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REVIEW

Advancements in molecular design and bioprocessing of recombinant adeno-associated virus gene delivery vectors using the insect-cell baculovirus expression platform

Pranav R.H. Joshi[1] | **Alina Venereo-Sanchez**[1] | **Parminder S. Chahal**[2] | **Amine A. Kamen**[1]

[1] Department of Bioengineering, McGill University, Montréal, Quebec, Canada

[2] Human Health Therapeutics Portfolio, National Research Council of Canada, Montréal, Quebec, Canada

Correspondence
Amine A. Kamen, Department of Bioengineering, McGill University, Montréal, QC, Canada.
Email: amine.kamen@mcgill.ca

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Abstract

Despite rapid progress in the field, scalable high-yield production of adeno-associated virus (AAV) is still one of the critical bottlenecks the manufacturing sector is facing. The insect cell-baculovirus expression vector system (IC-BEVS) has emerged as a mainstream platform for the scalable production of recombinant proteins with clinically approved products for human use. In this review, we provide a detailed overview of the advancements in IC-BEVS for rAAV production. Since the first report of baculovirus-induced production of rAAV vector in insect cells in 2002, this platform has undergone significant improvements, including enhanced stability of Bac-vector expression and a reduced number of baculovirus-coinfections. The latter streamlining strategy led to the eventual development of the Two-Bac, One-Bac, and Mono-Bac systems. The one baculovirus system consisting of an inducible packaging insect cell line was further improved to enhance the AAV vector quality and potency. In parallel, the implementation of advanced manufacturing approaches and control of critical processing parameters have demonstrated promising results with process validation in large-scale bioreactor runs. Moreover, optimization of the molecular design of vectors to enable higher cell-specific yields of functional AAV particles combined with bioprocess intensification strategies may also contribute to addressing current and future manufacturing challenges.

KEYWORDS
AAV-bioprocessing and gene therapy, insect cell-baculovirus expression vector system, One-Bac, Three-Bac, Two-Bac

Abbreviations: AAV, adeno-associated virus; AcMNPV, *Autographa californica* multinuclear polyhedrosis virus; BIIC, baculovirus infected insect cells; BV, baculovirus; Cap, capsid proteins; Cp, capsids; DOE, design of experiment; egt, ecdysteroid UDP-glucosyltransferase; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescence protein; GMP, good manufacturing practice; GOI, gene of interest; HEK293, human embryonic kidney 293; hGFP, humanised green fluorescence protein; Hi-5, High Five™ (*Trichoplusia ni*); hpi, hours post infection; hr2, homologous region 2; HSV, herpes simplex virus; IC-BEVS, insect cell baculovirus expression vector system; IE-1, immediate early 1; IL-2, interleukin 2; INF-β, interferon beta; ITR, inverted terminal repeat; IVP, infectious virus particle; MOI, multiplicity of infection; mRNA, messanger-RNA; NIIC, non-infected insect cells; ORF, open reading frame; pfu, plaque forming unit; PLA_2, phospholipase A_2; PTMs, post-translational modifications; rAAV, recombinant adeno-associated virus; RBE, Rep-binding element; rBV, recombinant baculovirus; Rep, replicase; Sf9, *Spodoptera frugiperda*; STB, stirred-tank bioreactor; TU, transducing units; USFDA, U.S. Food and Drug administration; VG, viral genome; VP, viral proteins; wtAAV, wild-type adeno-associated virus; wtITR, wild-type inverted terminal repeat

1 | INTRODUCTION

Since the establishment of the first successful continuous insect cell line from *Bombyx mori* in 1959,[1] sustained efforts have led to the establishment of insect cells as a workhorse for the expression of recombinant proteins for research and clinical applications. The discovery and isolation of *Autographa californica* multinuclear polyhedrosis virus (AcMNPV) in 1971 was instrumental in positioning the insect cell-baculovirus expression vector system (IC-BEVS) for heterologous protein expression.[2] Over the following years, extensive studies related to the biology of the baculovirus, infection kinetics, genome sequences, and structural variants were undertaken.[3,4] The first breakthrough was the discovery of the polyhedrin strong late promoter (*polh*) by Smith et al.[5] in 1983, which drives strong expression of the polyhedrin protein in the late infection phase in wild-type baculovirus (BV); however, it is not an essential element for a recombinant baculovirus vector. Smith et al.[5,6] replaced the polyhedrin gene with that of human interferon-beta (INF-β) and interleukin-2 (IL-2) and demonstrated a *polh*-driven robust expression of these proteins using IC-BEVS. A year later, another strong late promoter, *p10*, was discovered and its functionality in protein expression studies was demonstrated.[7]

