Review

Transient Transfection Methods for Clinical Adeno-Associated Viral Vector Production

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Abstract

Recombinant adeno-associated virus (AAV)-based vectors expressing therapeutic gene products have shown great potential for human gene therapy. One major challenge for translation of promising research to clinical development is the manufacture of sufficient quantities of AAV vectors that meet stringent standards for purity, potency, and safety required for human parenteral administration. Several methods have been developed to generate recombinant AAV in cell culture, each offering distinct advantages. Transient transfection-based methods for vector production are reviewed here, with a focus on specific considerations for development of AAV vectors as clinical products.

Introduction

GENE TRANSFER VECTORS based on adeno-associated virus (AAV) have demonstrated significant promise for human gene therapy, based on their excellent safety profile and ability to achieve long-term efficacy in animal models. Several clinical studies have been initiated, and some exciting early results have been reported (Carter, 2005; Warrington and Herzog, 2006; Fiandaca *et al.*, 2008; Maguire *et al.*, 2008). A member of the *Dependovirus* genus of the family *Parvoviridae*, AAV is a nonenveloped icosahedral virus particle about 26 nm in diameter and composed of a single-stranded 4680-nucleotide DNA genome encoding replication (*rep*) and encapsidation (*cap*) genes flanked by inverted terminal repeats (Berns and Parrish, 2007). AAV-based gene transfer vectors are generated by utilizing the capsid protein shell of the wild-type virus, but replacing the genome of the natural virus with a therapeutic transgene cassette, retaining only the viral inverted terminal repeats (ITRs). Effective clinical AAV vector manufacturing technologies are required to support clinical product development and eventual product licensure and commercialization. Critical aspects of vector product development include the need to establish manufacturing methods that ensure (1) consistently high purity, potency, and safety characteristics of the product; and (2) acceptable costs associated with large-scale manufacturing to meet the needs of clinical applications.

The first step in the clinical vector manufacturing process involves vector generation in cell culture. Vector generation typically requires a production cell line that provides the basic biosynthetic machinery, as well as additional genes that must be introduced to provide the full complement of gene products needed to direct vector generation. These additional genes include the vector genome, that is, the therapeutic transgene of interest and associated regulatory elements flanked by AAV ITRs, AAV *rep* and *cap* provided in *trans*, and helper virus genes, prototypically adenovirus *E1*, *E2a*, and *E4*, required to support vector genome replication and packaging. Various methods have been developed to introduce the vector genome template and requisite helper genes into production cells. These methods include transfection and/or infection of human cell lines such as HEK-293 and HeLa (Clark *et al.*, 1995, 1999; Conway *et al.*, 1999; Grimm *et al.*, 1998; Matsushita *et al.*, 1998; Xiao *et al.*, 1998) or more recently by infection of insect cells using recombinant baculoviruses (Urabe *et al.*, 2002; Negrete *et al.*, 2007). Each of these approaches provides certain advantages and limitations. Transient transfection provides great flexibility and speed, a significant benefit for the early stages of investigational product development. In contrast, development of stable transfected cell lines expressing a subset of the requisite genes, with additional genes provided by an infection process, is more complex and time-consuming, but ultimately provides better scalability and cost-effectiveness. The progression of a given AAV vector

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product candidate from exploratory research through clinical trials as an investigational new drug, to eventual licensure and commercialization, ideally requires a parallel vector production technology initially providing speed and flexibility, but eventually providing optimal cost-effectiveness and capacity. Several reviews provide an overview of various methods that have been developed for AAV vector production (Salveti *et al.*, 1998; Clark, 2002; Snyder and Flotte, 2002; Zolotukhin, 2005). The current review focuses on transient transfection-based methods for the generation of clinical-grade AAV vectors that provide maximal flexibility and increasingly promising potential for large-scale application.

Methods of Transfection

Expression of complex biologics such as licensed therapeutic monoclonal antibodies is typically performed in recombinant mammalian cell lines in which copies of the DNA encoding the protein of interest have been stably introduced and can be expressed for extended periods. The establishment of highly optimized producer cell lines for each candidate product is costly and time-consuming, but experience validates this approach as one of the best systems to generate recombinant products at commercial scale. However, for investigational new drug candidates at preclinical and early-phase clinical development, the flexibility of a system in which early-stage product candidates can be assessed quickly is a desirable feature. Transient gene expression of different candidate products in a single characterized production cell line, using easily modified plasmid DNA encoding the gene of interest, provides great flexibility and speed at early stages of product development (reviewed in Baldi *et al.*, 2007). Such flexibility is especially relevant to the generation of recombinant AAV. Producer cell line development for AAV vector products is complex because some of the genes required for vector biosynthesis, including AAV *rep* and some requisite helper virus genes, are cytotoxic. Transient transfection using a single characterized production cell line combined with multiple plasmids provides the ability to assess and optimize transgene regulatory elements and capsid serotypes. Although numerous chemical and physical methods have been developed to transfer DNA into culture mammalian cells for transient gene expression and production of recombinant products, three transfection methods most relevant to large-scale production are DNA coprecipitation with calcium phosphate, the use of polycations such as polyethylenimine (PEI), or cationic lipids.

Calcium phosphate coprecipitation

Coprecipitation of plasmid DNA with calcium phosphate, initially described by Graham and van der Eb (1973), is a well-established and simple method to achieve exogenous DNA transfer and expression in mammalian cells. When performed using carefully optimized procedures (Jordan *et al.*, 1996), calcium phosphate-mediated transfection enables transfer of DNA into up to 90% of HEK-293 cells (Meissner *et al.*, 2001). For the generation of recombinant AAV, the conditions and procedures used must be carefully optimized and consistently performed to achieve reliably high specific productivity. Efficient calcium phosphate-mediated transfection generally requires the presence of serum, a feature not recommended for late-stage investigational products. Protocols for transient transfection of adherent HEK-293 cells using calcium phos-

phate have been optimized in our laboratory over the course of several years to meet the demand of providing various recombinant AAVs for large-animal preclinical studies and early-phase clinical studies. We have found that this method is able to provide productivity exceeding 100,000 vector genomes (VG) per cell in HEK-293 cells, but this high productivity is critically dependent on HEK-293 cell quality at the time of transfection, and on characteristics of the transfection cocktail. Failure to generate and maintain the production cells in optimally "receptive" conditions can result in a dramatic (>10-fold) decrease in specific productivity. Regarding the preparation and use of the transfection cocktail, DNA-calcium phosphate precipitate formation is complex and acutely dependent on several variables including the concentrations of each reagent, the pH of the solution, the temperature, and the order of reagent addition. For example, a fine precipitate is optimal for transfection, and the efficiency of DNA precipitate uptake by cells decreases rapidly with increasing size of precipitate particles. Our laboratory has observed that relatively small excursions (e.g., >0.05 pH units) of transfection cocktail pH from the optimal range (pH 7.05–7.15) can dramatically affect precipitate size and lower transfection efficiency. Precipitate size also changes progressively from optimal to suboptimal over a relatively short period of time after its preparation, and therefore care must be taken to define and consistently implement an appropriate time frame for its addition to cells, a challenge particularly for large-scale transfections.

