

# Insect Cell-Based Recombinant Adeno-Associated Virus Production: Molecular Process Optimization

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## Abstract

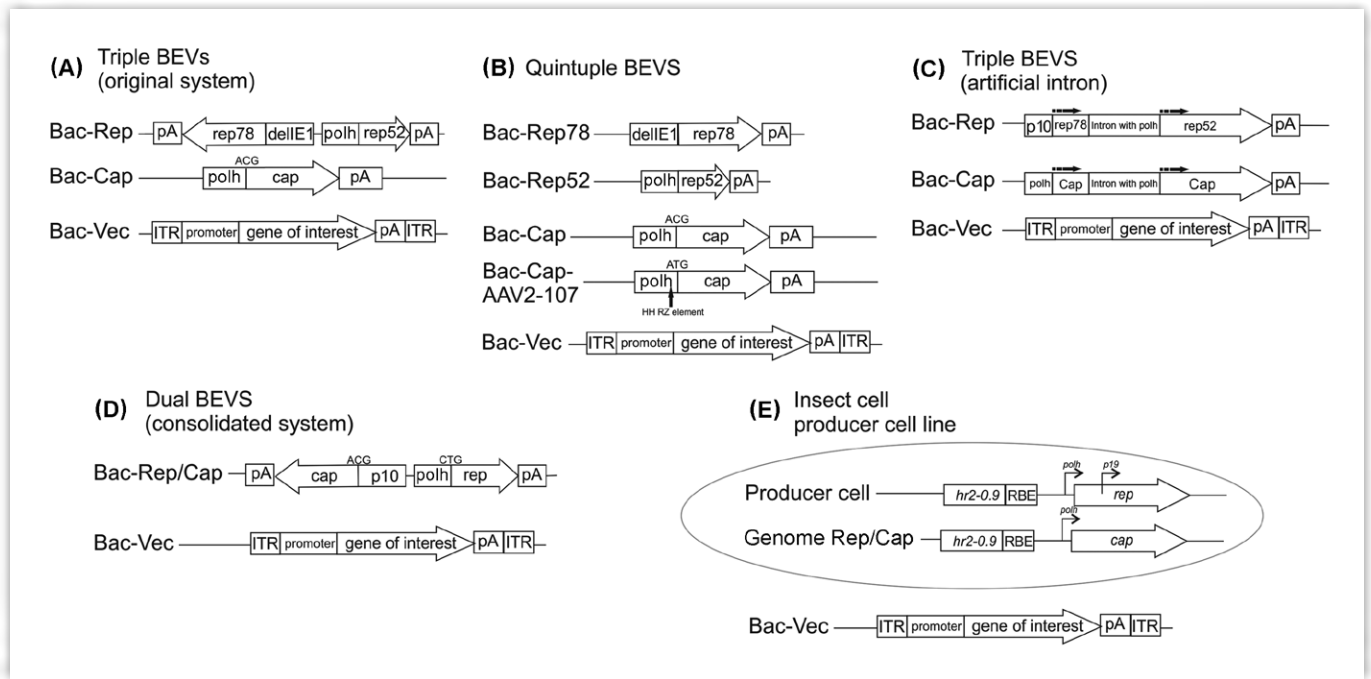
An increasing number of clinical trials, and the recent approval of the first gene therapy in Europe, alipogene tiparvovec (Glybera<sup>®</sup>, uniQure), holds promise for recombinant adeno-associated virus (rAAV) to become a mainstay in clinical practice. Since the molecular cloning of AAV in the 1980s, a plethora of production protocols/manufacturing systems for generating rAAV vectors have been developed. uniQure's manufacturing platform, which has received validation through regulatory approval, is also capable of supporting industrial-scale production based on the baculovirus expression vector system (BEVS) and insect cells. In this paper, we review the molecular process optimization of the various components of uniQure's rAAV production platform.

production of rAAV contains a number of the same necessary components: (1) a host cell, which serves as an environment for vector production; (2) AAV capsid proteins encoded by the *cap* gene; (3) AAV regulatory proteins needed for the replication and encapsidation, which are encoded by the *rep* gene; and (4) a transgene expression cassette flanked by AAV inverted terminal repeats (ITRs) (*i.e.*, the operon of interest used for gene transfer). In addition, a set of so-called "helper genes" are needed for the generation of rAAV, which can be provided by AAV helper virus (*e.g.*, adenovirus).