The cell line derived from the fall armyworm, *Spodoptera frugiperda* (Sf21 and derived clone Sf9) and the cabbage looper *Trichoplusia ni* (High Five; Hi5) were established as continuous cell lines and extensively used due to their susceptibility to baculovirus infection and favorable growth characteristics in adherent, and thereafter, suspension cell cultures.[8–12] Despite their routine use, both cell lines displayed notable differences. The Hi5 cells offered a comparatively higher yield of secretory proteins[13–15] and the addition of alpha1,3-fucose, a potentially immunogenic glycan structure, to the expressed proteins, which were absent in the proteins produced in Sf9 cells.[16] The Sf9 cells showed higher baculovirus susceptibility[17] and hence are often favored by researchers as host cells for both baculovirus and recombinant protein production. Despite these differences, both cell lines have been routinely used to produce therapeutic biologics with a history of regulatory approval for human use.[18,19]

In the early '90s, cell culture engineers took the lead in studying the insect cell growth kinetics and metabolism in the serum-supplemented and serum-free culture medium. Serum-free cell media with shear protective properties were developed, enabling insect cell growth in suspension cell cultures in shake flasks and bioreactors,[20–23] demonstrating the scalability and robustness of the IC-BEVS process for protein production.

Initially employed for the production of baculoviruses as biopesticides, IC-BEVS quickly gained popularity for the expression of a broad spectrum of recombinant proteins, including enzymes, glycoproteins, recombinant viruses, and vaccines.[13,24] The IC-BEVS platform has been used for the production of veterinary vaccines such as Porcilis pesti, Circumvent PCV, CircoFLEX,[25] and human vaccines such as Cervarix,[18,26] and Flublok.[11] Regulatory approval of Cervarix, a virus-like particle-based vaccine against cervical cancer, was a critical milestone as it was the first biologic produced in insect cells and approved for human use.

The adeno-associated virus (AAV) is currently gaining widespread popularity in gene therapy applications for the correction of monogenic disease conditions. In the last decades, there has been a steady growth in AAV-based gene therapy clinical studies, which have been supported by the accelerated development of IC-BEVS scalable production systems for AAV manufacturing. In addition to the approval of Glybera by the European Medicines Agency in 2012,[27] from a regulatory perspective, a more recent and significant milestone was the breakthrough designation by the USFDA of BioMarin's Hemophilia A gene therapy candidate, a recombinant adeno-associated virus 5 (rAAV5) gene delivery vector produced in insect cells using BEVS.[28] This event further contributed to aligning the IC-BEVS manufacturing process of AAV with the current standard of Good Manufacturing Practice (GMP).

In this review, focusing exclusively on the advancements of the IC-BEVS as a platform for AAV production, we provide insights into the evolution of molecular designs of baculovirus vectors and their key features. AAV manufacturing technologies using IC-BEVS are discussed from a process developer's standpoint. It is foreseeable that further vector optimizations combined with innovative process intensifications may significantly contribute to addressing current and future manufacturing challenges, enabling higher cell-specific and total yields of functional AAV gene delivery vectors of different serotypes.

2 | AAV TAXONOMY AND GENOME ORGANIZATION

AAV is a single-stranded DNA virus that belongs to the family *Parvoviridae* and the genus *Dependovirus* because of its replication-deficient nature. Members of this family have evolved over the years, expanding their spectrum of hosts, which includes mammals and insects.[29] Although mammalian cells are the natural host of adeno-associated virus, based on the common ancestral relationship (**Figure 1**A) and given that insect cells are the natural hosts of viruses belonging to the *Densovirinae* subfamily, they should facilitate the rescue and replication of AAV genes and support the expression of corresponding proteins. Ruffing et al.[30] in 1992 reported baculovirus-mediated AAV *cap* gene delivery, VP proteins expression, and capsids assembly in insect cells. However, the first report of Rep expression, Rep-mediated rescue of ITR-flanked *transgene*, and production of functional AAV vectors in insect-cell was published a decade later by Urabe et al.[31] Both reports confirmed the functionality of insect cells as a suitable host for adeno-associated virus production.

The wild-type AAV (wtAAV) genome is a linear, single-stranded DNA with a size of approximately 4.7 Kb.[32] Major coding regions consist of the genes responsible for the expression of non-structural regulatory Rep proteins and structural capsid proteins (Cap or VP). This coding region is flanked at both ends by an inverted terminal repeat (ITR) sequence, which acquires a T-shaped hairpin-like structure formed via the complementary base pairing of palindromic sequences. Figure 1B represents the genomic map of wtAAV2 and its transcriptional and translational profile.[32] The recombinant AAV (rAAV) vector retains only the ITR from the wild-type virus, the element essential for vector