Polyethylenimine coprecipitation

Use of the cationic polymer polyethylenimine (PEI) as a vector for gene and oligonucleotide transfer into cells in culture was first described by Boussif and colleagues (1995). In particular, 25-kDa PEI is well characterized and has been widely used to achieve efficient recombinant protein expression. Polyethylenimine undergoes ionic interactions with DNA, forming polyplexes that can be taken up by cells, traffic through endosomes and cytoplasm, and finally reach the nucleus through an incompletely understood mechanism (Merdan *et al.*, 2002). In contrast to the calcium phosphate method, PEI can mediate efficient DNA transfection to mammalian cells grown in suspension in serum-free medium, features that enhance process scalability and the safety of biologic products generated by this approach. However, PEI is nonbiodegradable and moderately cytotoxic. This method has been used to achieve transient expression of a variety of recombinant proteins (reviewed in Baldi *et al.*, 2007), and more recently to generate recombinant AAV. Drittanti and colleagues (2001) reported the production of 9000–12,000 VG-containing particles per cell, using 25-kDa PEI. Park and colleagues (2006) adapted HEK-293 cells into a serum-containing suspension culture, and using linear PEI obtained transfection efficiencies ranging from 54 to 99%, generating up to 10^{13} AAV vectors per 2-liter bioreactor. Durocher and colleagues (2007) reported production of AAV2 vectors at levels up to $\sim 10^{14}$ VG per 3.5-liter bioreactor by PEI-mediated transfection of suspension HEK-293 cells under serum-free conditions without the need for exchange of the cell culture medium. Hildinger and colleagues (2007) reported AAV2 and AAV5 vector titers ranging from 0.3×10^{13} to 1.6×10^{13} VG/liter in serum-free suspension-adapted HEK-293 cells, using PEI-mediated transfection, titers that were comparable to those

obtained by calcium phosphate-mediated transfection of adherent HEK-293 cells performed in parallel.

Cationic lipids

Transfection mediated by cationic liposomes was reported as a highly efficient DNA transfection procedure by Felgner and colleagues (1987). Although cationic lipids can achieve efficient gene transfer in suspension cultures, the cost of this class of reagents for large-scale applications remains a challenge. Liu and colleagues (2008) described the use of cationic lipids to achieve transient transfection of HEK-293 cells or Chinese hamster ovary (CHO) cells adapted to suspension, serum-free culture conditions, resulting in 50–80 mg of IgG per liter, although the volumetric productivity of recombinant human coagulation factor IX and erythropoietin was lower. Schlaeger and colleagues (2003) reported expression of secreted human placental alkaline phosphatase (SEAP) as up to 20 mg/liter after cationic liposome (Lipofectamine 2000)-mediated transfection of HEK-293EBNA cells grown in serum-free suspension culture. Although limited data are available for recombinant AAV production (Reed *et al.*, 2006), this approach appears to be comparable to PEI in terms of efficiency and utility for transfection of suspension, serum-free cultures, but remains cost-prohibitive for large-scale use.

Balancing Early-Stage Process Flexibility and Scalability

Although process scalability and large-scale cost-effectiveness are critical issues for successful commercial product development, maintaining process flexibility is an important objective at early stages. Investigational new gene therapy products can be expected to take 10 years or more to move from being a preclinical candidate to a licensed product. A strong emphasis on manufacturing process scalability early in investigational product development, although minimizing the need for process changes during the product development process, may significantly compromise the ability to evolve and incorporate the latest best practices in a rapidly evolving field. With new technologies continuously emerging, such as more efficient vector generation methods with the potential to enhance vector product quality and manufacturing scalability, changes in manufacturing processes should be anticipated and managed from the perspective of early-stage AAV vector product development to take advantage of these advances. It is arguably best to wait as long as possible before “locking in” the vector production process to enable incorporation of state-of-the-art technology into commercial-scale manufacturing processes. For example, vector production to support preclinical and early-stage clinical product development can reasonably be performed by transient transfection. Once a specific transgene construct and serotype have been validated as a candidate for later stage clinical product development, and it is determined that the early-stage methodology is not cost-effective with respect to anticipated clinical needs, the production system can be modified and optimized to achieve higher capacity. A critical prerequisite to ensure that clinical manufacturing changes do not adversely affect product quality and consistency during clinical development is the establishment and validation of a broad repertoire of product characterization assays to rigorously evaluate product comparability (Center for Biologics

Evaluation and Research, 2000, 2003). When process changes are implemented, comparison studies of representative pre- and post-change vectors must be rigorously performed and documented to ensure that safety and efficacy results observed at one phase of clinical development are valid for a subsequent phase.

