Manufacturing Clinical Grade Recombinant Adeno-Associated Virus Using Invertebrate Cell Lines

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Recombinant adeno-associated virus (rAAV) vectors are proving to be a reliable gene transfer system for several clinical applications, with an increasing body of evidence supporting safety and efficacy. Realizing the clinical and commercial potential of rAAV depends on a reliable source of high-quality, well-characterized rAAV lots. This requirement has been very challenging to achieve due to limits of manufacturing platforms, lot-to-lot variability, or differences in the rigor applied to quality-control assays. In addition to reliable, high-quality vectors, limited quantities of rAAV have hampered clinical development and discouraged investigations into applications that require large therapeutic doses or quantities needed to treat large patient populations. A minimal number of vector production runs should be sufficient to support all phases of clinical development, including non-clinical, pharmacological, and toxicological studies, as well as clinical studies and commercial supply. The production platform using the Sf9 invertebrate cell line has emerged as a scalable and economical source of rAAV. Access to larger quantities of rAAV has now enabled evaluation of gene therapeutics for diseases that require large doses per patient or diseases with large patient populations. The only licensed rAAV product, Glybera, was produced in Sf9 cells, and other rAAV products are in clinical trials in the United States and Europe. The development of the Sf9 rAAV genetics, processes, and overview of the current system are described.

Keywords: AAV vector, Sf9 cells

RECOMBINANT ADENO-ASSOCIATED VIRUS VECTORS FOR GENE TRANSFER

Recombinant adeno-associated virus (rAAV) vectors are a versatile and efficient platform used for somatic cell gene transfer. Currently, there are 28 open clinical trials using rAAV and 89 inactive studies (listed as either suspended, or no development reported, or discontinued withdrawn) that have utilized AAV vectors to date (Citeline, Pharma Intelligence; Informa). Efficacy, vector persistence, and safety have been observed in preclinical animal model studies and human trials. In some clinical trials, sustained or transient transgene expression and disease correction have been demonstrated in humans treated for alpha-1 antitrypsin deficiency,[1] lipoprotein lipase deficiency,[2] hemophilia B, hemophilia A (reviewed in High *et al*.[3] and Spencer *et al*.[4]), and two inherited forms of blindness: Leber's congenital amaurosis type 2, caused by mutations in the *RPE65* gene,[5,6] and Leber's heritary optic neuropathy, caused by mitochondrial genome mutations.[7] Reports of vector-related serious adverse events are rare, with the most common adverse events involving elevated liver enzymes and immune responses to transduced liver cells seen with large-dose intravenous administration, which can be managed with transient immunosuppression regimens.[8] Based on risk assessment, in 2012, the European Medicines Agency approved Glybera™ (*Alipogene tiparvovec*) for the treatment of familial hyperlipoproteinemia due to a genetic insufficiency of lipoprotein lipase.[9]

AAV vectors are based on the non-pathogenic *dependoparvovirinae* subfamily of the animal DNA *Parvoviridae*. In addition to the >12 AAV serotypes described, hundreds of naturally occurring capsid variants have been isolated and used as *in vitro* gene transfer experiments.[10] Synthetically derived

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DOI: 10.1089/hum.2017.042

AAV capsids produced by mixing or "shuffling" natural capsid variants have further diversified the variety and properties of the capsids.[11–14] In contrast to capsid shuffling and random combinatorial library approaches, rationally designed mutations have led to vectors with phenotypes that differ from the parental capsids. Presumably by interfering with protein degradation pathways, substitutions of capsid-surface exposed phosphorylation substrate residues (Y, S, T, and K), as well as SUMOylation and ubiquitination ligase acceptor lysine residues, resulted in enhanced biological activities for multiple serotypes and various target tissues.[15–19] Other processes for selecting specific capsid attributes, for example enhanced central nervous system transduction, have been developed in murine models. Selection strategies have been employed to elude pre-existing humoral immunity by preincubating capsid libraries with human immunoglobulin that either specifically or nonspecifically neutralizes many capsid variants ("neutralization" typically refers to a functional assay based on 50% transduction inhibition of reporter cell lines *in vitro*). However, the vectors that remain active may represent a set of capsids that with reduced immunodominant epitopes.