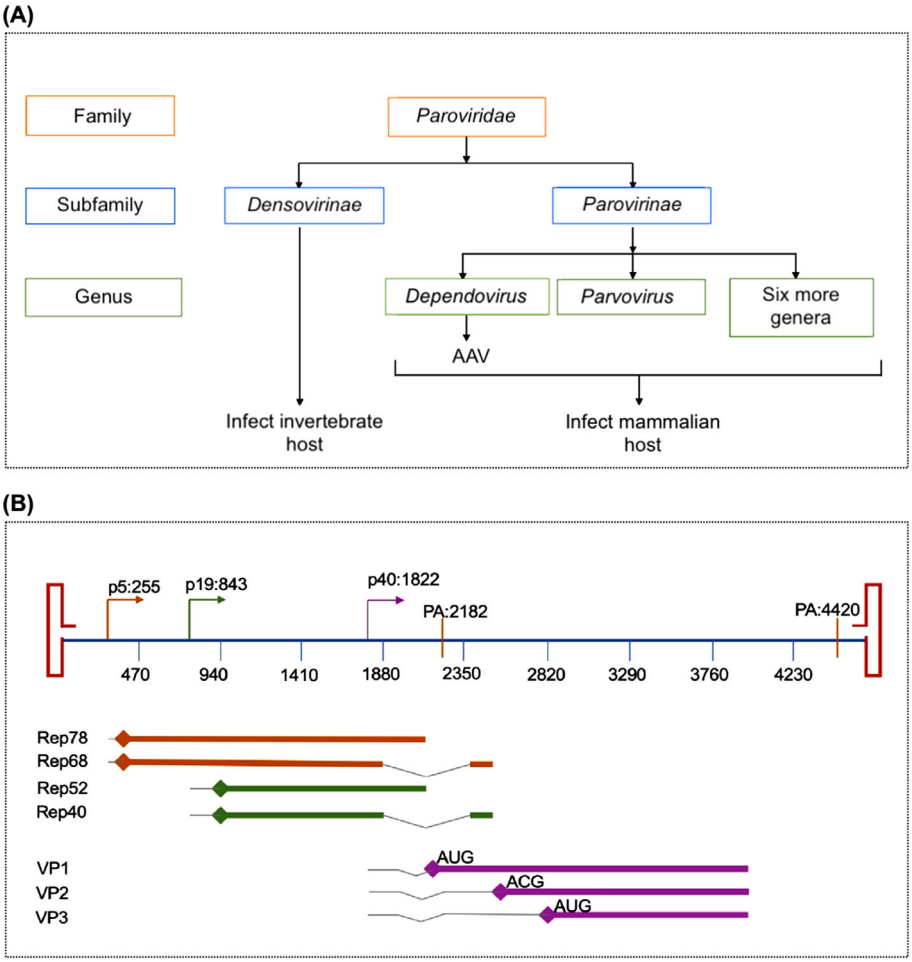

FIGURE 1 AAV family tree and wtAAV2 genome map and expression profile. (A) AAV family tree. The *Dependovirus* AAV mainly infects mammalian hosts and replicates in the presence of a helper virus. Though evolutionary distant yet related to the *Densovirinae* subfamily, it shares common structural and functional features with viruses from this subfamily, which mainly infect invertebrate hosts such as insects. (B) wtAAV2 genome map and protein expression profile. The AAV genome schematic illustrated is based on a previous publication.[39] The numbers represent the nucleotide position. The viral genome is flanked by two ITRs, one at each end. Two promoters in the left ORF drive the expression of four regulatory Rep proteins, whereas a single promoter in the right ORF drives the expression of three capsid protein subunits (VP1, VP2, and VP3) from a single mRNA transcript. Intron splicing generates four Rep proteins of different sizes from two mRNA transcripts, whereas the leaky scanning of weak translational codons results in the production of three VP proteins in a stochastic ratio of 1:1:10. The diamond shape represents the N-terminal of the peptide. Two polyadenylation (PA) signal sequences are shown via the orange line

genome encapsidation, whereas the *rep/cap* genes are replaced by an expression cassette consisting of a gene of interest flanked by ITR. The *rep/cap* genes essential for rAAV production are supplied in *trans* via a suitable vector, such as baculovirus vector in case of insect cell-based production system.

3 | MOLECULAR DESIGN OF BACULOVIRUS EXPRESSION VECTORS AND CELL LINES FOR THE PRODUCTION OF RAAV

Since insect cells are the heterologous host of AAV, substantial efforts have been made in the last two decades in applying molecular engineering approaches to achieve efficient AAV production employing the IC-BEVS platform. Building upon the intrinsic advantages of the IC-BEVS platform, efforts have been directed towards achieving higher expression stability, improved AAV yield, and streamlining manufacturing processes. Below, we summarize the progressive development of various strategies and highlight features that might be of consideration when using IC-BEVS for AAV production. The bioprocessing features are discussed separately in Section 4.