Optimizing Recombinant AAV Productivity

An important goal for AAV vector production is to achieve consistent, high vector productivity. It has often been a challenge to generate sufficient amounts of this complex biologic to support large-animal studies and early-phase clinical studies. Measured as vector genomes generated per cell, AAV vector specific productivity (defined here as the total amount of the recombinant product generated) using transient transfection can be highly variable, ranging from $\sim 10^3$ to $\sim 10^5$ VG/cell, corresponding to approximately 0.006 to 0.6 pg of vector per production cell. A comparison of productivity levels achieved by transient transfection for rAAV with levels reported for other recombinant products is potentially instructive to formulate objectives for AAV vector production. Representative data for specific productivity for rAAV and selected recombinant proteins are shown in Table 1. In comparison, specific productivity ranged from 1.3 to 150 pg/cell for recombinant proteins, suggesting an ~ 100 -fold specific productivity “gap” between rAAV and other recombinant products generated by transient transfection. Part of this productivity gap is certainly due to the unique requirement during rAAV biosynthesis for concurrent synthesis of helper products that are absorbing a portion of production cell biosynthetic capacity, and the cellular toxicity of some helper gene products. A second part of the productivity gap can be attributed to the known inefficiency of packaging of vector genome DNA into preformed capsids. Grimm and colleagues (1999) first reported that only a small percentage (1.7–20%) of the AAV particles generated in cell culture by transient transfection actually contain vector genomes, the majority corresponding to empty capsids. This result has been confirmed by various groups using transfection- or infection-based production methods, as described later. Although optimization of production plasmid design, including attenuation of large Rep protein expression to increase recombinant AAV production, has been reported (Li *et al.*, 1997; Vincent *et al.*, 1997; Grimm *et al.*, 1998; Ogasawara *et al.*, 1998), further improvement in packaging efficiency is needed. Identification of molecular features required for efficient packaging, and modification of production plasmids and culture conditions accordingly to insert additional vector genomes into the more abundantly produced empty capsids, could enhance specific productivity severalfold. A third component of the productivity gap is likely attributable to the fact that recombinant proteins such as monoclonal antibodies have been subjected to intense cell culture process development and optimization to define improved media, nutrients, and conditions (Pham *et al.*, 2005; Backliwal *et al.*, 2008a,b). Additional efforts using these approaches may substantially improve AAV specific productivity in cell culture. By better utilizing the full production cell biosynthetic capacity and improving vector genome packaging efficiency, a suggested productivity goal for recombinant AAV produced by transient transfection is 10^6 VG/cell, an approximately 10-fold increase above best reported current

TABLE 1. YIELDS OF RECOMBINANT ADENO-ASSOCIATED VIRUS AND OTHER RECOMBINANT PRODUCTS, USING TRANSIENT TRANSFECTION OF HEK-293 CELLS

Product	Transfection reagent	Culture format	Yield			Ref.
			mg/liter	VG/liter	pg/cell (posttransfection)	
IgG	CaPi	Suspension	5	—	2.5 (10 days)	Girard <i>et al.</i> (2002)
SEAP	PEI	Suspension	20	—	10 (4 days)	Durocher <i>et al.</i> (2002)
SEAP	Liposome	Suspension, SF	15–20	—	10 (4 days)	Schlaeger <i>et al.</i> (2003)
Various recombinant proteins	CaPi	Suspension	2.5–28	—	1.3–14 (5–7 days)	Baldi <i>et al.</i> (2005)
	PEI	Suspension, SF	2.6–27	—	1.3–14 (6–7 days)	
IgG	Liposome	Suspension	50–80	—	25 (4 days)	Liu <i>et al.</i> (2008)
IgG	PEI	Suspension	300	—	150 (10 days)	Backliwal <i>et al.</i> (2008b)
rAAV	CaPi	Adherent	—	—	~0.09 (2 days)	Drittani <i>et al.</i> (2001)
	PEI	Adherent	—	—	0.09 (2 days)	
rAAV	PEI	Suspension	—	5×10^{12}	~0.02 (2–3 days)	Park <i>et al.</i> (2006)
rAAV	PEI	Suspension, SF	—	2.6×10^{13}	~0.1 (3–4 days)	Durocher <i>et al.</i> (2007)
rAAV	CaPi	Adherent	—	9×10^{13}	0.8 (4 days)	Hauck <i>et al.</i> (2007)
rAAV	CaPi	Adherent	—	$0.5\text{--}1.9 \times 10^{13}$	0.05–0.17 (2 days)	Hildinger <i>et al.</i> (2007)
	PEI	Suspension, SF	—	$0.3\text{--}0.6 \times 10^{13}$	0.02–0.07 (2 days)	

Abbreviations: CaPi, calcium phosphate; PEI, polyethylenimine; rAAV, recombinant adeno-associated virus; SEAP, secreted human placental alkaline phosphatase; SF, serum free.

levels (Hauck *et al.*, 2007). In addition to the obvious benefit of cost-effectiveness, an important advantage of higher productivity is the reduced burden on purification when the starting material (crude vector harvest) has a higher ratio of the vector product to total harvest biomass.

GMP Manufacturing Considerations

Clinical AAV vector manufacturing requires knowledge of the complex methods required to generate, purify, and characterize AAV vectors, combined with implementation of current Good Manufacturing Practice (GMP) (U.S. Department of Health and Human Services, 2006a,b). At the early stages of biologic product development, U.S. Food and Drug Administration (FDA) draft guidance indicates that an incremental approach to manufacturing controls be taken in a stage-specific manner during drug development, with full GMP compliance at licensure (Center for Biologics Evaluation and Research, 2006). Biological components and raw materials used to support vector generation in cell culture must meet rigorous specifications. Raw materials that are animal derived should be minimized or, whenever possible, avoided because they are generally incompletely defined, demonstrate significant lot-to-lot variation, and may harbor difficult-to-detect adventitious agents. If used, animal-derived products such as porcine trypsin and bovine serum should be obtained from reliable sources and extensively tested to document high levels of safety and characterization, and process development efforts should be initiated to replace them. Cell lines used for production of rAAV must have a defined history, and be generated, characterized, and stored in a manner ensuring safety, adequate supply, and consistency of performance over a period of many years. Once established, a master cell bank (MCB) of the production cell line chosen for clinical vector manufacturing is subjected to extensive characterization using validated test methods to verify identity and ensure absence of viral and microbial

contamination (Food and Drug Administration, 1998). At early stages of product development, it is not unusual to use the MCB directly for vector production. For later stage clinical trials, and once the production cell line for prospective commercial process has been defined, production of a two-tiered production cell banking system composed of the license-stage MCB and working cell bank(s) should be established to ensure that consistent cells are used over time for cGMP product manufacturing. Appropriate comparability studies must be performed to qualify changes. Analogous considerations are required for plasmids, which are critical biological components used in transient transfection-based production of clinical-grade AAV vectors. Plasmids should be produced with a system that avoids β -lactam antibiotics (e.g., ampicillin), commonly used in research settings, because of the potential sensitivity of human recipients to trace residual levels of the antibiotic (Center for Biologics Evaluation and Research, 2004). Production plasmids should be designed to help achieve the highest possible final product purity, potency, and safety, including reduction or elimination whenever possible of the potential for generation of wild-type AAV and other vector-related impurities that, once produced, are difficult to remove by purification process steps.