AAV MOLECULAR GENETICS AND VECTOR PRODUCTION

The linear single-stranded DNA *Dependoparvovirinae* genome is approximately 4.7 kb, with two extended open reading frames (ORFs), Rep and Cap, that encode the four nonstructural and three structural proteins, respectively. Rep 78 and Rep 68 are expressed from the p5 promoter, and Rep 52 and Rep 40 are expressed from the p19 promoter.[20] The two smaller variants arise from a common 3′-splicing event of the p5 and p19 transcripts. *In vitro* replication studies indicate that either of the p5 proteins and either of the p19 proteins are sufficient to support DNA replication and virus or vector production.[21] The p19 Rep proteins are amino-terminal truncated versions of the p5 Rep proteins. In addition to the super family (SF) III helicase activity common to the four Rep proteins,[22–25] the p5 Rep proteins have conserved RCR protein motifs 2 (uHuHuuu) and 3 (uuuu D/E, D/E),[26] where u represents a bulky hydrophobic residue (F, Y, W) that provide the activities necessary for terminal resolution.[23,27,28] The peptide encoded in the spliced sequence functions as a competitive inhibitor of cyclic-adenosine monophosphate activated protein kinases A and X (PKA and PrKX, respectively).[29–31] However, the role in wild-type AAV infection/co-infection has not been fully described.

The assembled capsid consists of three structural proteins—VP1, VP2, and VP3—in approximately 1:1:10 relative abundance.[32] VP1, VP2, and VP3 are translated from a single primary transcript expressed from the p40 promoter that utilizes alternative splice acceptors: the mRNA resulting from the utilization of the upstream splice acceptor retains the VP1 AUG codon, leading to translation of VP1.[33–35] The majority of the transcripts utilize the slightly downstream splice acceptor that excises the VP1 methionine codon, which are translated into VP2 and VP3. VP2 translation initiates inefficiency at a non-canonical start codon, often ACG that is a codon for threonine, whereas the first AUG encountered by the scanning ribosomes is the initiation codon for VP3, the major capsid protein. Within the VP1u (unique) region is phospholipase A2 (PLA2) activity, essential for escape from late endosomes following uptake and internalization.[36,37]

Recently, the assembly activating protein (AAP) was identified as a second ORF within the *cap* gene, limited to the *dependoparvovirinae*.[38,39] Cap ORF codon optimization and synthetic combinatorial libraries disrupt the AAP ORF, preventing capsid assembly, thereby constraining approaches to generate diverse capsid libraries. However, although AAP is not essential for all serotype capsid assembly, expression enhances particle assembly.[40] Until the AAP is more fully characterized, capsid modification approaches are selective for the second ORF that produces intact, functional AAPs.

Flanking the ORFs are 145 nt terminal palindromes, often referred to as inverted terminal repeats (ITRs), which, in the single-stranded form, consist of three interrupted palindromes typically designated as A, B, and C and the unpaired D sequence. When folded into the most energetically stable duplex conformation, the B and C sequences form opposing hairpins, with the A palindrome forming the stem of a T-shaped structure. As a full duplex, for example either in a plasmid or as replicative intermediates, the p5 Rep protein subunits bind as a hexamer to the $(GCTC)_3$ repeats in the A stem and, oriented by contact with the unpaired TTT at the tip of one of the cross-arms, unwind a smaller palindromic motif at the A and D junction, exposing the single-stranded T-T substrate of the p5 Rep-nickase.[41–46] The catalytic tyrosine (Y156 in AAV2) forms a covalent phosphotyrosine with the 5′-thymidine of the nicking site.[23,28,47,48] The nascent 3′-end of the nicked strand then serves as a primer for cellular DNA polymerase and producing two complementary full-length virus genomes with intact but inverted termini. A subsequent,

uncharacterized, phosphodiesterase cleavage reaction resolves the nucleoprotein complex, allowing the Rep complex to reform and process additional replicative intermediate ITR substrates.

Vector DNA encapsidation, which has not been separated from replication, is dependent on Rep helicase and ATPase activities. Although approximately 110% of the wild-type virus genome can be packaged, the yield of vector rapidly diminishes once the full-length genome size is exceeded. Since packaging and replication are inseparable, encapsidation "signals" may consist of dynamic, actively replicating vector genomes rather than static *cis* acting motifs.