Protocols for the generation of rAAV can be classified into two main groups based on the use of either mammalian or insect cells for production. Further subdivision comes from the type of method for the DNA delivery. The first protocols for rAAV generation combined the infection of cells with adenovirus and transfection with plasmids carrying the AAV genome, which resulted in the generation of AAV progeny.<sup>[4,5]</sup> Subsequently, after the identification of helper genes, the transfection-only methodologies were developed, which extended the flexibility and modularity of rAAV production further.<sup>[6-9]</sup> It is the flexibility and relatively short time needed from plasmid to rAAV that makes the transfection-based approaches for generating AAV the preferred method for research purposes. On the other hand, a lack of consistency, which is intrinsically linked to transfection-based methods, and challenges related to scale-up of attachment-dependent mammalian cells limited the use of transfection-based protocols for an industrial implementation. To overcome these obstacles, alternatives were developed. Almost 14 years after the first protocols for generating AAV were established, Urabe, Ding, and Kotin proposed the use of BEVS and insect cells to produce AAV.<sup>[10]</sup> Because the process of culturing insect cells in suspension was already being used for the production of number of proteins successfully<sup>[11]</sup>, it seemed like a good candidate for generating rAAV. Therefore, it quickly became evident that the BEVS system had the potential to provide an alternative to mammalian cells for rAAV generation.

## Introduction

AAV was detected in 1966 and designated an impurity in adenovirus stocks.<sup>[1]</sup> The development of DNA recombination techniques allowed for cloning and molecular analysis of the AAV genome some 15 years later.<sup>[2,3]</sup> A number of AAV features such as the lack of association with a human disease, transduction of post-mitotic tissues, and a low immunogenicity made it an interesting candidate for gene transfer. The first protocols for a preparation of rAAV as a gene delivery vehicle dates back to 1988.<sup>[4,5]</sup> Since then, the production process, and our understanding of it, has been shaped by various rationales such as safety, yields per cell, scalability, impurities profile, simplicity, modularity, robustness, clinical application, and finally, commercial and regulatory requirements. In general, every protocol for the



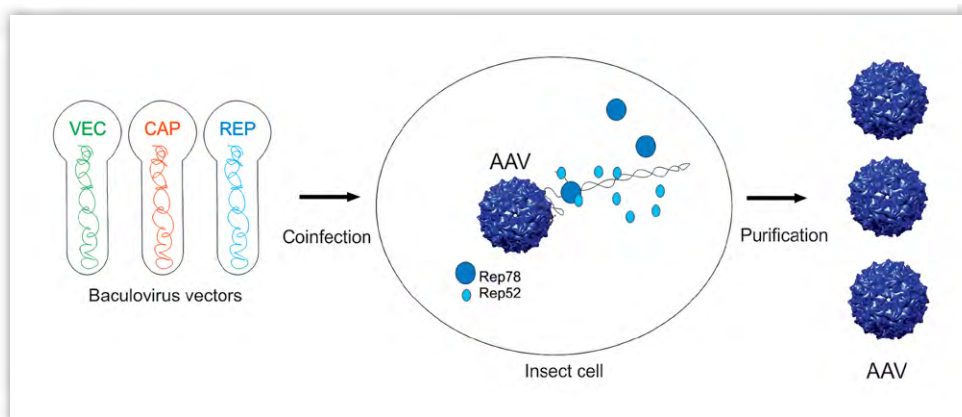
**FIGURE 1. Comparison of insect cells based rAAV production systems.** The various rAAV insect cell-based production systems are shown and classified based on the number of baculovirus constructs used.

