for perfusion operation. The outlet tube extends all the way down to the bottom half of the bellow. The upper support screen confining the carriers in BelloCell-500 is removed in BelloCell-500-AP, thus inverting BelloCell-500-AP on seeding enables the settlement of carriers to the reactor neck and lid.

to submerge the carriers, thus allowing for nutrient transfer. After a period of delay time at the top, the relaxation drops the medium to the lower bellow, thus exposing the carriers to air for oxygen transfer. After another delay at the bottom, the cycle is repeated. Note, however, that the BelloCell-500-AP is different from the BelloCell-500 in that the BelloCell-500-AP has extra inlet and outlet tubes for perfusion operation and has no upper support screen to confine the carriers, thus inverting the BelloCell-500-AP leads to the stacking of BioNOC II carriers near the lid and neck (Fig. 1B).

For cell seeding to the BelloCell-500-AP,  $1 \times 10^8$  HEK-293 cells suspended in 150 ml of DMEM were added. The reactor was inverted, swirled gently so that the cells and carriers settled evenly to the reactor neck and lid (normal lid without the 0.22- $\mu\text{m}$  pore size filter), and incubated in the  $\text{CO}_2$  incubator at 37°C. After 3 hr, the BelloCell-500-AP was replenished with 350 ml of fresh medium, replaced with a new lid (with a 0.22- $\mu\text{m}$  pore size filter), and mounted to the BelloStage. Throughout the culture period, the linear moving rate was set at 1.5 mm/sec (delay times at the top and bottom were 0 and 30 sec, respectively). Moreover, the inlet and outlet tubes were connected to a BelloFeeder (CESCO Bioengineering) pump and a 2-liter external medium reservoir. Twenty-four hours post-seeding, the BelloFeeder was turned on and operated intermittently at a perfusion rate of 999 ml of medium per 24 hr. The perfusion rate was elevated to 1999 ml of medium per 24 hr after the cells entered the exponential growth phase. During culture, six pieces of BioNOC II carriers were taken to measure cell number by the crystal violet dye method (Chen *et al.*, 2005).

#### *Baculovirus-mediated transduction and rAAV production*

Virus-mediated transduction on 10-cm dishes was performed as described (Chen *et al.*, 2005) with modifications. Depending on the MOI used, HEK-293 cells ( $5 \times 10^6$  cells per dish) were incubated with unconcentrated virus, using phosphate-buffered saline (PBS, pH 7.4) as the surrounding solution to adjust the final volume to 3 ml. The dishes were shaken on a rocking plate at 27°C for 6 hr. After transduction, the cells were washed with PBS, replenished with 10 ml of DMEM, and continued in culture at 37°C.

HEK-293 cells cultured in the BelloCell-500-AP were co-transduced with Bac-LacZ, Bac-RC, and Bac-Helper as described (Chen *et al.*, 2005), but with some modifications. The cell number was determined by the crystal violet dye method and the three viruses for cotransduction were mixed. The volume of each virus depended on the MOI (see Results). The transduction solution was prepared by mixing 167 ml of virus solution with 333 ml of  $\text{NaHCO}_3$ -deficient DMEM (serving as the surrounding solution), which gave a volumetric ratio (surrounding solution to virus solution) of  $\approx 2$ , which favored VLP production (Chen *et al.*, 2005). Before transduction, the pH of the transduction solution was adjusted to pH 7.7 with 1 N NaOH and the immobilized cells were washed with 400 ml of PBS for 5 min. After washing, the PBS was discarded and transduction was initiated by adding 500 ml of transduction solution. The reactor was gently swirled to allow uniform contact between the cells and viruses, and the transduction continued for 6 hr

by operating the reactor on the BelloStage at 27°C (linear moving rate, 1.5 mm/sec; delay time at top and bottom, 20 sec). The transduction solution was then poured and 470 ml of fresh medium containing 2.5 mM sodium butyrate was added. The rAAV production phase commenced by operating the reactor on the BelloStage at 37°C, as in the cell culture phase. Approximately  $1 \times 10^8$  cells were sampled daily from the reactor for CsCl gradient centrifugation purification and subsequent analyses. Production was terminated 4 days posttransduction.

#### Purification of rAAV particles

Cotransduced HEK-293 cells were lysed by three cycles of freeze–thawing and treated with 2  $\mu$ l of DNase I at 37°C for 30 min. The lysates were treated with 0.5% sodium deoxycholate at 37°C for 30 min and centrifuged at  $13,000 \times g$  for 30 min. The supernatant was then mixed with 40% CsCl solution and centrifuged at  $181,000 \times g$  for 24 hr. Fractions (0.7 ml) were analyzed by PCR, using primers probing the CMV promoter region to detect the presence of the rAAV genome. rAAV-positive fractions were pooled and dialyzed against PBS to remove CsCl.

#### Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot

To detect rAAV proteins, samples were resolved by 10% (for Rep proteins) or 12% (for Cap proteins) gel electrophoresis and electrotransferred to nitrocellulose membranes for Western blotting, according to the instructions of the manufacturer (Bio-Rad, Hercules, CA). The primary antibody was mouse monoclonal antibody (1:50 dilution; American Research Products, Belmont, MA) specific for AAV Rep or Cap proteins. The secondary antibody was horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The membranes were developed with chemiluminescence reagent (ECL Western blotting substrate; Pierce Biotechnology, Rockford, IL). Western blot analysis of baculovirus vp39 and gp64 proteins was performed as described previously (Yang *et al.*, 2007).

#### Quantification of rAAV titer

The expression of  $\beta$ -galactosidase ( $\beta$ -Gal) was detected with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) staining (Sollerbrant *et al.*, 2001). Titration of rAAV was performed according to the instructions included with the AAV helper-free system (Stratagene). Briefly, rAAV samples were diluted 10-fold serially and HT-1080 cells in 24-well plates ( $6 \times 10^4$  cells per well) were incubated with DMEM containing 240 mM hydroxyurea and 2.5 mM sodium butyrate for 4 hr. The diluted virus was then used to transduce the HT-1080 cells. After transduction, the cells were cultured in fresh DMEM for 3 days and then subject to X-Gal staining. The rAAV titer was calculated and expressed as infectious viral particles per milliliter (IVP/ml).

rAAV vector genome (VG) copy numbers were measured by quantitative real-time PCR (qPCR) as described (Urabe *et al.*, 2002) with slight modifications. Briefly, rAAV genome was extracted with a blood and tissue extraction miniprep kit (Viogene, Taipei, Taiwan). Primers targeting the CMV promoter re-

gion (nucleotides 363–437) within the vector genome were designed as follows: forward, 5'-GCCCCACTTGGCAGTACATCAA-3'; reverse, 5'-GGGCCATTTACCGTCATTGAC-3'. Primers targeting the internal control gene  $\beta$ -actin were designed as follows: forward, 5'-TTCTTTGCAGCTCCTTCGTTGCCG-3'; reverse, 5'-TGGATGGCTACGTACATGGCTGGG-3'. qPCRs were performed in an ABI 7300 (Applied Biosystems, Foster City, CA) as described (Lee *et al.*, 2007). For each PCR, a no-template reaction was included as negative control. Vector genome numbers were quantified on the basis of an external standard curve, using pAAV-LacZ as the control. pAAV-LacZ was quantified with a spectrophotometer and then serially diluted (150, 15, 1.5, 0.15, and 0.015  $\mu$ g) so as to create the standard curves. The number of baculoviral genomes was similarly quantified by qPCR except that the primers (forward, 5'-CTCACGACCGAAAAGATCAAGA-3'; reverse, 5'-GCTCGGCCCTGACCAAGT-3') were designed to target the gentamicin resistance gene incorporated into the baculoviral genome, and the standard curve was generated by serially diluting pBac-LacZ (30, 3, 0.3, 0.03, and 0.003  $\mu$ g).

#### Statistical analysis

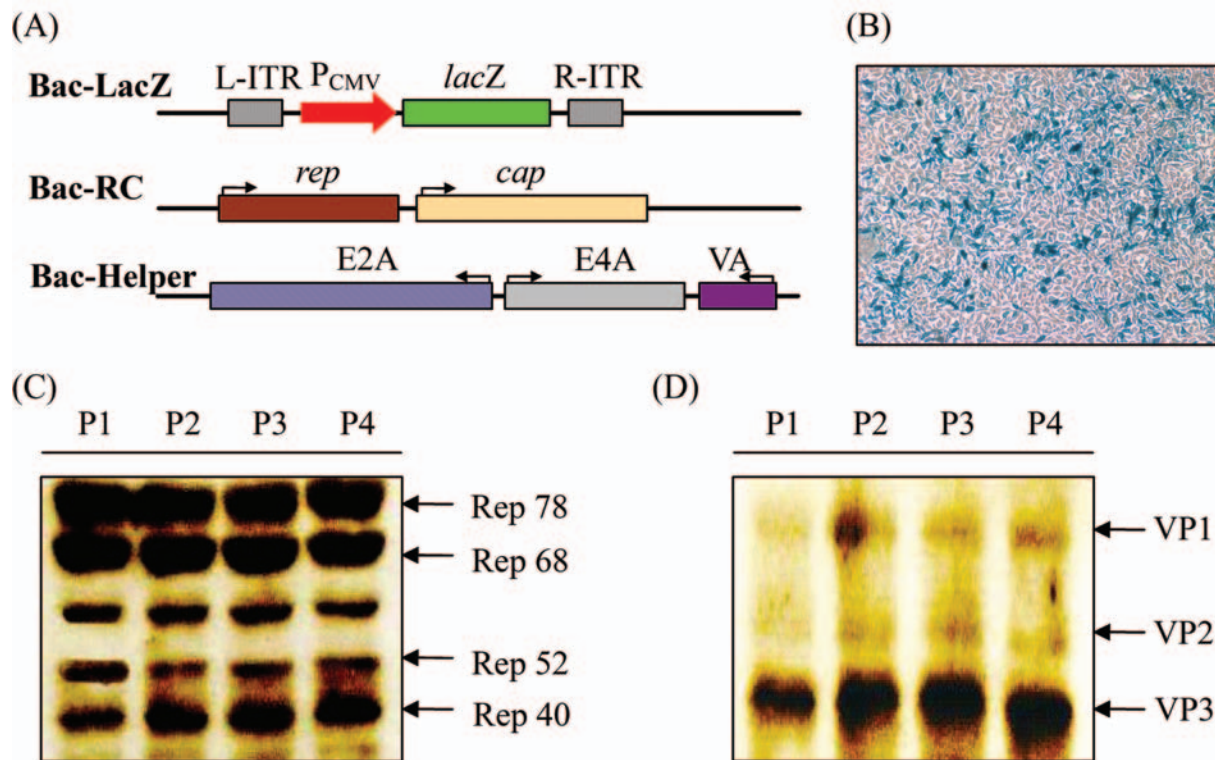
All data were analyzed by independent samples *t* tests and are expressed as mean values of three independent experiments. *p* values less than 0.05 were considered significant.