The utility of rAAV for human applications involving large single doses or large patient populations has been hindered by the inability to produce sufficient quantities of vector that can be used to support investigational new drug–enabling, nonclinical, large-animal dose escalation and toxicological studies, as well as clinical studies and commercial supply. Depending on the application, a single patient dose may range from ≤1E + 12 to ≥1E + 16 particles or vg, vector genome–containing particles. The main obstacle to producing large quantities of clinical grade rAAV is a consequence of the biphasic life cycle of AAV. The wild-type virus may cause either a productive or latent infection, and in cultured cells, the provirus is frequently localized (ca. 70%) to the AAVS1 locus on human chromosome 19.[49,50]

Alternatively, a productive AAV infection results from a host cell co-infected with a helper virus, such as adenovirus, that initiates a lytic infection and cytopathic effects.[51] Although many details of the effect of the helper-virus on the host cell are unknown, a number of the AAV and helper-virus interactions have been recently described.[52–54] The requirement for cytotoxic helper-virus gene expression limits rAAV production to transient processes, including co-transfection[55] and adenovirus infection of stable cell lines,[56,57] and recombinant herpes simplex virus,[58] which challenges the scalability of production.

The conventional method of producing rAAV vectors for gene transfer is based on co-transient transfection of human embryonic kidney (HEK) 293 cells. The plasmids supply the AAV ORFs that encode the AAV structural and nonstructural proteins. The "helper" virus ORFs, usually adenovirus E2a, E2b, E4orf6, and VARNA, are often expressed from a co-transfecting plasmid.[59,60] Because *Escherichia coli* does not express the AAV Rep proteins during plasmid amplification, the vector genome may be *in cis* with either set of virus genes. The nonstructural, rolling-circle replication-like Rep proteins are found in all members of the *Parvoviridae*, and SFIII helicase activity is required for replication of the linear, single-stranded DNA genome. The helicase activity is critical for unwinding the ends as part of the terminal resolution process. In addition, it is expected that the Rep helicase activities are involved in packaging or encapsidating the virion DNA into preformed capsids.[61,62] The two p5 proteins, Rep 68 and Rep 78, also have sequence-specific DNA binding and nickase activities. For vector production (and virus propagation *in vitro*), either the spliced or unspliced forms of the p5 and p19 Rep proteins are sufficient.

BACKGROUND: INVERTEBRATE CELL-BASED rAAV PRODUCTION

The dependoparvovirus genus of the *Parvoviridae* family is comprised of two subfamilies: the *Densovirinae* that infect invertebrate hosts, and the *Parvovinae* that infect vertebrate hosts.[63] Based on resemblances between the two subfamilies and specialization of host–virus relationships, a reasonable conjecture is that the *Parvoviridae* are the descendants of an ancient virus lineage that predated the invertebrate/vertebrate split, which occurred approximately 650 million years ago.

The *Parvoviridae* lack genes that encode polymerases and other macromolecular synthetic pathway proteins. Therefore, during a lytic infection, *Parvoviridae* genomes are replicated in the infected cell nucleus using cellular DNA polymerase complex and virus nonstructural protein activities specific to amplify linear single-stranded DNA virion genomes. Basic biochemical characterization of a *Junonia coenia* densovirus nonstructural (NS1) protein[64] confirmed that the replication of *Densovirinae* and *Parvovidinae* genomes mechanistically share these fundamental features: leading strand DNA synthesis initiated via primer extension of the 3′-terminal palindrome into a duplex replicative intermediate followed by resolution of the two strands. Thus, an invertebrate host, *Spodoptera frugiperda*, was anticipated to function as a suitable surrogate for AAV vector DNA replication. A preliminary test of this hypothesis was performed with Sf9 cells transfected with plasmids with or without AAV2 ITRs flanking a β-galactosidase ORF (LacZ gene) cassette then infected with recombinant baculovirus expression vector (BEV) that expressed AAV2 Rep proteins. In the absence of AAV DNA replication, electrophoretic fractionation of the baculovirus DNA (ca. 135 kbp)

appeared as low-mobility bands on agarose gels. However, in the presence of Rep protein expression, in addition to the low mobility DNA bands, additional bands appeared corresponding in size to conformers of the LacZ vector genome. Subsequent co-infection experiments confirmed the transfection results and demonstrated that the AAV2-eGFP vector genome was "rescued" from the recombinant baculovirus genome and replicated as AAV DNA in Sf9 cells in the presence of Rep-expressing BEVs. In addition to the monomeric vector genome, on agarose gels, the DpnI-resistant replication products appeared as a ladder corresponding to the replicative forms (Ding C, Kotin RM. 1999. Unpublished results).