3.1 | First generation IC-BEVS: Three-Bac

The production of functional rAAV2 vectors in insect cells was first reported in 2002 by Urabe et al.[31] This system required co-infection of *S. frugiperda* (Sf9) cells with three baculovirus expression vectors (BEVs), each delivering one of the three essential genes (*rep*: Bac-Rep, *cap*: Bac-Cap, and ITR-flanked gene of interest: Bac-ITR-GOI) for AAV

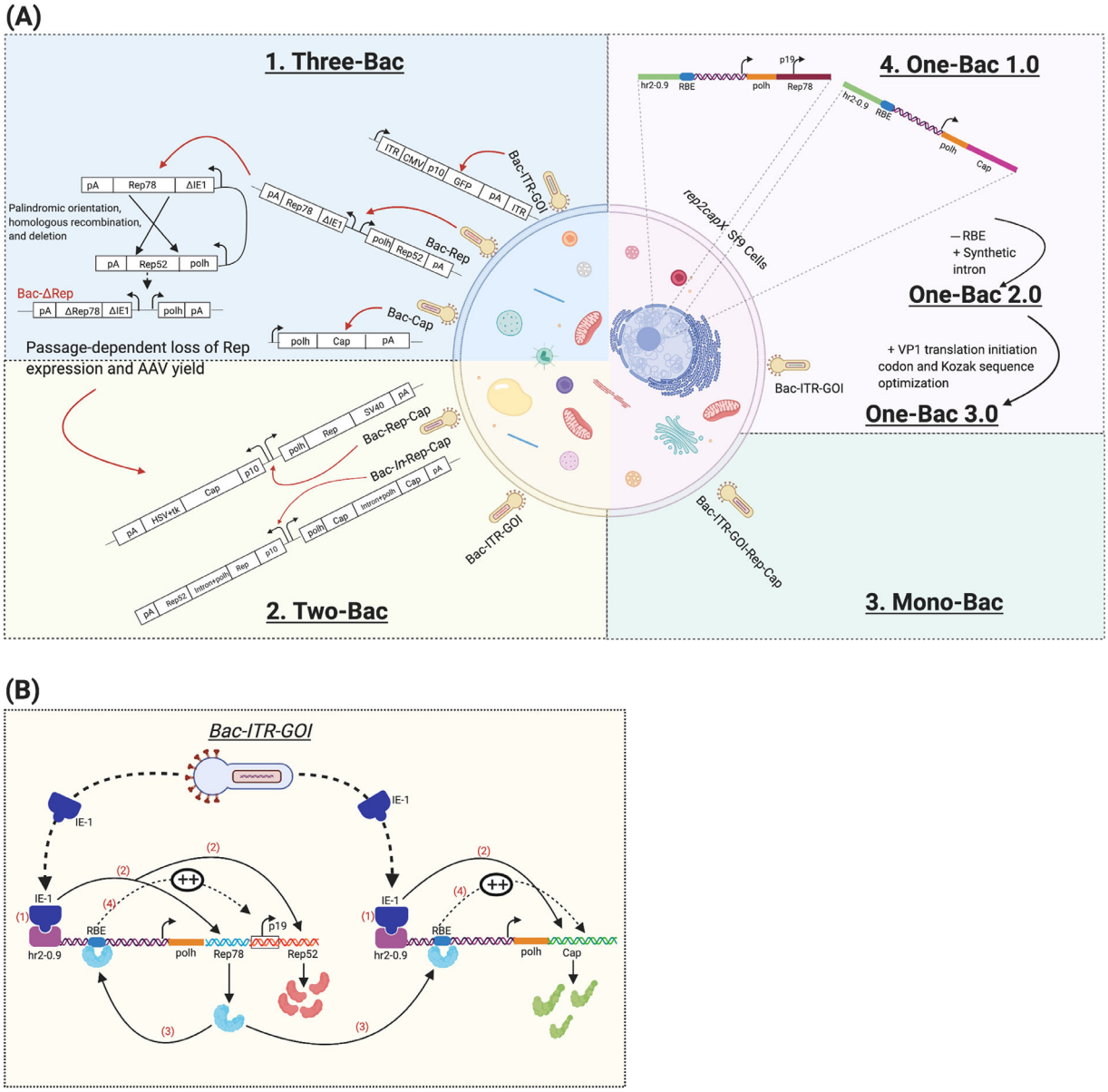

FIGURE 2 AAV insect cell baculovirus expression systems for AAV production and mechanism of inducible expression in One-Bac. (A) Four major systems for rAAV production using the IC-BEVS. The Three-Bac system consisted of three rBEVs vectors, each carrying a specific gene sequence.[31] Further study with this system identified critical shortcomings, related to the expression stability of rBEVs and the underlying mechanism.[33] The follow-on systems exhibited better expression stability of rBEVs overextended passage numbers and required only two rBEVs' co-infections for AAV production. The systems such as One-Bac or Mono-Bac further simplified the manufacturing process requiring only a single rBEV co-infection. One-Bac consists of *a rep2capX* packaging cell line and a Bac-GOI vector,[38] whereas in Mono-Bac, a single BEV carries all the necessary gene (Bac-ITR-GOI-Rep2-CapX) sequences.[37] (B) The postulated mechanism of induction and amplification of *rep/cap* genes in One-Bac1.0. Bac-ITR infection provides IE-1, which activates hr2-0.9 (1) and induces Rep78/52 or Cap expression (2). The expressed Rep78 further forms a complex with RBE (3) and induces the second round of amplification of Cap or Rep52 expression (4), resulting in a higher Rep52:Rep78 ratio which is reported to favor a higher vector yield.[38] The schematics of Bac-vector expression cassettes depicted in this figure were adapted from the original publications and recreated using Biorender.com