## RESULTS

#### Recombinant baculovirus construction and confirmation of protein expression

Three recombinant baculoviruses were constructed in this study (Fig. 2A). Bac-LacZ carries the vector genome, composed of the CMV promoter-driven *lacZ* gene flanked by AAV ITRs. Bac-RC harbors the AAV-2 *cap* and *rep* genes for capsid production and genome replication. Bac-Helper harbors the Ad E2A, E4, and VA RNA genes to provide helper functions. Bac-RC contains no polyhedrin promoter, so that the *rep* and *cap* genes are expressed under the control of their endogenous promoters. The construction of Bac-LacZ was verified by transducing HEK-293 cells at an MOI of 10 and detecting  $\beta$ -Gal expression by X-Gal staining at 1 day posttransduction. The significant fraction of cells that stained blue (Fig. 2B) indicated the correct expression of CMV-driven  $\beta$ -Gal by Bac-LacZ.

It was previously shown that the baculoviral genome carrying the *rep* gene was genetically unstable and that the expression of Rep proteins rapidly diminished on baculovirus passaging (Aucoin *et al.*, 2006). To confirm the correct construction and genome stability of Bac-RC, HEK-293 cells were transduced with Bac-RC of different passages (up to P4) and analyzed by Western blot 2 days posttransduction. Figure 2C and D shows that Bac-RC transduction led to the expression of Rep78, Rep68, Rep52, and Rep40 as well as the essential capsid proteins VP1, VP2, and VP3, thus confirming the function of Bac-RC virus. Furthermore, the expression levels did not decrease with virus passage, suggesting that the AAV-associated gene cassette was not appreciably truncated on baculovirus passaging, at least within four passages. The construction of Bac-



**FIG. 2.** Confirmation of recombinant baculovirus construction and protein expression. (A) Schematic illustration of the genes harbored by Bac-LacZ, Bac-RC, and Bac-Helper. (B) Detection of Bac-LacZ-mediated  $\beta$ -Gal expression by X-Gal staining. (C) Detection of Rep protein expression. (D) Detection of Cap protein expression. HEK-293 cells were transduced with Bac-LacZ (MOI of 10) in 10-cm dishes cells and stained with X-Gal 1 day posttransduction. Alternatively, HEK-293 cells were transduced with Bac-RC of various passages (P1, P2, P3, or P4) at an MOI of 15 and subjected to Western blotting at 2 days posttransduction, using anti-Rep or anti-Cap antibodies. The membranes were developed by chemiluminescence.

Helper was not verified at this stage because of the lack of proper assays.

#### rAAV production by baculovirus cotransduction

To confirm the usefulness of baculovirus for rAAV production, HEK-293 cells were either mock transduced, singly transduced with Bac-LacZ (MOI of 10), or cotransduced with Bac-LacZ, Bac-RC, and Bac-Helper (MOI of 5 for each virus). Transduced cell lysates were purified by CsCl gradient centrifugation and purified samples were used to transduce HT-1080 cells. Figure 3A and B reveals negative X-Gal staining of cells incubated with samples purified from mock-transduced (Fig. 3A) and Bac-LacZ-transduced (Fig. 3B) cell lysates, indicating that mock transduction or Bac-LacZ transduction did not result in false positives in the HT-1080 transduction experiments. In contrast, samples purified from cotransduced cells resulted in a high proportion of HT-1080 cells staining blue (Fig. 3C), confirming the presence of the *lacZ* gene in the purified samples and hence the production of rAAV vector by baculovirus triple transduction. This experiment also indirectly confirmed the functionality of Bac-Helper virus.