In a published paper, Urabe *et al.*<sup>[10]</sup> described the original system using three baculoviruses encoding: (1) Rep proteins, which are responsible for replication/packaging; (2) Cap proteins, whose expression leads to the formation of a select vector capsid; and (3) a transgene, which contains the **therapeutic gene of interest** flanked by the ITRs. ITRs are the only AAV DNA genome fragments that are needed in *cis* for replication and packaging of the transgene cassette (Figure 1). Since insect cells are not natural hosts for AAV, a molecular redesign of the operons encoding *rep* and *cap* is necessary to achieve high yields of potent rAAV. This includes a change of the promoters for both *rep* and *cap*, and balancing the expression of all components by genetic engineering. After the initial publication of the three-baculovirus system<sup>[10]</sup> various other designs have

followed (Figure 1). This includes the five-baculovirus system<sup>[12]</sup>, the redesigned three-baculovirus system<sup>[13]</sup>, and the consolidated two-baculovirus system.<sup>[14, 15]</sup> Following these, a packaging insect cell line was reported.<sup>[16]</sup>

### Optimized rAAV Production Platform

uniQure developed their advanced rAAV production platform based on the original three-baculovirus technology<sup>[10]</sup> which they licensed from the National Institutes of Health (NIH) in the US. It consists of the three baculoviruses, each of which delivers a separate component to the cell necessary for a rAAV production. Bac-Cap encodes for capsid proteins of a given serotype, Bac-Rep encodes for Rep proteins involved in the replication and packaging, and Bac-Vec is carrying a transgene cassette flanked by ITRs (Figure 2).



**FIGURE 2. uniQure's rAAV manufacturing platform.** Three baculoviruses: Bac-Vec, Bac-Cap, and Bac-Rep are used to coinfect insect cells for the generation of rAAV. Bac-Vec harbors the expression cassette used for gene transfer. Bac-Cap and Bac-Rep drive expression of capsid and Rep proteins, respectively. (The AAV image was prepared with the software package, UCSF Chimera, a visualization system for exploratory research and analysis<sup>[23]</sup>, using AAV5 capsid structure file 3NTT.)

Simultaneous infection of insect cells with all three baculoviruses results in expression of the system components (*i.e.*, Rep and Cap proteins). The transgene cassette, which is flanked by ITRs, is replicated, forming the substrate for subsequent encapsidation into the assembled vector particles. The expression of VP1, VP2, and VP3 from the *cap* gene leads to formation of vector particles. Preformed capsids are filled with the transgene cassettes by Rep enzymes, ultimately leading to the formation of a mature rAAV (Figure 2). In order to adapt the system for a large-scale industrial application, a number of molecular process optimization were performed, including a redesign of the *rep* and *cap* operons.