Vector Quality Characteristics

AAV vectors prepared for use in clinical studies must be extensively characterized, and meet predetermined specifications for vector quality characteristics pertaining to vector identity, safety, purity, potency, and stability (Center for Biologics Evaluation and Research, 2004; Gombold *et al.*, 2006). Vectors prepared for use in Investigational New Drug (IND)-supporting preclinical pharmacology/toxicology studies must be comparable to vectors prepared for use in the planned clinical study. Vector quality characteristics are a function of both upstream (vector generation in cell culture)

and downstream (vector purification) processes. Many vector quality control tests, for example, those for sterility, mycoplasma, adventitious viral agents, endotoxin, and residual host cell and culture medium constituents, represent challenges that are generally common to other biological products generated in cell culture (Center for Biologics Evaluation and Research, 2000, 2004; reviewed in Wright, 2008), and are not discussed further here. However, vector-related impurities, a class of impurities corresponding to AAV particles that closely resemble bona fide vectors, and are influenced by the method of vector generation used, are discussed in additional detail here. Vector-related impurities include (1) AAV particles that contain nucleic acid sequences other than the intended vector genome, and that are inadvertently packaged during vector production (e.g., wild-type AAV, encapsidated residual plasmid, and host cell DNA); and (2) empty AAV capsids. The amounts of vector-related impurities generated are influenced by the nature of the biological reagents and conditions of the vector production process. Because of their close resemblance to the actual vector product, removal of vector-related impurities from bona fide vectors by subsequent scalable purification steps ranges from difficult (for empty capsids) to essentially impossible (for some encapsidated DNA impurities). If not removed, vector-related impurities add excess viral antigen and heterogeneous, potentially immunogenic or otherwise deleterious nucleic acid sequences to the clinical vector product. In contrast to most previously licensed virus-based clinical products (e.g., vaccines) that are intended to induce immune responses, investigational AAV vectors are generally intended to achieve long-term gene expression, and must avoid activation of transgene-limiting immune responses to be effective. Therefore minimizing or preventing formation of vector-related impurities during vector biosynthesis in cell culture is an important objective for vector production.

Wild-type AAV

The formation of wild-type AAV (including helper virus-dependent replication-competent pseudo-wild-type species) during vector biosynthesis can involve homologous or non-homologous (Muzyczka, 1992; Allen *et al.*, 1997; Wang *et al.*, 1998) recombination events between AAV ITRs, and *rep* and *cap* sequences present in *trans* in helper plasmids. In non-optimized systems wild-type AAV can account for a large percentage of the total particles generated (Samulski *et al.*, 1989), and cannot subsequently be separated from the vector product. Although wild-type AAV is not a known human pathogen after natural infection, as an impurity in AAV vector administered *in vivo* its potential for replication, as a result of subsequent adventitious infection with a helper virus, could lead to expression of viral antigens and immune-mediated clearance of transduced cells. Modification of production plasmids to eliminate homologous sequences (Samulski *et al.*, 1989), inactivation or replacement of the p5 promoter region implicated in deleterious recombination events (Grimm *et al.*, 1998), separation of AAV *rep* and *cap* sequences (Allen *et al.*, 1997), and engineering oversized *rep-cap* helper plasmid cassettes to reduce the efficiency of inadvertent self-packaging (Cao *et al.*, 2000) are approaches that have been reported to substantially eliminate the generation of wild-type AAV during vector production.

Other AAV-encapsidated DNA impurities

Other encapsidated DNA impurities in vector preparations can be derived from production plasmids used for transient (or stable) transfection (Committee for Medicinal Products for Human Use, 2005). After initial characterization of this type of impurity (Allen *et al.*, 1997; Wang *et al.*, 1997), Nony and colleagues (2003) reported packaged *rep-cap* sequences in the absence of inverted terminal repeats at levels up to 2% of vector genomes in AAV2 vectors, and implicated a role for a Rep-binding motif (CARE [*cis*-acting replication element]) in their generation. Chadeuf and colleagues (2005) reported encapsidated prokaryotic DNA impurities derived from production plasmids at levels ranging from 1.2 to 6.3% in recombinant AAV vectors generated by transfection of HEK-293 cells or by helper virus infection of stable producer cell lines. Gao and colleagues (2008) reported residual *cap* at levels ranging from 0.4 to 1.0% in 17 lots of recombinant AAV 2, 7, and 8, and that was transcriptionally active. Hauck and colleagues (2009) reported similar levels of packaged DNA impurities, and described approaches to reduce them. Packaging of fragments of mammalian production cell genomic DNA has been reported to range from ~1% of total DNA in highly purified vectors to ~3% in vectors that are copurified with empty capsids (Smith *et al.*, 2003), levels that, for high vector doses, are predicted to exceed regulatory guidelines developed for biological products (WHO Expert Committee on Biological Standardization, 1998). The mechanism of generation of heterogeneous encapsidated DNA impurities is not well understood, but is likely related to imperfect fidelity of the DNA-packaging mechanism mediated by the helicase function of Rep52/40 that translocates single-stranded DNA genomes into preformed AAV capsids (King *et al.*, 2001). But taken together, the available data indicate that encapsidated DNA impurities are abundant subsequent to recombinant AAV generation in cell culture, and may exceed 10% of vector genome-containing AAV particles in crude harvests, even using strategies to eliminate wild-type AAV. The risk for a candidate clinical vector product includes the possibility that encapsidated DNA impurities may contribute to expression of viral antigens such as AAV *cap* (Recombinant DNA Advisory Committee, 2007) or inadvertently provide immune adjuvant prokaryotic CpGs. Li and colleagues (2007) reported that capsid-specific cytotoxic T lymphocytes (CTLs) eliminated liver and muscle cells that endogenously expressed *cap* in cell culture and *in vivo*; however, they found that that cells transduced with AAV2 vectors were rarely eliminated by capsid-specific CTLs in a mouse model. Wang and colleagues (2007) reported that although cross-presentation of AAV2 capsid protein could activate CTLs, vector-transduced hepatocytes were not targets for capsid-specific CTLs in mice. Hauck and colleagues (2009) demonstrated that trace *cap* impurities present in clinical vectors were not transcriptionally active. Together these data suggest that the risk associated with residual DNA impurities such as AAV *cap* depends on the amount of impurity present, and emphasize the importance of minimizing residual DNA impurities including encapsidated DNA in clinical vectors. Additional concerns about encapsidated DNA impurities include their tumorigenic potential (Committee for Proprietary Medicinal Products, 2001), and potential for transfer of prokaryotic sequences such as antibiotic resistance genes (Chadeuf *et al.*,