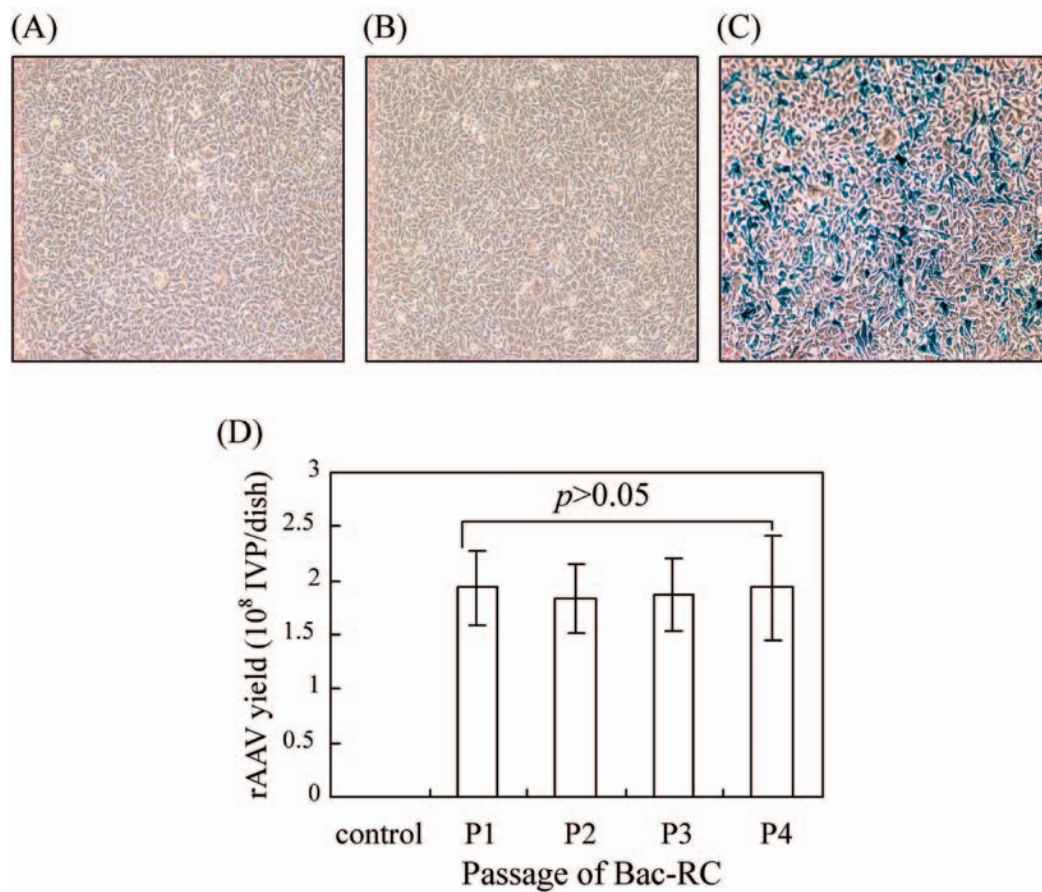
To evaluate whether passaging Bac-RC affected the rAAV yield, HEK-293 cells were transduced in 10-cm dishes ( $5 \times 10^6$  cells per dish) with Bac-LacZ (MOI of 20), Bac-Helper (MOI

of 5), and Bac-RC of different passages (MOI of 20). rAAV samples were purified as described above and the yield was determined by titration. Figure 3D reveals that the rAAV yields resulting from Bac-RC of various passages were statistically similar ( $1.8\text{--}1.9 \times 10^8$  IVP/dish;  $p > 0.05$ ) and confirms that Bac-RC virus passage (up to P4) did not affect the subsequent rAAV yield.

#### Improvement of baculovirus-mediated rAAV production

Because the Rep expression level is crucial to rAAV yield (Li *et al.*, 1997), and the baculovirus dose profoundly influences the level of expression of this protein (Chen *et al.*, 2005), we sought to manipulate the baculovirus dose to improve rAAV yield. To this end, HEK-293 cells were cotransduced in 10-cm dishes ( $5 \times 10^6$  cells per dish) with various relative doses of Bac-LacZ and Bac-RC. To simplify the design of the experiment, the Bac-Helper dose (MOI of 5) and total baculovirus dose (MOI of 45) were fixed.

Both the qPCR (Fig. 4A) and titration (Fig. 4B) data measured 3 days posttransduction indicate that rAAV yield increased with elevated Bac-RC dose. In comparison with a dose ratio of 1:1, the maximal rAAV yield resulting from a dose ratio of 1:6 was 4.2-fold higher in terms of vector genome ( $\approx 1.3 \times 10^{11}$  VG/dish) and 2.6-fold higher in terms of titer



**FIG. 3.** Confirmation of rAAV production by baculovirus cotransduction. HT-1080 cells were transduced with samples purified from HEK-293 cells that were either (A) mock transduced, (B) singly transduced with Bac-LacZ (MOI of 10), or (C) cotransduced with Bac-LacZ, Bac-RC, and Bac-Helper (MOI of 5 for each virus). All HEK-293 cells were transduced in 10-cm dishes, harvested and lysed 4 days posttransduction, and subjected to CsCl gradient centrifugation. Fractions collected from the cotransduced samples were analyzed by PCR to locate rAAV-positive fractions. For mock-transduced and singly transduced samples, fractions were collected at the identical location. The fractions were pelleted, resuspended in 3.5 ml of PBS, and used to transduce HT-1080 cells. The HT-1080 cells were subjected to X-Gal staining 2 days posttransduction. Original magnification,  $\times 100$ . (D) Variation of rAAV yield with Bac-RC passage. HEK-293 cells were transduced in 10-cm dishes ( $5 \times 10^6$  cells per dish) with Bac-LacZ (MOI of 20), Bac-Helper (MOI of 5), and Bac-RC of various passages (MOI of 20). rAAV samples were purified as described above and the yield was determined by titration. Data represent means  $\pm$  SD of three independent experiments.