generation, hence the name Three-Bac (**Figure 2**A). The successful AAV vector production demonstrated that AAV genes, when delivered as an integral part of the baculovirus genome under the transcriptional control of insect promoters, can successfully undergo DNA and protein processing pathways in insect cells. They adequately provided the components and cellular machinery required for the rescue and replication of AAV genes, AAV protein expression, capsid assembly, and viral genome encapsidation. Moreover, unlike mammalian cells, no additional helper virus (adenovirus or herpes simplex virus) superinfection or supplementation of helper genes was necessary for AAV production, indicating that the baculoviral vector provided all or any essential helper function(s).

3.2 | Second generation IC-BEVS: Two-Bac, Mono-Bac, and One-Bac1.0

Although initially successful for AAV2 production, the Three-Bac did not achieve widespread application because of the genetic instability of BEVs and consequent loss of AAV yield, and the inability to produce functional AAV vectors of other serotypes.[33,34] These limitations were partially resolved in the follow-on systems based on two and one BEV, namely, Two-Bac and One-Bac/Mono-Bac. In the Two-Bac system reported by Smith et al.[35] and Chen,[36] *rep* and *cap* expression cassettes were introduced into a single BEV (Bac-Rep-Cap or Bac-*In*-Rep-Cap) (Figure 2A), therefore now requiring co-infection of only two baculoviruses. AAV production with Mono-Bac required Sf9 cells to undergo infection with a single BEV harboring all three essential genes.[37] In contrast to the Mono-Bac, the One-Bac1.0 reported by Aslanidi et al.[38] consisted of baculovirus (BV) inducible stable Sf9 packaging cells harboring the *rep2* (AAV2 *rep*) and *capX* (X = serotype of interest) genes. Infection of these cells with a single baculoviral vector carrying an ITR-flanked *transgene* cassette resulted in an inducible and amplified expression of AAV proteins offering up to 10-fold higher cell-specific yield of AAV2 vector particles compared to the Three-Bac. Comprehensive reviews providing detailed insights into the molecular design of each of these systems have been published previously.[39,40]

3.3 | Advancements in IC-BEVS

The development of the first- and second-generation BEV systems and their subsequent improvements were driven by the combined aims of achieving sustained expression of AAV proteins (Rep and Cap) in stoichiometric proportions, improved AAV production yield and functionality, and overall vector quality. The following sections summarize the various strategies undertaken to achieve each of these goals.

3.3.1 | Enhancement in AAV Rep expression

The head-to-head orientation of Rep78 and Rep52 expression cassettes of the Three-Bac's Bac-Rep vector was found to be genetically unstable due to palindromic orientation (Figure 2A), causing passage dependent loss of Rep78/Rep52 expression and substantially low functional AAV titer.[33] Kohlbrenner et al.[33] restored expression stability by isolating the Rep78 and Rep52 sequences onto different baculovirus vectors, although quadruple infection was now required, complicating the Three-Bac-based AAV production process even more.

Chen[36] reported stable Bac-Rep expression by introducing a synthetic intron in the Rep coding region. The *polh* promoter of this synthetic intron drove the Rep52 expression independent of Rep78, resulting in stable expression of both proteins from two different mRNAs for extended passage numbers. In contrast, in the Two-Bac system, Smith et al.[35] modified the nucleotide sequences of the Rep78 translational initiation codon providing weak Kozak consensus and the codons downstream of Rep78. The resulting leaky ribosomal scanning of Rep78 and concomitant expression of both Rep78/Rep52 proteins from a single mRNA transcript showed Bac-Rep stability up to seven passages.

A more recent modification utilized the weak initiation codon for Rep78, which enabled partial exon skipping and subsequent expression of downstream Rep52 from a single expression cassette and reportedly enhanced Bac-Rep stability for at least five passages.[41]

In the Mono-Bac,[37] the *rep-cap* expression cassette reported in Two-Bac[35] was integrated into the *egt* (ecdysteroid UDP-glucosyltransferase) locus of the baculovirus genome. The very late phase expression of Rep proteins, specifically after baculovirus DNA replication, minimized Rep-induced excision of the *rep-cap* expression cassette from the baculovirus genome, resulting in sustained expression stability. Furthermore, this late phase expression of Rep proteins was also critical to avoid Rep-induced excision of AAV expression cassette[42] and its integration stability in baculovirus genome as this can ultimately affect the stability of Mono-Bac BEV and overall production titer of AAV vectors.