2005). The major source of the encapsidated plasmid DNA in vectors generated by transfection has been shown to be the backbone of ITR-containing vector plasmid (Chadeuf *et al.*, 2005; Hauck *et al.*, 2009). Hauck and colleagues (2009) reported that the use of a vector plasmid modified with a stuffer sequence, so that the backbone exceeded the packaging capacity of AAV2, reduced the levels of encapsidated plasmid DNA impurities 7.6-fold.

AAV empty capsids

As mentioned previously, Grimm and colleagues (1999) reported that empty capsids were the predominant type of AAV particle generated in cell culture by transient transfection or infection protocols, and accounted for 80 to 98.3% of total particles generated. Other groups subsequently confirmed these findings: Drittanti and colleagues (2001) reported that the ratio of total particles to VG-containing particles in rAAV prepared by transfection or infection methods ranged from 2.85 to 7.65, corresponding to 65 to 87% empty capsids; Sommer and colleagues (2003) reported ratios of AAV capsid particles (CP) to VG-containing particles ranging from 9.7 to 31.1, corresponding to 90 to 97% empty capsids in vector purified by a method that did not resolve vectors from vector-related impurities; Park and colleagues (2006) reported total CP-to-VG ratios ranging from 7 to 22, corresponding to 86 to 96% empty capsids. Together these data indicate that AAV empty capsids are typically present at a level corresponding to an ~10-fold excess over vector genome-containing AAV particles in crude harvests. The inclusion of empty capsids in clinical AAV vector preparations is undesirable because they represent an unnecessarily high amount of viral antigen. Capsid-specific CTLs were implicated in an efficacy-limiting immune response after successful expression of human coagulation factor IX using recombinant AAV2-mediated gene transfer in humans in a clinical study for hemophilia B (Manno *et al.*, 2006; Mingozzi *et al.*, 2007). Although the vector used in that study was free of empty capsids, Hauck and colleagues concluded that the preformed capsid protein component of the vector inoculum was the source of capsid epitopes recognized by CD8⁺ T cells. This result indicates that even the amount of capsid protein associated with highly purified, empty capsid-free vector can be sufficient to sensitize vector-transduced cells to immune effector functions in human subjects. Therefore the reduction of capsid protein to the lowest possible level required to achieve therapeutic gene transfer, through improved vector design combined with optimized vector generation and purification methods, is supported as an important goal to enhance clinical vector safety and the potential for long-term efficacy. Parameters and features of production cells and plasmids that influence the level of empty capsid generation are not well understood. Our laboratory has observed that vector transgene cassettes that exceed the natural packaging capacity of AAV (~4.7 kb) result in higher empty capsid generation (our unpublished observations). Cell culture conditions are also implicated on the basis of the observation that the proportion of empty capsids can vary widely among independent preparations of a single vector (Sommer *et al.*, 2003). Grimm and colleagues (1999) investigated the effect of expressing more AAV2 *cap* in their transfection system, and found that although more empty capsids were generated, the specific yield

of vector was actually reduced. They concluded that undertermined elements(s) involved in DNA replication and encapsidation, and that are present on the wild-type virus (which demonstrated higher packaging efficiency), are missing in recombinant AAV gene transfer vectors. Because of the limited ability to prevent the generation of all vector-related impurities during vector generation by transient transfection as well as cell culture methods, purification methods should be designed to further reduce these impurities (Qu *et al.*, 2007; Hauck *et al.*, 2009).

Conclusions

The therapeutic potential of recombinant AAV-based gene transfer for the treatment of many human diseases is great, emphasized by a strong safety record and promising results in clinical studies. Careful design and implementation of vector production methods are a critical consideration for successfully navigating the investigational product development pathway from early-phase clinical development through eventual product licensure. Transient transfection for vector production is well suited to support early-stage development, which benefits from flexibility, including the option to assess multiple vector candidates. For later stage investigation product development and prospective licensure, the transition to vector production systems that ensure sufficiently high capacity and cost-effectiveness must be carefully considered and implemented to maintain high vector quality and comparability. Developments in large-scale transient transfection methodologies performed with serum-free suspension mammalian cells may provide a vector production system that combines both early-stage flexibility as well as excellent scalability.

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Author Disclosure Statement

The author is the Director of a clinical vector core laboratory at an academic institute, and consults in the area of AAV vector manufacturing.

References

- Allen, J.M., Debekak, D.J., Reynolds, T.C., and Miller, A.D. (1997). Identification and elimination of replication-competent adeno-associated virus (AAV) that can arise by nonhomologous recombination during AAV vector production. *J. Virol.* 71, 6816–6822.
- Backliwal, G., Hildinger, M., Chenuet, S., DeJesus, M., and Wurm, F.M. (2008a). Coexpression of acidic fibroblast growth factor enhances specific productivity and antibodies titers in transiently transfected HEK293 cells. *New Biotechnol.* 2/3, 162–166.
- Backliwal, G., Hildinger, M., Kuettel, I., Delegrange, F., Hacker, D.L., and Wurm, F.M. (2008b). Valproic acid: A viable alternative to sodium butyrate for enhancing protein expression in mammalian cell cultures. *Biotechnol. Bioeng.* 100/1, 182–189.
- Baldi, L., Muller, N., Picasso, S., Jacquet, R., Girard, P., Thanh, H.P., Derow, E., and Wurm, F.M. (2005). Transient gene