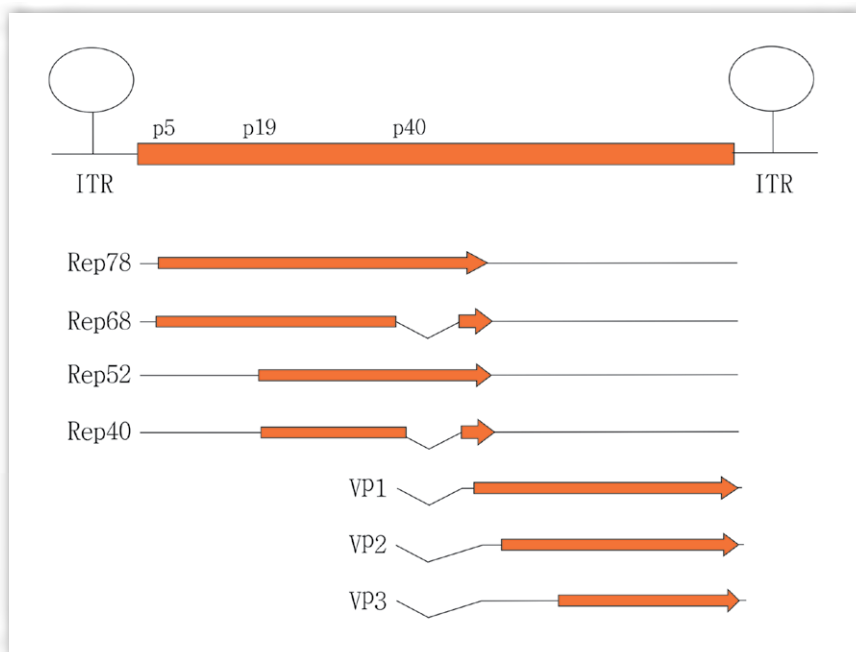
### Molecular Process Optimization

AAV is a small virus of approximately 22 nm in diameter. Its genome is only ~4.7 kilobase pairs (kbp) in length but encodes eight different proteins responsible for the capsid formation, genome replication, and packaging (Figure 3). In order to express all of its genes from a rather compact genome, AAV uses three different promoters, an alternative splicing, and a leaky ribosomal scanning. To replicate the expression of all the system components in a different host cell, a thorough molecular adaptation was necessary. The expression of AAV Rep proteins in the native host is driven by the two separate promoters (*i.e.*, *p5* and *p19*). This results in the generation of the four transcripts (the products of an alternative splicing) that encode the two large Rep78

and Rep68 proteins and the two small proteins, Rep52 and Rep40. To express Rep proteins in insect cells, Urabe and colleagues used the two separate baculovirus promoters (*i.e.*, the partially deleted promoter for the immediate-early 1 gene ( $\Delta ie-1$ )) which drive the expression of Rep78 and the polyhedrin promoter (*polh*) driving Rep52 (Figure 1).<sup>[10]</sup> The *cap* operon also needed adjustments. In mammalian cells, AAV expresses the three viral proteins (*i.e.*, VP1, VP2, and VP3) from the *p40* promoter, which generates a messenger that is spliced alternatively to a transcript that encodes VP1 and a second transcript that drives VP2 and VP3. The expression of the two proteins from the second transcript is achieved by a leaky ribosomal scanning of a non-canonical start of VP2, which is an ACG codon. Due to the differences in splicing between the natural AAV host and the insect cells, the simple adaptation of the *cap* operon by promoter exchange did not result in the generation of potent capsids.<sup>[10]</sup> Therefore, Urabe *et al.* modified the translational start of VP1 to resemble that of VP2 in order to rely on the ribosomal scanning mechanism principle (Figure 1).<sup>[10]</sup> These alterations resulted in an expression of three VPs in nearly correct stoichiometry and assembly of the potent vectors.

### Tailoring the AAV Capsid for BEVS

The first modifications to AAV capsids for expression in insect cells were demonstrated with serotype 2.<sup>[10]</sup> However, upon adaptation of other AAV serotypes (*e.g.*, serotype 1), it was noticed that more fine tuning was required to balance