Robust Rep-dependent AAV DNA replication in baculovirus-infected Sf9 cells was the critically important discovery that subsequently resulted in rAAV production in Sf9 cells. Production of virus-like particles in Sf9 cells for structural studies and as vaccines had been described for a variety of virus genera, including parvoviruses and human papilloma virus (reviewed in Thompson *et al.*[65]). However, achieving stoichiometric expression of two or more structural proteins had not been utilized routinely.

DEVELOPMENT OF THE FIRST-GENERATION BACULOVIRUS/SF9 CELL PRODUCTION SYSTEM

The first successful baculovirus/Sf9 cell production process was developed using a triple co-infection: Rep-Bac, Cap-Bac, and (name of vector)-Bac. The AAV2 non-structural proteins, Rep 78 and Rep 52, were expressed from two separate opposing cassettes in the Rep-Bac. The three structural proteins were expressed as a single cassette from a second Cap-Bac, and the vector genome was introduced into cells on a third baculovirus. The ratio of the two Rep proteins was modeled on the relative levels attained in mammalian cells transfected with pDG[59] where the level of Rep 78 was attenuated by replacing the AAV p5 promoter with the mouse mammary tumor virus long terminal repeat, a steroid inducible promoter. In the absence of dexamethasone, "leaky" expression resulted in low levels of Rep 78 relative to Rep 52. The stoichiometry of Rep 52 and Rep 78 in pDG-transfected HEK 293 cells substantially improved the rAAV yields. A similar Rep 52:Rep78 ratio in Sf9 cells was achieved by designing a baculovirus that expressed Rep 52 from the baculovirus major late p10 promoter and separately regulated expression of Rep78 by a modified *Orgyia pseudotsugata* nuclear polyhedrosis virus immediate early (Op ΔIE1) promoter.[66] The triple infection effectively generated rAAV in Sf9 cells. However, the two opposing Rep ORFs formed a large inverted repeat in the Rep-Bac that proved to be genetically unstable, and Rep expression diminished after passage 3 or 4.[67]

SECOND-GENERATION BACULOVIRUS/SF9 CELL PRODUCTION SYSTEM

Generating rAAV in Sf9 cells using the first-generation process required simultaneous infection with three different baculoviruses. At a multiplicity of infection (MOI) of 5, the probability of infecting any cell with at least three baculoviruses is 0.84. However, when multiple virus types are required for a productive infection, the Poisson distribution does not determine the probability of a cellular infection with at least one of the necessary viruses, that is, ≥1 Rep-Bac, ≥1 Cap-Bac, and ≥1 vector-Bac. However, late in the infection cycle when the MOI becomes very large, there is a near certainty that each cell is exposed to at least one of the three different baculoviruses required for rAAV production.

A major advancement in the production process was achieved by consolidating the Rep and Cap cassettes into a single baculovirus, thereby reducing by one the number of unique infectious particles required to generate rAAV. The consolidated baculovirus configuration improved the genetic stability of the Rep cassettes and increased the rAAV yields.[68]

In contrast to the first-generation system, the second-generation system required only two rather than three different baculoviruses to infect a cell simultaneously to generate rAAV. This increases the probability that more of the cells in the culture will produce rAAV. For example, at a theoretical MOI_e of 5 (i.e., each cell is infected with exactly five virus particles), there are 21 combinations of three virus types arranged in groups of five. Of the 21 combinations, six will be productive, that is, there is at least one virus type of the three different baculoviruses represented in the group of five. Combinations are calculated using the standard formula:

$$nCr = (r = n - 1)!/r!(n - 1)!$$

where nCr represents the possible combinations, r is the number of virus particles infecting a cell (MOI_e), and n is the number of virus types needed for a productive infection. Thus, only about a third of the cells infected with five particles have at least one Rep-Bac, one Cap-Bac, and one vector-Bac, and are competent to produce rAAV. Whereas the second-generation system requires two different

baculovirus types to produce rAAV, at a MOI_e of 5, four of the six combinations will be productive.

Productive combinations can be calculated using the following equation:

$$nCr_p = (r_p + n - 1)!/(r - n)!(n - 1)$$

or

$$nCr_p = (r_p - 1)!/(r_p)!(n - 1)!$$

where nCr_p represents the possible *productive* combinations and $r_p = r - n$.