In contrast to some of the above systems where a separate strong *polh* promoter was used for Rep52 expression, Aslanidi et al.[38] retained the partially active native *p19* promoter of Rep52 to drive its expression in One-Bac 1.0 insect cells. The introduction of the Rep-binding element (RBE) resulted in Rep52 expression higher than Rep78 via a feed-forward loop achieving a desirably higher Rep52:Rep78 ratio (Figure 2B). The modified version of the One-Bac Sf9 cell line harboring a stably integrated *rep-cap* cassette demonstrated higher expression stability than previously described Bac-Rep vectors, up to at least 35 cell-culture passages.[43]

3.3.2 | Restoration of VP1 expression proportion for improved AAV functionality

In mammalian cells, the natural host of AAV, the combination of alternate mRNA splicing and leaky ribosomal scanning of a weak VP2 translational initiation codon enables the expression of three VP proteins in their prototypic ratio (VP1:VP2:VP3 = 1:1:10) from a single mRNA transcript.[32] Sufficient expression and subsequent incorporation of VP1 subunit, harboring phospholipase A_2 (PLA_2) like enzymatic domain, in the AAV capsid are critical as the PLA_2 is reportedly responsible for late endosomal escape and consequent perinuclear localization which are essential steps for the transduction efficiency of AAV vectors.[44,45] Initial failures at attaining the expression of three VP proteins in given prototypic proportions using Three-Bac necessitated the use of a weak VP1 translational initiation codon, which, leveraging leaky ribosomal scanning, produced three VP proteins in the desired ratio.[31] Although successful for AAV2, the Three-Bac was later reported to produce sub-optimal expression levels of VP1 of other clinically relevant serotypes such as AAV5 and AAV8, generating defective/non-transducing vector particles.[33,34]

Based on the original findings by Kozak,[46,47] Urabe et al. hypothesized that, compared to AAV2 VP1, a single nucleotide difference at a critical position in the Kozak sequence surrounding the AAV5 VP1

translational initiation codon may be responsible for sub-optimal ribosomal scanning efficiency and reduced VP1 expression levels. Building on this hypothesis, complete or partial VP1 domain swapping strategies have been shown to generate AAV chimeric vectors (AAV2/5 or AAV2/8) with restored VP1 expression levels and improved transduction efficiency.[33,34]

Chen achieved sufficient VP protein expression by placing VP1 under the transcriptional control of a strong *polh* promoter, whereas the VP2/3 expression was governed by a synthetic intron.[36] As a result, a higher and stable expression of all VP proteins was achieved from two separate mRNA transcripts.

Similar to the original Three-Bac, the Two-Bac[48] and the One-Bac systems, harboring the weak VP1 translational initiation codon, also showed suboptimal expression levels of VP1 proteins in certain serotypes other than AAV2.[49] Bosma et al.[48] reported that the selection of a modified optimal VP1 translation initiation codon and the associated downstream nucleotide sequence resulted in optimal stoichiometric expression of all three VP proteins generating an AAV5 vector with improved functionality in the Two-Bac system. In One-Bac2.0, the second-generation One-Bac, the original strong VP1 codon, and a synthetic intron were introduced to restore the expression level of AAV5 VP1.[50] In a more recent modification, the third-generation One-Bac3.0, a combination of the modified translational initiation codon and Kozak sequences offered suboptimal translation efficiency yet sufficiently high VP1 expression levels in more than one serotype, reportedly in both AAV5 and AAV9, resulting in highly transducing vectors.[51]

Differently from previous reports, Galibert et al.[52] found that baculovirus protease-driven degradation of VP1 led to the production of defective/VP1 deficient AAV vectors of serotype 1, 6, and 8 in insect cells. Deletion of cathepsin expressing *v-cath* locus from the baculovirus genome or the use of the E64 protease inhibitor improved the post-expression stability of VP1 and restored the potency of the vector.

A novel strategy involving the use of the 5′-untranslated region (5′-UTR) to regulate AAV VP protein expression levels was reported.[53] The engineered 5′-UTR sequence, forming a hairpin-like secondary structure, was introduced upstream of the VP1 codon to generate AAV2 and AAV9 vectors with the desired VP1 level.

3.3.3 | Improved efficiency and specificity of vector genome packaging

Concerns regarding the co-packaging of non-vector nucleotide sequences, including antibiotic selection marker genes from the bacmid backbone, were first raised during the regulatory evaluation of Glybera.[54] The baculovirus DNA sequences proximal to the AAV ITR in the Bac-ITR vector are also prone to reverse packaging due to ITR's role as a packaging signal.[55] Even though collaterally packaged baculoviral gene sequences, in general, are not expressed in mammalian cells, the delivery of such non-transgene elements may evoke a potential immunogenic response in vivo and hence is considered a safety risk. Therefore, dedicated efforts have been invested in improving packaging efficiency and specificity.