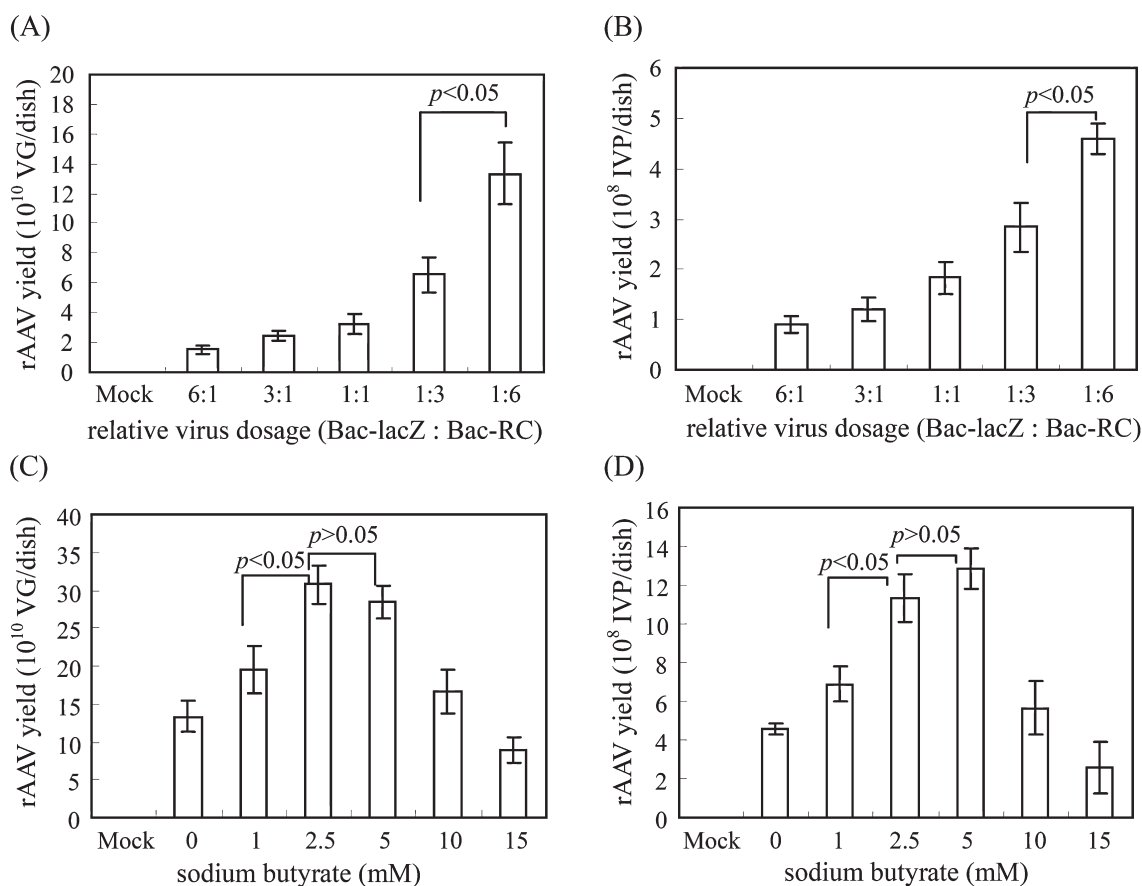
( $\approx 4.6 \times 10^8$  IVP/dish). These data underscored the significance of relative expression levels and indicated that a higher dose of Bac-RC relative to Bac-LacZ favored rAAV production.

To further elevate rAAV yield, HEK-293 cells were cotransduced under the best conditions identified above and then cultured with DMEM containing various concentrations of sodium butyrate, a histone deacetylase inhibitor that enhances baculovirus-mediated expression in mammalian cells (Condreay *et al.*, 1999; Chiang *et al.*, 2006). Both the qPCR (Fig. 4C) and titration (Fig. 4D) data revealed that rAAV yield increased with ascending butyrate concentrations, reaching a plateau and then declining, probably because of the elevated cytotoxicity imparted by butyrate (Hu *et al.*, 2003). In comparison with 0 mM butyrate, 2.5 mM butyrate resulted in a  $\approx 1.3$ -fold increase in vector genome number ( $\approx 3.1 \times 10^{11}$  VG/dish, corresponding to  $\approx 6.2 \times 10^3$  VG/cell) whereas 5 mM butyrate gave rise to a  $\approx 2.0$ -fold increase in biologically active particles ( $\approx 1.4 \times 10^9$

IVP/dish or  $\approx 280$  IVP/cell). Because the rAAV yields obtained at 2.5 and 5 mM were statistically similar ( $p > 0.05$ ), 2.5 mM butyrate was used in subsequent experiments.