Thus, regardless of the MOI, only two of the possible combinations of viruses would be nonproductive: Rep/Cap-Bac only, or vector-Bac only. In the first-generation system, three different baculoviruses (Rep-Bac, Cap-Bac, and vector-Bac) coinfecting a cell are needed to produce rAAV, so for example at a MOI_e of 10, 36/66 are productive combinations, and at a MOI_e of 12, 55/91 are productive combinations, and so on. Using the second-generation, two baculovirus process, it was apparent that rAAV yields were routinely ≥3-fold greater than the triple infection. Not only were the probabilities greater that cells were productively infected, the productive (simultaneous) infection occurred earlier than with the three baculovirus system, resulting in longer rAAV time of production before the cells die. In contrast, the first-generation process may result in sequential infections with the three virus types, resulting in a temporal insufficiency of the three components necessary for rAAV generation, that is, AAV structural and nonstructural proteins, and vector genomes. Reducing the required types of baculovirus to one improves the probability of a productive infection. However, maintaining the AAV vector genome in *cis* in a Rep expression baculovirus may be problematic because in the presence of Rep 78, the AAV vector genome will replicate, which may compromise baculovirus genome propagation.

By duplicating the strategy of using the native VP2 non-canonical start codon for VP1, the structural proteins were expressed as a single transcript that generated VP1, VP2, and VP3 in the appropriate ratio. In addition to achieving the stoichiometry of the three structural proteins, this Cap-Bac configuration proved to be genetically stable through six passages. Based on 100-fold amplification for each passage, starting with 1 mL of P1, the increased stability theoretically can generate 10^9 L of baculovirus stock.

A second important advancement occurred when the titerless infected cells and scale-up (TIPS) process was adapted for rAAV production. Rather than titering baculovirus infectious units, the infectious materials were cryopreserved as baculovirus-infected insect cells (BIICs). The frozen single-use aliquots stably preserves the infectivity and facilitates scalability, leading to highly reproducible rAAV yields in volumes that ranged between 0.2 and 500 L.[69]

The baculovirus manufacturing platform for rAAV vectors is amenable to supporting clinical trials and commercial production for a variety of indications that require large product lots, such as those involving large doses and/or large populations. The baculovirus manufacturing platform is also one that can be incorporated into current Good Manufacturing Practices (cGMP)-compliant processes that are controlled, aseptic, and perform consistently. The final rAAV drug product generated meets release specifications for identity, safety, purity, potency, and stability.

STABLE SF9 CELL LINES FOR rAAV PRODUCTION

To reduce further the complexity of the BEV infection required to generate rAAV particles, Zolotukin *et al*. developed an alternative approach using stable cell lines that inducibly express the AAV *rep* and *cap* genes upon baculovirus infection.[70,71] Thus, the production requires infection with a single BEV bearing the vector genome. Recently, cell lines were established that optimized expression of VP proteins, resulting in rAAV with biological activity equivalent to HEK 293-produced rAAV but with substantially reduced quantities of encapsidated non-vector DNA.[72,73]

UPSTREAM PROCESS

Banks of Sf9 cells, helper BIICs, and vector BIICs can be generated, cryopreserved, and qualified for long-term use in manufacturing.

To produce AAV, a vial of Sf9 cells is thawed, grown in commercially available culture media, expanded in suspension in shaker flasks and/or wave bioreactors, transferred to a stirred-tank bioreactor, and, at terminal volume, infected with BIICs in a 1:1:10,000 (helper BAC:vector BAC:cell) ratio. Following incubation for 48–72 h, yields on average of 2.7E14 vg/L can be achieved, resulting in average total yields in the 1E17 vg range at 400 L scale with the two vector serotypes shown that harbored 12 different transgenes (Table 1). The 40–60 L processes are carried out as process development (PD) runs toward the large-scale GMP batches. During maturation of the production