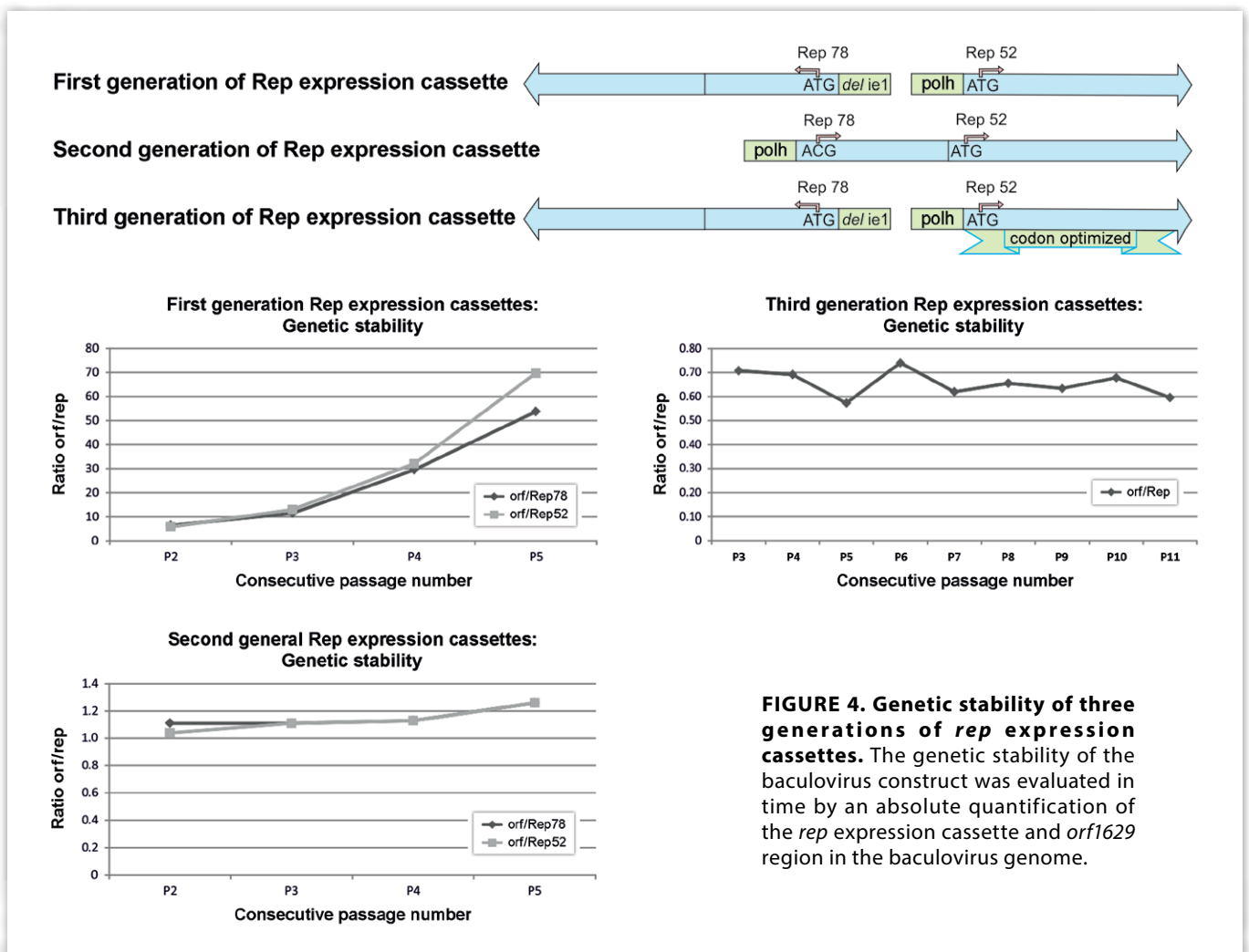


**FIGURE 3. Genetic organization of AAV.** The AAV genome is composed of two open-reading frames, *rep* and *cap*, located between ITRs, encoding four nonstructural proteins (Rep) involved in replication/packaging, and three proteins that form the capsid: VP1, VP2, and VP3.

the ratio between VP1 and VP2.<sup>[17]</sup> Therefore, a number of AAV type 1 variants were created to increase the expression of VP1 protein by modifying the VP1 initiation triplet and its surroundings. Varieties of non-canonical start codons were used. These subtle changes to the initiation start of VP1 resulted in altered levels of VP1 protein expression, which was reflected in the assembled capsids. Likewise, the initial development results showed low-level VP1 incorporation into capsids whereas alternative suboptimal initiation codons led to equal or improved VP1/VP2 ratios.<sup>[17]</sup> VP1 plays a crucial role in intracellular trafficking. It contains so-called phospholipase A<sub>2</sub> domain (PLA<sub>2</sub>) domain, which is normally hidden inside of the capsid particle. It is exposed at a low pH level usually found in the endosomes. Once this N-terminal part of VP1 is exposed, it can display its enzymatic activity and hydrolase, specifically the 2-acyl ester (*sn*-2) bond of phospholipid substrates, resulting in an release of lysophospholipids and free fatty acids allowing, in turn, endosomal escape of AAV.<sup>[18,19]</sup> Therefore, the levels of VP1 incorporation have a great impact on the vector potency.