#### Culture of HEK-293 cells and rAAV production in the BelloCell-500-AP

To transfer the production process to BelloCell reactors, we first evaluated HEK-293 cell growth in the BelloCell-500. Unfortunately, unlike BHK cells, which adhered well to the carriers, HEK-293 cells attached poorly to the carriers, thus leading to settlement of cell clumps at the reactor bottom and inferior cell growth (our unpublished observations). On the other hand, the BelloCell-500-AP resulted in a shorter lag phase ( $< 1$  day), faster cell growth, and a higher cell yield ( $\approx 3.4 \times 10^9$  cells per reactor) as delineated in Fig. 5A (solid squares). The improvement was attributed to the perfusion culture and inversion of



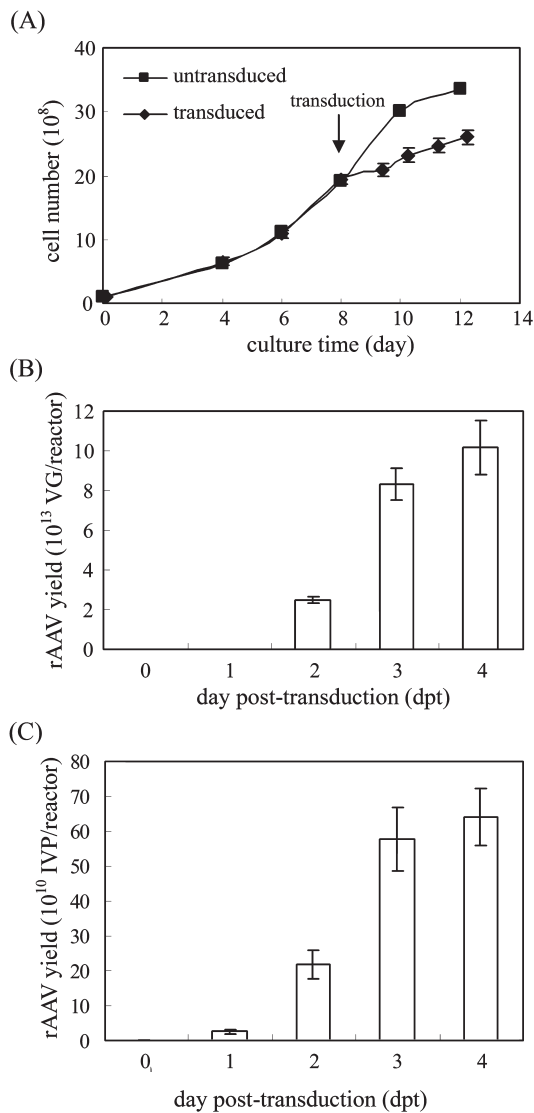
**FIG. 4.** Improvement of rAAV yield. (A and B) Variation of vector genome (VG) copy number and infectious viral particles per dish with relative doses of virus (Bac-LacZ:Bac-RC). HEK-293 cells were transduced in 10-cm dishes ( $5 \times 10^6$  cells per dish) at a given Bac-Helper dose (MOI of 5) and total baculovirus dose (MOI of 45). Mock, mock transduction. (C and D) Variation of vector genome (VG) copy number and infectious viral particles per dish with sodium butyrate concentration. HEK-293 cells were transduced in 10-cm dishes ( $5 \times 10^6$  cells per dish) with Bac-LacZ (MOI of 6), Bac-RC (MOI of 36), and Bac-Helper (MOI of 5). All rAAV samples were harvested 3 days posttransduction and purified by CsCl gradient. All data represent means  $\pm$  SD of three independent experiments.

the BelloCell-500-AP on seeding, which allowed for the settlement of carriers to the lid and neck of the reactor and substantially augmented HEK-293 cell attachment to the carriers ( $\approx 89\%$  attachment rate; data not shown). Therefore, HEK-293 cells were cultured and cotransduced with Bac-LacZ (MOI of 6), Bac-RC (MOI of 35), and Bac-Helper (MOI of 5) when the cell number reached  $2.0 \times 10^9$  per reactor, and then cultured with DMEM containing 2.5 mM butyrate. Figure 5A (solid diamonds) shows that transduced cells remained capable of growth in the reactor, although at a slower rate, which was ascribed to the shift of metabolic activities from growth to protein overexpression (Chen *et al.*, 2005). It should be noted that during transduction  $\text{NaHCO}_3$ -deficient DMEM was used as the surrounding solution in lieu of PBS (Chen *et al.*, 2005) because PBS resulted in detachment of HEK-293 cells (our unpublished observation). Replacement of PBS with  $\text{NaHCO}_3$ -deficient DMEM considerably alleviated the cell detachment problem while maintained the high transduction efficiency (Shen *et al.*, 2007).

During the rAAV production phase,  $\approx 1 \times 10^8$  cells were collected from the reactor daily and the samples were purified by CsCl gradient centrifugation. qPCR (Fig. 5B) and virus titration (Fig. 5C) analyses of the purified rAAV samples confirmed that the yield increased with time but that the production rate slowed by 3 days posttransduction as the yield on days 3 and 4 was statistically similar ( $p > 0.05$ ). The maximal specific yield at 4 days posttransduction reached  $\approx 3.8 \times 10^4$  VG/cell and  $\approx 247$  IVP/cell, respectively. These yields corresponded to  $\approx 1 \times 10^{14}$  VG (Fig. 5B) and  $\approx 6.4 \times 10^{11}$  IVP (Fig. 5C) per reactor run.