Table 1. *Summary of production runs for rAAV5 and rAAV6*

Serotype	*# of Runs*	*Scale (L)*	*Average total yield (vg)*	*Average cells/mL*	*Average yield vg/cell*
AAV5	8	400	1.3E17 ± 8.2E16	4.0E6 ± 9.6E5	8.4E4 ± 6.0E4
	3	200	2.0E16 ± 6.1E15	2.7E6 ± 9.1E5	3.9E4 ± 2.1E4
	4	40–50	5.8E15 ± 3.5E15	2.7E6 ± 7.0E5	4.6E4 ± 2.2E4
AAV6	13	400	9.0E16 ± 8.3E16	3.2E6 ± 5.1E5	7.3E4 ± 6.9E4
	8	200	5.7E16 ± 5.0E16	2.7E6 ± 5.3E5	1.1E5 ± 8.2E4
	6	40–60	2.8E15 ± 2.2E15	3.3E6 ± 2.9E6	2.7E4 ± 2.8E4

The initial process is performed in 40–60 L scale during process development. The parameters are then transferred to the 200 L pilot production scale bioreactors. For current Good Manufacturing Practices production, the process is transferred to 400 L bioreactors. The average values for total vector yield (as vector genome-containing particles [vg]), average cell density, and average vg yield per cell are shown. The range for each value is given as ±value. Notably, as the process undergoes optimization, the vector yield trends higher.

process from development (40–60 L scale) to pilot runs (200 L) to GMP batches (400 L), process improvements are incorporated, and together with appropriate controls, a trend toward higher yields is observed.

DOWNSTREAM PROCESSES

Several options are available for downstream processing of rAAV from the serum-free Sf9 cell culture. Cecchini *et al*. described a process that recovers both intracellular and extracellular rAAV by processing the entire bioreactor contents using mechanical cell disruption.[69] Alternatively, detergents may be used to lyse the cells either *in situ* or following tangential flow filtration (TFF) to concentrate the cells. Through a series of decreasing pore-size membrane filtration steps, the insoluble materials in the culture/lysate are removed. The bulk material is typically treated with nuclease to degrade cellular DNA that could interfere with the column chromatography steps, and to reduce the residual DNA in the final product. The virus particles are captured from the lysate using immune-affinity chromatography or ion exchange chromatography.[69] The vector in the column elution fractions is concentrated and formulated to the bulk-drug substance using TFF.

QUALITY-CONTROL ASSAYS

As with any cGMP-compliant production system, assay development, assay qualification, and testing of raw materials, reagents, product intermediates, and final product is necessary. Baculovirus-specific assays for baculovirus tittering, Sf9 host cell protein and DNA residuals, baculovirus protein, and DNA residuals need to be utilized. Once produced, purified, formulated, and dispensed into containers, the vector batch is characterized using qualified assays to ensure it meets preset specifications for safety, identity, purity, potency, and stability.[74,75]

Assays for protein purity and identity

Batches are analyzed by silver, fluorescent dye, or Coomassie blue staining of capsid proteins separated on reduced and non-reduced SDS polyacrylamide gels. Three capsid proteins (VP1, VP2, and VP3) should be visible in the correct stoichiometry of approximately 1:1:10, have the correct molecular weights (~87, 72, 62 kDa), and free of non-AAV proteins. Identification of protein impurities using specific antibodies, mass spectrometry, or by protein sequencing can help trace the source (cellular, serum protein, etc.) in order to modify the manufacturing process to eliminate the impurity. Identity release testing involves not only confirmation by DNA sequencing of the un-rearranged vector genome that is packaged, but also the serotype of the capsid.

Assays for rAAV genome titer and the particle-to-infectivity ratio

Real-time polymerase chain reaction (PCR)-based assays are becoming the accepted standard to determine the vector genome titer, including quantitative PCR and digital PCR methods. Identifying a suitable PCR target in the vector is important (good efficiency, avoiding collinearity with endogenous cellular sequences, etc.). Plasmid and unpackaged vector DNA is digested with nuclease, and the nuclease is inactivated by heat. Treated virus is added directly to the PCR reaction, or encapsidated rAAV vector genomes are liberated by protease digestion, phenol extraction, and ethanol precipitation prior to PCR analysis.

Assay for infectious rAAV

To obtain an infectious titer, an endpoint dilution based on a $TCID_{50}$ format is paired with real-time PCR. Rep/cap cells in a 96-well plate format are infected with adenovirus helper, and serial dilutions of rAAV are made in replicates. Following infection, the wells are evaluated by real-time PCR

(see PCR target design above), and the Karber method is used to calculate the infectious titer.[76–79]

Assay for replication competent AAV

Dilutions of the rAAV preparation are used to infect 293 cells (or another suitable cell type that lacks AAV genes) in the presence of adenovirus (or another suitable helper virus) for 48 or 72 h. Since no additional wild-type AAV is added, only particles in the rAAV preparation that have packaged a wild-type or pseudo-wt AAV genome are capable of replication. Real-time PCR is conducted using primers in the AAV ITR and *rep* gene.