The inverted terminal repeat sequence of the AAV genome is the only element from the wild-type AAV that is maintained in a recombinant vector and is present in *cis* in a transgene expression cassette. A recent report by Savy et al.[56] showed that the unmodified AAV2 wild-type ITR (wtITR) sequence promotes enhanced and specific encapsidation of the rAAV8 vector sequence in contrast to truncated ITRs, which is found in traditional packaging plasmids such as pSUB201 and its variants. The latter was found to be responsible for non-specific packaging. Specifically, with the use of intact wtITR, two-dimensional improvements were reported. First, the relative proportion of packaged capsids increased to 40% from 10%, and second, the packaging of non-specific sequences was reduced by 10-fold.[56]

The incorporation of RBE in the original One-Bac1.0 was responsible for high-frequency co-packaging of *rep/cap* sequences in AAV capsids, confirming its role as a packaging signal.[50] Excision of the RBE component in the One-Bac2.0/AAV5 cell line resulted in more than 3- and 4-log reductions in the frequency of *rep* and *cap* sequence co-packaging, respectively, with final *rep/cap* packaging levels reaching as low as 0.001% and 0.02%, respectively.[50] The AAV vector produced using RBE-negative One-Bac3.0 also demonstrated a lower frequency of the co-packaging of all non-vector-specific sequences (*rep/cap*, baculoviral, and Sf9 genome) in the range of 0.003%–0.4%.[51]

Recently, BEVs generated via homologous recombination, in contrast to the traditional Tn-7 transposition protocol, showed improved expression stability and lack of co-packaging of bacterial or transposase-derived sequences, providing a high-quality AAV vector preparation.[57]

4 | BIOPROCESSING OF rAAV USING THE IC-BEVS MANUFACTURING PLATFORM

In addition to the molecular design of expression systems, the bioprocessing aspect of IC-BEVS is equally essential for the successful manufacturing of AAV. AAV production in insect cells is inducible upon BV infection, a process based on the complex dynamics of insect cell baculovirus interaction and kinetics of baculoviral and AAV gene expression, which in turn affects the extent and kinetics of AAV protein production and overall AAV quality and yield. This process has been primarily and extensively documented in peer-reviewed research publications with the Three-Bac system, which has been the workhorse of AAV production and bioprocessing research for more than a decade. In the following sub-sections, a detailed discussion on AAV bioprocessing from the Three-Bac standpoint is provided, highlighting important process intensification strategies. The key features of recent improvements in the AAV production process related to the Two-Bac and the One-Bac systems and critical areas of future improvements are discussed in later sections. **Table 1** summarizes the AAV yield at various scales employing different IC-BEVS production systems. A schematic overview of the AAV production process employing the IC-BEVS platform is shown in **Figure 3**.