- expression in suspension HEK-293 cells: Application to large scale production. *Biotechnol. Prog.* 21, 148–153.
- Baldi, L., Hacker, D.L., Adam, M., and Wurm, F.M. (2007). Recombinant protein production by large-scale transient gene expression in mammalian cells: State of the art and future perspectives. *Biotechnol. Lett.* 29, 677–684.
- Berns, K.I., and Parrish, C.R. (2007). Parvoviridae. In D.M. Knipe, P.M. Howley, D.E. Griffin, R.A. Lamb, and M.A. Martin, eds. *Fields Virology*, 5th ed. (Lippincott Williams & Wilkins, Philadelphia) pp. 2437–2477.
- Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., and Behr, J.-P. (1995). A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: Polyethylenimine. *Proc. Natl. Acad. Sci. U.S.A.* 92, 7297–7301.
- Cao, L., Liu, Y., During, M.J., and Xiao, X. (2000). High-titer, wild-type free recombinant adeno-associated virus vector production using intron-containing helper plasmid. *J. Virol.* 74, 11456–11463.
- Carter, B.J. (2005). Adeno-associated virus vectors in clinical trials. *Hum. Gene Ther.* 16, 541–550.
- Center for Biologics Evaluation and Research, Center for Drug Evaluation and Research, Food and Drug Administration, U.S. Department of Health and Human Services. (2000). Guidance for industry: Analytical procedures and methods validation—chemistry, manufacturing and controls documentation. Available at <http://www.fda.gov/cber/guidelines.htm> (accessed May 2009).
- Center for Biologics Evaluation and Research, Center for Drug Evaluation and Research, Food and Drug Administration, U.S. Department of Health and Human Services. (2003). Guidance for industry: Comparability protocols—protein drug products and biological products—chemistry, manufacturing, and controls information. Available at <http://www.fda.gov/cber/guidelines.htm> (accessed May 2009).
- Center for Biologics Evaluation and Research, Center for Drug Evaluation and Research, Food and Drug Administration, U.S. Department of Health and Human Services. (2004). Guidance for FDA and sponsors: Content and review of chemistry, manufacturing, and control (CMC) information for human gene therapy Investigational New Drug applications (INDs). Available at <http://www.fda.gov/cber/guidelines.htm> (accessed May 2009).
- Center for Biologics Evaluation and Research, Center for Drug Evaluation and Research, Food and Drug Administration, U.S. Department of Health and Human Services. (2006). Guidance for industry: INDs—approaches to complying with CGMP during phase 1. Available at <http://www.fda.gov/cber/guidelines.htm> (accessed May 2009).
- Chadeuf, G., Ciron, C., Moullier, P., and Salvetti, A. (2005). Evidence for encapsidation of prokaryotic sequences during recombinant adeno-associated virus production and their *in vivo* persistence after vector delivery. *Mol. Ther.* 12, 744–753.
- Clark, K.R. (2002). Recent advances in recombinant adeno-associated virus vector production. *Kidney Int.* 61, S9–S15.
- Clark, K.R., Voulgaropoulou, F., Fraley, D.M., and Johnson, P.R. (1995). Cell lines for the production of recombinant adeno-associated virus. *Hum. Gene Ther.* 6, 1329–1341.
- Clark, K.R., Liu, X., McGrath, J.P., and Johnson, P.R. (1999). Highly purified recombinant adeno-associated virus vectors are biologically active and free of detectable helper and wild-type virus. *Hum. Gene Ther.* 10, 1031–1039.
- Committee for Medicinal Products for Human Use (CHMP), European Medicines Agency. (2005). Report from the CHMP Gene Therapy Expert Group meeting. EMEA/CHMP/183989/2004. Available at www.emea.europa.eu/pdfs/human/genetherapy/18398904en.pdf (accessed May 2009).
- Committee for Proprietary Medicinal Products (CPMP), European Agency for the Evaluation of Medicinal Products. (2001). Position statement on the use of tumorigenic cells of human origin for the production of biological and biotechnological medicinal products. CPMP/BWP/1143/00. Available at <http://www.emea.europa.eu/pdfs/human/press/pos/114300en.pdf> (accessed May 2009).
- Conway, J.E., Rhys, C.M.J., Zolotukhin, I., Zolotukhin, S., Muzyczka, N., Hayward, G.S., and Byrne, B.J. (1999). High-titer recombinant adeno-associated virus production utilizing a recombinant herpes simplex virus type 1 vector expressing AAV-2 Rep and Cap. *Gene Ther.* 6, 986–993.
- Drittanti, L., Jenny, C., Poulard, K., Samba, A., Manceau, P.L., Soria, N., Vincent, N., Danos, O., and Vega, M. (2001). Optimised helper virus-free production of high-quality adeno-associated virus vectors. *J. Gene Med.* 3, 59–71.
- Durocher, Y., Perret, S., and Kamen, A. (2002). High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. *Nucleic Acids Res.* 30, E9.
- Durocher, Y., Pham, P.L., St-Laurent, G., Jacob, D., Cass, B., Chahal, P., Lau, C.J., Nalbantoglu, J., and Kamen, A. (2007). Scalable serum-free production of recombinant adeno-associated virus type 2 by transfection of 293 suspension cells. *J. Virol. Methods* 144, 32–40.
- Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., and Danielsen, M. (1987). Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. U.S.A.* 84, 7413–7417.
- Fiandaca, M., Forsayeth, J., and Bankiewicz, K. (2008). Current status of gene therapy trials for Parkinson's disease. *Exp. Neurol.* 209, 51–57.
- Food and Drug Administration, U.S. Department of Health and Human Services. (1998). International conference on harmonisation; guidance on viral safety evaluation of biotechnology products derived from cell lines of human or animal origin. *Fed. Regist.* 63, 51074–51084. Available at <http://www.fda.gov/cber/gdlns/virsafe.pdf> (accessed May 2009).
- Gao, G., Wang, Q., Wang, L., Vandenbergh, L., Lock, M., Grant, R., and Wilson, J.M. (2008). Inadvertent gene transfer of co-packaged *rep* and *cap* sequences during the production of AAV vector and its potential impact on vector performance. *Mol. Ther.* 16, S105.
- Girard, P., Drouazi, M., Baumgartner, G., Bourgeois, M., Jordan, M., Jacko, B., and Wurm, F.M. (2002). 100-liter transient transfection. *Cytotechnology* 38, 15–21.
- Gombold, J., Peden, K., Gavin, D., Wei, Z., Baradaran, K., Mire-Sluis, A., and Schenerman, M. (2006). Lot release and characterization testing of live-virus-based vaccines and gene therapy products. 1. Factors influencing assay choices. *Bio-Process Int.* 4, 46–56.
- Graham, F.L., and van der Eb, A.J. (1973). A new technique for the assay of infectivity of human adenovirus 45 DNA. *Virology* 52, 456–467.
- Grimm, D., Kern, A., Rittner, K., and Kleinschmidt, J.A. (1998). Novel tools for production and purification of recombinant adeno-associated virus vectors. *Hum. Gene Ther.* 9, 2745–2760.
- Grimm, D., Kern, A., Pawlita, M., Ferrari, F.K., Samulski, R.J., and Kleinschmidt, J.A. (1999). Titration of AAV-2 particles via