Assay for determination of intact AAV capsids and full capsids

The capsid enzyme-linked immunosorbent assay for several AAV serotypes is available and may be used to quantify total (infectious, non-infectious, and empty) viral particles. Alternatively, the optical density can be measured to calculate a total capsid titer. Together with the PCR assay for vector genome titer value, the total capsid titer can be used to determine the full particle number. Alternative methods using isopycnic density gradients or analytical ultracentrifugation (AUC) physically separate full and empty particles based on buoyant density or sedimentation velocity, respectively. AUC more directly determines the relative quantities of the full and empty capsids and, by acquiring the data in real time, does not involve gradient fractionation and subsequent analysis for capsid antigens and DNA.

Safety testing

Safety testing is conducted to ensure that process intermediates or the final product is free of detectable contaminating agents or process residuals. The primary safety tests include: adventitious agents (*in vitro* and *in vivo*), mycoplasma, endotoxin, sterility, and general safety.

Stability studies

A stability study should be designed to generate data for a purified rAAV viral vector at the proper storage temperature, formulation, and fill volume, and in the storage container used for animal and patient doses. The study is designed to demonstrate genetic and physicochemical stability and container integrity for at least the duration of the toxicology or clinical trial.

DISCUSSION

In the ensuing 15 years since the first-generation baculovirus/Sf9 system for rAAV production was published, the process has been gaining increasing acceptance, especially for applications requiring large vector doses or with large patient populations. Despite the growing popularity of the platform, the challenges remain, particularly with demonstrating comparability with vectors produced in mammalian cells. Independent analyses comparing rAAV produced in Sf9 and HEK293 cells demonstrated that the particles had similar properties and activities.[73,80] Often the disparity has been attributed to suboptimal ratio of the capsid proteins: virions deficient in VP1 have lower biological activity than virions with the VP1:VP2:VP3 model ratio of 1:1:10. However, enhancing the incorporation of VP1 into the capsid has been accomplished by modifying the VP1 initiation codon and the Kozak context.[73]

In addition to the improvements to the molecular reagents, process improvements have resulted in higher consistency of quality and yields. Establishing baculovirus clones and banks that are genetically stable and screened for higher AAV yields is important. Upstream processes using the TIPS facilitates a highly amplified scale-up process,[69,81] and downstream processes for recovery and purification, for example large-scale affinity chromatography, have been applied to the production of both clinical and non-clinical rAAV of a variety of serotypes.

As the utilization of the baculovirus platform becomes more widespread for recombinant protein, vaccine, and AAV vector manufacturing, issues general to the platform as well as specific to AAV production are being identified and addressed. Three examples are discussed below.

Endogenous rhabdovirus of Sf9 cells

The recent description of persistant *Spodoptera* rhabdovirus[82] present in Sf9 and Sf21 cells necessitates additional virus removal, virus inactivation, and quality assays to demonstrate the reduction of the Sf-rhabdovirus from the rAAV product. Although Sf-rhabdovirus does not infect mammalian cells and is considered to be nonpathogenic to humans, the virus and constituent proteins are known process impurities requiring qualified assays for removal or log-reduction. An alternative Sf-rhabdovirus–free cell line, Sf-RVN (rhadovirus negative), has been developed[83] and used for rAAV production, and this simplifies the downstream processing. Furthermore, adventitious viral agent testing includes not only human virus targets but also tests for viruses of insect origin, such as nodaviruses.

scAAV packaging

Although not experimentally demonstrated, the conversion of the single-stranded (ss) rAAV virion

DNA to transcriptionally competent duplex form (ds) has been inferred to be rate limiting for the onset of gene expression.[84] The formation of ds DNA occurs either by annealing the complementary plus and minus vector genomes or extension of the 3′-terminus by host cell DNA polymerase. When packaging vector genomes that are approximately 2.3–2.4 kb in length (~50% of the full-length AAV genome), a high percentage of the vector produced in Sf9 cells contains the 4.7 kb genome, and a lower percentage contains the 2.3 kb genome. The 4.7 kb genome is from the unprocessed replicative intermediate present in the Sf9 cells.[85] In contrast, producing scAAV vectors in HEK 293 cells utilizes a trs-defective ITR to prevent processing of the sc-replicative intermediate. This approach with a single functional ITR per genome limits the availability of templates and compromises vector yields. In addition, the rAAV virion genomes were often heterogeneous with truncations, a large content of plasmid DNA, and unidentified non-vector DNA.[72,86,87]