### Rep Improvements: Stability, Yields, and Purity

The genetic stability of baculovirus vectors is one of the key parameters for an industrial-scale production, which allows the preparation of vector seed banks and the use of their consecutive passages in the generation of rAAV. The original, first-generation Rep expression cassette baculovirus vector consists of two Rep expression units cloned in opposite orientations, forming a large inverted repeat.<sup>[10]</sup> The repetitive nature of this construct results in genetic instability over time.<sup>[12]</sup> In order to prevent the gradual losses of Rep expression due to recombination between the Rep78 and Rep52, a redesign of the original construct was performed. This resulted in the generation of the second-generation Rep expression cassette, which contains a single, overlapping operon-encoding Rep78 and Rep52 driven by the polyhedrin promoter.<sup>[20]</sup> The translation initiation start site of Rep78 was altered to non-canonical initiation codon (Figure 4). As a result, polycystronic mRNA was generated, and due to leaky ribosomal scanning, the translation of both Rep78 and Rep52 was allowed.<sup>[20]</sup> This



**FIGURE 4. Genetic stability of three generations of rep expression cassettes.** The genetic stability of the baculovirus construct was evaluated in time by an absolute quantification of the rep expression cassette and orf1629 region in the baculovirus genome.

baculovirus vector turned out to be genetically stable during the serial amplification and therefore, suitable for an industrial-scale production of rAAV (Figure 4). The genetic stability of the baculovirus construct was evaluated in time by an absolute quantification of the expression cassette and *orf1629* region in the baculovirus genome. To this aim, the baculovirus was serially amplified in Sf9 cells after which quantitative polymerase chain reaction (qPCR) analysis was performed on baculovirus DNA. The constant ratio of the baculovirus genome versus the expression cassette DNA indicates recombinant baculovirus vector stability during the serial amplification steps. Rep enzymes have crucial role in the replication and packaging of the transgene cassette of interest into the preformed AAV capsids. Due to the central role they play in the rAAV production process, even a slight modification of these enzymes may have profound effects on the quality of the produced vectors. Therefore, a library containing various designs of the two Rep expression units was created and tested for both improvements in AAV vector yields and industrial quality requirements of vectors that are subject to pharmaceutical market authorization.<sup>[21, 22]</sup> As a result of the above-mentioned strategies for vector production, a third-generation Rep expression cassette was generated. This cassette contains codon-optimized versions of Rep52<sup>[22]</sup>, for example, engineered in order to prevent a homologous recombination that was noted with the first generation of Rep expression cassette.<sup>[10]</sup> The newly created third-generation

Rep expression cassette was suitable for industrial rAAV production due to robust genetic stability for at least 11 serial baculovirus passages, and improved rAAV features (Figure 4).<sup>[22]</sup> In particular, the following parameters were improved: an increased number of full particles compared to the number of total particles<sup>[22]</sup>, and a significantly improved DNA impurities profile (data not shown).

## Discussion

The current and future development of AAV-based gene therapies and its industrial applications are tightly linked to the design of their manufacturing systems. The scalability, production cost-effectiveness, and vector quality are important considerations. For the generation of rAAV, BEVS appears to solve many of these hurdles. Insect cells, in combination with baculovirus transduction, offer scalability, robustness, and consistency. Vector safety is reinforced by use of non-pathogenic vectors to deliver the DNA components during production. The process-related DNA contaminants are low, and due to their origins (*i.e.*, invertebrate cells and baculoviruses), are unlikely to be transcriptionally active in mammalian cells. Another level of safety relates to the use of baculovirus promoters for expression of AAV *cap* and *rep* genes, and the redesign of their operons. Therefore, the theoretical risk of expressing these proteins, driven from putative DNA contamination or the generation of replication-competent AAV (rcAAV) due to genetic recombination, is extremely low.

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PROOF 2: 25 SEPT 2014

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