- a novel capsid ELISA: Packaging of genomes can limit production of recombinant AAV-2. *Gene Ther.* 6, 1322–1330.
- Hauck, B., Liu, X., Joyce, S., Tai, A., Bennicelli, J., Zeleniaia, Z., High K.A., and Wright, J.F. (2007). Process characterization and GMP manufacture of a gene transfer vector for Leber congenital amaurosis. *Mol. Ther.* 15, S167.
- Hauck, B., Murphy, S.L., Smith, P.H., Liu, X., Zeleniaia, O., Mingozzi, F., Sommer, J.M., High, K.A., and Wright, J.F. (2009). Undetectable transcription of *cap* in a clinical AAV vector: implications for pre-formed capsids in immune responses. *Mol. Ther.* 17, 144–152.
- Hildinger, M., Baldi, L., Stettler, M., and Wurm, F.M. (2007). High-titer, serum-free production of adeno-associated virus vectors by polyethylenimine-mediated plasmid transfection in mammalian suspension cells. *Biotechnol. Lett.* 29, 1713–1721.
- Jordan, M., Schallhorn, A., and Wurm, F.M. (1996). Transfecting mammalian cells: Optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res.* 24, 596–601.
- King, J.A., Dubielzig, R., Grimm, D., and Kleinschmidt, J.A. (2001). DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsids. *EMBO J.* 20, 3282–3291.
- Li, C., Hirsch, M., Asokan, A., Zeithaml, B., Ma, H., Kafri, T., and Samulski, R.J. (2007). Adeno-associated virus type 2 (AAV2) capsid-specific cytotoxic T lymphocytes eliminate only vector-transduced cells coexpressing the AAV2 capsid *in vivo*. *J. Virol.* 81, 7540–7547.
- Li, J., Samulski, R.J., and Xiao, X. (1997). Role for highly regulated *rep* gene expression in adeno-associated virus vector production. *J. Virol.* 71, 5236–5243.
- Liu, C., Dalby, B., Chen, W., Kilzer, J.M., and Chiou, H.C. (2008). Transient transfection factors for high-level recombinant protein production in suspension cultured mammalian cells. *Mol. Biotechnol.* 39, 141–153.
- Maguire, A.M., Simonelli, F., Pierce, E.A., Pugh, E.N., Mingozzi, F., Bennicelli, J., Banfi, S., Marshall, K.A., Testa, F., Surace, E.M., Rossi, S., Lyubarsky, A., Arruda, V.R., Konkle, B., Stone, E., Sun, J., Jacobs, J., Dell-Oso, L., Hertle, R., Ma, J., Redmond, T.M., Zhu, X., Hauck, B., Zeleniaia, O., Shindler, K.S., Maguire, M.G., Wright, J.F., Volpe, N.J., McDonnell, J.W., Auricchio, A., High, K.A., and Bennett, J. (2008). Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N. Engl. J. Med.* 358, 2240–2248.
- Manno, C.S., Arruda, V.R., Pierce, G.F., Glader, B., Ragni, M., Rasko, J., Ozelo, M.C., Hoots, K., Blatt, P., Konkle, B., Dake, M., Kaye, R., Razavi, M., Zajko, A., Zehnder, J., Nakai, H., Chew, A., Leonard, D., Wright, J.F., Lessard, R.R., Sommer, J.M., Tigges, M., Sabatino, D., Luk, A., Jiang, H., Mingozzi, F., Couto, L., Ertl, H.C., High, K.A., and Kay, M.A. (2006). Successful transduction of liver in hemophilia by AAV-factor IX and limitations imposed by the host immune response. *Nat. Med.* 12, 342–347.
- Matsushita, T., Elliger, S., Elliger, C., Podsakoff, G., Villarreal, L., Kurtzman, G.J., Iwaki, Y., and Colosi, P. (1998). Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther.* 5, 938–945.
- Merdan, T., Kunath, K., Fischer, D., Kopecek, J., and Kissel, T. (2002). Intracellular processing of poly(ethylene imine)/ribozyme complexes can be observed in living cells by using confocal laser scanning microscopy and inhibitor experiments. *Pharm. Res.* 19, 140–146.
- Mingozzi, F., Maus, M.V., Hui, D.J., Sabatino, D.E., Murphy, S.L., Rasko, J.E., Ragni, M.V., Manno, C.S., Sommer, J., Jiang, H., Pierce, G.F., Ertl, H.C., and High, K.A. (2007). CD8⁺ T-cell responses to adeno-associated virus capsid in humans. *Nat. Med.* 13, 419–422.
- Meissner, P., Pick, H., Kulangara, A., Chatellard, P., Friedrich, K., and Wurm, F.M. (2001). Transient gene expression: Recombinant protein production with suspension-adapted HEK293-EBNA cells. *Biotechnol. Bioeng.* 75, 197–203.
- Muzyczka, N. (1992). Use of adeno-associated virus as a general transduction vector for mammalian cells. *Curr. Top. Microbiol. Immunol.* 158, 97–129.
- Negrete, A., Yang, L.C., Mendez, A.F., Levy, J.R., and Kotin, R.M. (2007). Economized large-scale production of high yield of rAAV for gene therapy applications exploiting baculovirus expression system. *J. Gene Med.* 9, 938–948.
- Nony, P., Chadeuf, G., Tessier, J., Moullier, P., and Salvetti, A. (2003). Evidence for packaging of *rep-cap* sequences into adeno-associated virus (AAV) type 2 capsids in the absence of inverted terminal repeats: A model for generation of *rep*-positive AAV particles. *J. Virol.* 77, 776–781.
- Ogasawara, Y., Urabe, M., and Ozawa, K. (1998). The use of heterologous promoters for adeno-associated virus (AAV) protein expression in AAV vector production. *Microbiol. Immunol.* 42, 177–185.
- Park, J.Y., Lim, B.-P., Lee, K., Kim, Y.-G., and Jo, E.-C. (2006). Scalable production of adeno-associated virus type 2 vectors via suspension transfection. *Biotechnol. Bioeng.* 94, 416–430.
- Pham, P.L., Perret, S., Cass, B., Carpentier, E., St-Laurent, G., Bisson, L., Kamen, A., and Durocher, Y. (2005). Transient gene expression in HEK293 cells: Peptone addition posttransfection improves recombinant protein synthesis. *Biotechnol. Bioeng.* 90, 332–344.
- Qu, G., Bahr-Davidson, J., Prado, J., Tai, A., Cataniag, F., McDonnell, J., Zhou, J., Hauck, B., Luna, J., Sommer, J.M., Smith, P., Zhou, S., Colosi, P., High, K.A., Pierce, G.F., and Wright, J.F. (2007). Separation of adeno-associated virus type 2 empty particles from genome-containing vector by anion-exchange column chromatography. *J. Virol. Methods* 140, 183–192.
- Recombinant DNA Advisory Committee, National Institutes of Health. (2007). AAV Symposium: Immune Responses to Adeno-Associated Virus (AAV) Vectors. Available at <http://www4.od.nih.gov/oba/RAC/meeting.html> (accessed May 2009).
- Reed, S.E., Staley, E.M., Mayginnes, J.P., Pintel, D.J., and Tullis, G.E. (2006). Transfection of mammalian cells using linear polyethylenimine is a simple and effective means of producing recombinant adeno-associated virus vectors. *J. Virol. Methods* 138, 85–98.
- Salvetti, A., Oreve, S., Chadeuf, G., Favre, D., Cherel, Y., Champion-Arnaud, P., David-Ameline, J., and Moullier, P. (1998). Factors influencing recombinant adeno-associated virus production. *Hum. Gene Ther.* 9, 695–706.
- Samulski, R.J., Shang, L.S., and Shenk, T. (1989). Helper-free stocks of recombinant adeno-associated viruses: Normal integration does not require viral gene expression. *J. Virol.* 61, 3096–3101.
- Schlaeger, E.-J., Kitas, E.A., and Dorn, A. (2003). SEAP expression in transiently transfected mammalian cells grown in serum-free suspension culture. *Cytotechnology* 42, 47–55.
- Smith R.H., Ding, C., and Kotin, R.M. (2003). Serum-free production and column purification of adeno associated virus type 5. *J. Virol. Methods* 114, 115–124.
- Smith, P.H., Wright, J.F., Qu, G., Patarroyo-White, S., Parker, A., and Sommer, J.M. (2003). Packaging of host cell and plasmid