#### Detection of residual baculovirus

Purified rAAV particles were further analyzed to detect residual baculovirus. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 6A) revealed the presence only of VP1, VP2, and VP3 regardless of the sampling timing, and Western blot analyses (Fig. 6B) detected no baculovirus-



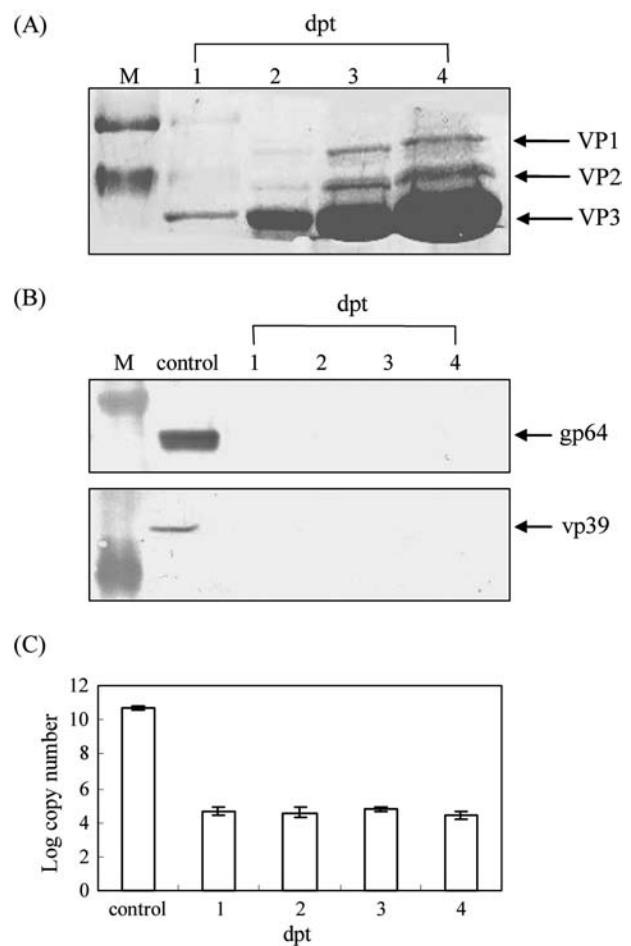
**FIG. 5.** Culture of HEK-293 cells and rAAV production in the BelloCell-500-AP. **(A)** Growth curves of transduced and untransduced cells. **(B)** Variation of rAAV yield with time as measured by qPCR. **(C)** Variation of rAAV yield with time as measured by virus titration. Approximately  $1 \times 10^8$  HEK-293 cells were seeded in the BelloCell-500-AP and the reactor was inverted during the seeding period. During culture, six BioNOC II carriers were taken daily from the top, middle, and bottom layers of the bed and evenly distributed to three microcentrifuge tubes to measure cell numbers by the crystal violet dye method. Cell numbers calculated from the three tubes were averaged and converted to the total cell number in the reactor. The cells were either untransduced or transduced with Bac-LacZ (MOI of 6), Bac-RC (MOI of 35), and Bac-Helper (MOI of 5) when the cell number reached  $2.0 \times 10^9$ , and then cultured with DMEM containing 2.5 mM butyrate. rAAV particles were purified from  $\approx 1 \times 10^8$  cells. Data represent means  $\pm$  SD of three independent experiments.

specific gp64 and vp39 proteins in the purified samples, thus indicating highly pure rAAV samples. Meanwhile, the qPCR analysis (Fig. 6C) revealed a low amount of baculoviral DNA in the purified sample ( $>10,000$ -fold less) than in the control

(the unconcentrated baculoviral stock). These data collectively eased concerns regarding baculoviral contamination of purified rAAV preparations.

## DISCUSSION

Low plasmid transfection efficiency and difficulty in process scale-up have impeded the large-scale production of rAAV vectors. To address the problems of inefficient plasmid transfection, baculovirus has been employed to deliver the ITR-flanking cassette and AAV *rep* gene into HEK-293 cells (Sollerbrant *et al.*, 2001), which leads to rAAV production with a yield approaching  $1 \times 10^6$  IVP/ml. This method, however, requires Ad coinfection, which complicates subsequent purification and is yet to be scaled up. Alternatively, a new rAAV production method based on a bac-



**FIG. 6.** Detection of residual baculovirus in purified rAAV samples. **(A)** SDS-PAGE analysis. **(B)** Western blot analysis using anti-gp64 and anti-vp39 antibodies. **(C)** qPCR analysis. At 1, 2, 3, and 4 days posttransduction, rAAV particles were extracted from  $\approx 1 \times 10^8$  cells and purified by CsCl gradient as in Fig. 5. Bands in the Western blot and SDS-PAGE assays were visualized with BCIP/NBT and Coomassie blue, respectively. Baculoviral stock was analyzed as the control. Data are representative of three independent reactor runs. M, molecular weight marker.



ulovirus expression vector–insect cell system (BEVS) has been developed (Urabe *et al.*, 2002), in which the genes encoding Rep and Cap and the AAV ITR genomes are carried by three separate baculoviruses. Coinfection of insect cells (Sf-9) with these three baculoviruses yields high-titer rAAV vectors.