Packaging of non-vector DNA

Encapsidation of non-vector DNA occurs to a varying extent depending on the production process. Regardless, DNA *in cis* with the ITRs is the most frequently packaged non-vector DNA. To explain the packaging of DNA proximal to the AAV ITR, the input DNA substrates are duplex molecules where the extruded ITRs are capable of forming energetically stable cruciform structures.[88,89] These cruciform are resolved or "rescued" from the backbone via Rep-mediated non-homologous recombination occurring independently at each ITR. A plasmid where one ITR but not the other is resolved generates a molecule with the plasmid backbone covalently attached to the vector genome.[90] Initiating at the 3′-OH generated by p5 Rep protein nickase activity, the DNA is packaged until the process stalls due to increasing compressive force that exceeds the force generated by the ATPase/helicase complex.[91]

Robust replication of the vector genome requires accurate rescue of the vector genome from the backbone that retains functional origins of DNA synthesis. Ideally, the resulting geometric amplification of the template "enriches" the vector genome with two functional ITRs from the aberrantly rescued templates that do not efficiently replicate. Reported values for encapsidation of pDNA in vector produced by transient co-transection range up to 26% for scAAV and 2.9% for ssAAV.[87] Similarly, stable cell lines maintain integrated copies of the vector genome that are rescued and replicated following rAAV infection[92] and packaged into AAV capsids. In this case, cell-DNA is *in cis* with the ITRs. Thus, when inaccurate or aberrant rescue occurs, the vectors tend to package the adjoining cell DNA in measurable quantities.[86]

In the Sf9/baculovirus production process, the AAV vector genome is introduced into the Sf9 cell as an infectious baculovirus. The AAV vector genome is rescued from the baculovirus genome and amplified to very high copy number, as evidenced from cell extracts during the production process. Based on ethidium-stained agarose gels of the extracts, the replicated AAV genome copy number is estimated to exceed the baculovirus genome by >100-fold. Arguably, the independently replicating AAV vector genome was accurately rescued from the duplex form, and therefore would no longer be *in cis* with baculovirus DNA. Although low levels of DNAse-resistant baculovirus DNA remain, presumably representing encapsidated DNA, the levels are <1% of the total vg. Primer "walking" around the baculovirus genome indicates that the ITR proximal sequences are overrepresented and sequences further from the vector genome are in lower quantities.[9] The data agree with the robust amplification of the vector genome in Sf9 cells infected with Rep-expressing baculoviruses.

CONCLUSIONS

The Sf9/baculovirus platform for manufacturing infectious and potent rAAV gene transfer vectors is proving to be appropriate for several products that are currently in development, in early and late clinical trials, as well as for commercial distribution. Demonstration of scalability, adoption into cGMP processes, and compatibility with analytical methods supports the growing use of the system for manufacturing complex advanced gene therapies. Furthermore, rAAV vectors made in the Sf9/baculovirus system perform similarly to rAAV vectors made in mammalian cells when evaluated in animal models and humans. Continued improvements to the system itself and to upstream and downstream process manipulations will result in higher rAAV vector productivity, scale, yields, potency, and purity.

ACKNOWLEDGMENTS

We acknowledge the Brammer Bio process development, manufacturing, quality control, and quality assurance departments that have worked together to establish this robust platform. We are grateful to the past and current laboratory members (R.M.K.) who have contributed to developing the first- and second-generation processes.

AUTHOR DISCLOSURE

R.O.S. and R.M.K. are inventors on patents related to recombinant AAV technology and own equity in gene therapy-related companies. To the extent that the work in this manuscript increases the value of these commercial holdings, the authors have a conflict of interest. Portions of the technology described in this report are covered by United States and European patents assigned to the Secretary of the Department of Health and Human Services. A fraction of the licensing fees and royalty payments made to the National Institutes of Health is distributed to the inventors (R.M.K.) in accordance with U.S. Government and National Institutes of Health policy.

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Received for publication March 8, 2017;
accepted after revision March 25, 2017.

Published online: March 27, 2017.