- DNA into recombinant adeno-associated virus particles produced by triple transfection. *Mol. Ther.* 7, S348.
- Snyder, R.O., and Flotte, T.R. (2002). Production of clinical-grade recombinant adeno-associated virus vectors. *Curr. Opin. Biotechnol.* 13, 418–423.
- Sommer, J.M., Smith, P.H., Parthasarathy, S., Isaacs, J., Vijay, S., Kieran, J., Powell, S.D., McClelland, A., and Wright, J.F. (2003). Quantification of adeno-associated virus particles and empty capsids by optical density measurement. *Mol. Ther.* 7, 122–128.
- Urabe, M., Ding, C., and Kotin, R.M. (2002). Insect cells as a factory to produce adeno-associated virus type 2 vectors. *Hum. Gene Ther.* 13, 1935–1943.
- U.S. Department of Health and Human Services. (2006a). Current Good Manufacturing Practice in Manufacturing, Processing, Packing, or Holding of Drugs. 21 CFR 210. Available at <http://www.gmp1st.com/drreg.htm> (accessed May 2009).
- U.S. Department of Health and Human Services. (2006b). General and Current Good Manufacturing Practice for Finished Pharmaceuticals. 21 CFR 211. Available at <http://www.gmp1st.com/drreg.htm> (accessed May 2009).
- Vincent, K.A., Piraino, W.T., and Wadworth, S.C. (1997). Analysis of recombinant adeno-associated virus packaging and requirements for *rep* and *cap* gene products. *J. Virol.* 71, 1987–1995.
- Wang, L., Figueredo, J., Calcedo, R., Lin, J., and Wilson, J.M. (2007). Cross-presentation of adeno-associated virus serotype 2 capsids activates cytotoxic T-cells but does not render hepatocytes effective cytolytic targets. *Hum. Gene Ther.* 18, 185–194.
- Wang, X.S., Qing, K., Ponnazhagan, S., and Srivastava, A. (1997). Adeno-associated virus type 2 DNA replication *in vivo*: Mutation analyses of the D sequence in viral inverted terminal repeats. *J. Virol.* 71, 3077–3082.
- Wang, X.S., Khuntirat, B., Qing, K., Ponnazhagan, S., Kube, D.M., Zhou, S., Dwarki, V.J., Yoder, M.C., and Srivastava, A. (1998). Characterization of wild-type adeno-associated virus type 2-like particles generated during recombinant viral vector production and strategies for their elimination. *J. Virol.* 72, 5472–5480.
- Warrington, K.H., and Herzog, R.W. (2006). Treatment of human disease by adeno-associated viral gene transfer. *Hum. Genet.* 119, 571–603.
- WHO Expert Committee on Biological Standardization. (1998). Requirements for the use of animal cells as *in vitro* substrates for the production of biologicals. WHO Technical Report Series, No. 878. (World Health Organization, Geneva, Switzerland).
- Wright, J.F. (2008). Manufacturing and characterizing AAV-based vectors for use in clinical studies. *Gene Ther.* 15, 840–848.
- Xiao, X., Li, J., and Samulski, R.J. (1998). Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J. Virol.* 72, 2224–2232.
- Zolotukhin, S. (2005). Production of recombinant adeno-associated virus vectors. *Hum. Gene Ther.* 16, 551–557.

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