In this study, we adopted a different approach by exploiting baculovirus-mediated gene delivery into mammalian cells and developed a triple transduction system. Baculovirus-mediated transduction of HEK-293 cells was efficient and able to mediate the expression of Rep and Cap proteins for rAAV production (Figs. 2 and 3), indicating that despite the large baculoviral DNA ( $\approx 130$  kb) the genes required for rAAV production could be expressed and the vector genome (the ITR-flanking expression cassette) harbored by baculovirus could be processed, replicated, and packaged into rAAV particles. Intriguingly, the highest yield was obtained when the Bac-LacZ:Bac-RC dose ratio was 1:6 (Fig. 4A and B), which was inconsistent with the suggested molar ratio (1:1) for pAAV-LacZ and pAAV-RC in the AAV helper-free system. The discrepancy might stem from the large baculoviral genome relative to the plasmid DNA. Because baculovirus does not replicate within HEK-293 cells, the large Bac-RC genome might make it more difficult to uncoil for *rep* and *cap* transcription. In addition, the large Bac-LacZ genome might impose steric hindrance that impeded the Rep proteins from accessing the ITR-flanking cassette for rAAV genome replication, thus leading to the requirement for a higher Rep expression level. This hypothesis is at least partly supported by the fact that the rAAV yield was significantly enhanced by sodium butyrate, which relaxes the chromatin structure (Fig. 4C and D).

Furthermore, baculovirus-mediated rAAV production was readily scaled up to the BelloCell-500-AP. Unlike BHK cells, which grew well in the BelloCell-500 (Chen *et al.*, 2005), HEK-293 cells attached to the carriers loosely, thus giving rise to additional difficulty in cell culture. Nonetheless, by adjusting the seeding and transduction process, in conjugation with the perfusion operation, we were able to improve the growth characteristics and obtained high cell yield ( $3.4 \times 10^9$  cells) in the BelloCell-500-AP (Fig. 5A), which corresponded to a volumetric yield of  $6.8 \times 10^6$  cells per milliliter of culture and was equivalent to the cell yield obtainable in  $\approx 170$  150-cm<sup>2</sup> flasks (assuming  $1.5 \times 10^7$  cells per flask). With the improved culture and transduction conditions, the specific rAAV yield amounted to  $3.8 \times 10^4$  VG/cell, or 247 IVP/cell, which corresponded to  $\approx 1 \times 10^{14}$  VG or  $6.4 \times 10^{11}$  IVP per reactor run (Fig. 5B and C). In comparison with the common production systems relying on plasmid transfection, this system gave comparable or superior specific yield and also allowed for high-density culture on a larger scale, thus enhancing the volumetric yield and reducing the cost. One BelloStage accommodates up to four BelloCell-500-AP reactors, thus allowing for straight-forward process scale-up. Furthermore, the BelloCell-500-AP is disposable and easy to operate, thus obviating sterilization and requiring minimal personnel training.

Compared with the new BEVS system that results in high specific yield (253 IVP/cell or  $2.1 \times 10^5$  VG/cell) (Meghrouh *et al.*, 2005), this system also gave similar specific yield in terms of infectious viral particles. However, baculovirus replicates in insect cells and could complicate rAAV purification should the BEVS be used for production. In contrast, in this system bac-

uloviral DNA degrades, rather than replicates, within the mammalian cells over time (Ho *et al.*, 2004; Wang *et al.*, 2005), thus it is unlikely that baculoviral proteins and DNA persist abundantly in the rAAV preparations after extensive purification. This notion is confirmed by the trace amounts of baculoviral proteins (Fig. 6B) and DNA (Fig. 6C) in the purified rAAV samples, and eases concerns that baculovirus used for transduction would contaminate the final rAAV preparations.

In summary, we have developed a novel process for rAAV production based on baculovirus-mediated transduction of HEK-293 cells. The rAAV yield is significantly elevated by adjusting the baculovirus dose and adding sodium butyrate, and, when combined with the BelloCell-500-AP reactor, amounts to  $1 \times 10^{14}$  VG and  $\approx 6.4 \times 10^{11}$  IVP. The yield is comparable to the yields obtained by other methods, and may be further improved by optimizing the MOI of the three baculoviruses, the seeding and transduction conditions, and the bioreactor process parameters. The transduction process can be performed in biosafety level 1 facilities because baculovirus is nonpathogenic to humans, and eliminates the need for expensive transfection reagents and time-consuming baculovirus ultracentrifugation. Therefore, the combination of baculovirus-mediated transduction and BelloCell-500-AP culture provides a novel and economically viable approach for rAAV production.

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## AUTHOR DISCLOSURE STATEMENT

The authors declare no competing interests.

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