TABLE 1 Summary of AAV production in IC-BEVS platform

Author, year and production system	Serotype	Bac-MOIs (absolute values) Rep: Cap: ITR	Transgene	Culture volume	Cell density[a] [cells per mL]	VG per cell	VG mL^{-1}	IVP or TU per cell	IVP or TU per mL	Cp: VG: IVP(TU)	Specific notes
Urabe et al.[31] (2002) Three-Bac	2	5:5:5	GFP	200 mL	2×10^{6}[b]	4.5×10^{4}[c]	NA	NA	NA	NA: 1344:1[d]	First report of rAAV production in Sf9 cells
Meghrous et al.[63] (2005) Three-Bac	2	1.6:1.6:1.6	GFP	60 mL	3×10^{6}[b]			168	4.75×10^{8}[f]	NA	AAV production in Sf9 cells at high MOI
	2	1.6:1.6:1.6	GFP	60 mL	1×10^{6}[e]	NA	NA	185	4.38×10^{8}[f]	NA	AAV production in Hi5 cells at high MOI
	2	5:5:5	GFP	60 mL	2.5×10^{6}[b]			75	3.73×10^{8}[f]	NA	Low cell density, batch mode production
	2	5:5:5	GFP	60 mL	2.5×10^{6}[b]			122	5.90×10^{8}[f]	NA	Low cell density with medium exchange
	2	5:5:5	GFP	60 mL	5.0×10^{6}[b]			105	8.78×10^{8}[f]	NA	High cell density with medium exchange
	2	5:5:5	GFP	60 mL	7.5×10^{6}[b]			116	11.60×10^{8}[f]	NA	High cell density with medium exchange
	2	5:5:5	GFP	3L	2.6×10^{6}[b]			250	6.52×10^{8}[f]	1640[g]: NA: 1	Infection in a fresh Ex-Cell 420 medium
	2	5:5:5	GFP	3L	3.08×10^{6}[b]			132	4.09×10^{8}[f]	830[g]: NA: 1	AAV production process scalability
	2	5:5:5	GFP	20L	1.7×10^{6}[b]			253	4.46×10^{8}[f]		demonstration
Urabe et al.[34] (2006) Three-Bac	5	1:1:1	hGFP	NA	2×10^{6} 2×10^{6}	5.6×10^{4}[h] 7.67×10^{4}[i]	NA	NA	NA	NA	Bac-Cap modification, VP1 Domain swapping to produce functional AAV5 vector particle
Aucoin et al.[72] (2006) Three-Bac	2	9:1:1	GFP	20 mL	NA	NA	2×10^{10}[j]	NA	5×10^{7}[f]	3400[g]:400:1	MOI optimization study.
	2	9:9:9	GFP	20 mL			2×10^{9}[j]		1.7×10^{8}[f]	3000[g]:12:1	
Negrete et al.[71] 2007 Three-Bac	2	0.3:0.3:0.3	GFP	20 mL	2×10^{6}	NA	2×10^{12}[c]	NA	2×10^{11}[k]	NA: 10: 1	Screening of low Bac-MOIs and cell density to economize AAV production
	2	3:3:3	GFP	20 mL	2×10^{6}		3.6×10^{12}[c]		3.2×10^{12}[k]	NA: 1.12: 1	
	2	0.03:0.03:0.03	GFP	10 L	1×10^{6}		2×10^{11}		NA	NA	
Aucoin et al.[82] (2007) Three-Bac	2	3:3:3	GFP	200 mL	2×10^{6}[b]	NA	$\sim 3 \times 10^{10}$[j]	NA	$\sim 1 \times 10^{9}$[k]	NA: 30:1	Three-Bac system with a modified Bac-Cap sequence. Demonstration of AAV process scalability at a large scale
	2	3:3:3	GFP	5L-Wave	2×10^{6}[b]		$\sim 9 \times 10^{9}$[j]		$\sim 5 \times 10^{8}$[k]	NA: 18:1	
	2	3:3:3	GFP	20L-Wave™	2×10^{6}[b]		$\sim 5 \times 10^{9}$[j]		$\sim 3 \times 10^{8}$[k]	NA: 16:1	
	2	3:3:3	GFP	10L-STB	2×10^{6}[b]		$\sim 2 \times 10^{10}$[j]		$\sim 5 \times 10^{8}$[k]	NA: 40:1	
	2	3:3:3	GFP	40L-STB	2×10^{6}[b]		$\sim 4.33 \times 10^{10}$[j]		$\sim 7.5 \times 10^{8}$[k] ($\pm 2.5 \times 10^{8}$)	NA: 20:1	
Aucoin et al.[82] (2007) Three-Bac	2	10:10:3	GFP	25 mL	$\sim 2 \times 10^{6}$	~12000	NA	200[f]	4×10^{8}	4600:79:1	Temperature modulation study and its effect on AAV yield

(Continues)

TABLE 1 (Continued)

Author, year and production system	Serotype	Bac-MOIs (absolute values) Rep: Cap: ITR	Transgene	Culture volume	Cell density[a] [cells per mL]	VG per cell	VG mL^{-1}	IVP or TU per cell	IVP or TU per mL	Cp: VG: IVP(TU)	Specific notes
Mena et al.[68] (2010) Three-Bac	2 2 2 2 2 2	0.1:0.1:0.1 3:3:3 0.1:0.1:0.1 3:3:3: 0.1:0.1:0.1 0.1:0.1:0.1	GFP GFP GFP GFP GFP GFP	20 mL 20 mL 20 mL 20 mL 20 mL 3L	1×10^6 1×10^6 5×10^6 5×10^6 9.43×10^{6l} 9.5×10^{6l}	 NA 	4.8×10^{10j} 1.3×10^{10j} 2.9×10^{10j} 3.34×10^{10j} 1.17×10^{11j} 2.2×10^{11j}	NA	4.7×10^{8jf} 1.8×10^{8jf} 6.8×10^{8jf} 7.2×10^{8jf} 2.9×10^{9jf} 2.35×10^{9jf}	NA: 102:1 NA: 72: 1 NA: 43: 1 NA: 46: 1 NA: 40:1 NA: 94:1	Low MOI, Batch production High MOI, Batch production High cell density, medium exchange before the infection High cell density, medium exchange before the infection Low MOI, Fed-batch production Low MOI, Fed-batch production
Liu et al.[77] (2010) Three-Bac	2 2	45:45:5 45:45:5	GFP GFP	50 mL 50 mL	1×10^6 1×10^6	NA	NA	122 240	1.22×10^8 2.4×10^8	NA NA	Batch production Fed-batch production
Cecchini et al.[76] (2011) Three-Bac	9 9 8 6 6 6	BIIC: NIIC (1:10,000)	GFP U7smOPT GFP U7smOPT PLS PLS	10L 20L 100L 200L 20L	3.55×10^{6l} 3.23×10^{6l} 3.88×10^{6l} 4.29×10^{6l} 4.41×10^{6l} 3.44×10^{6l}	2.24×10^4 2.41×10^4 1.48×10^4 2.0×10^4 1.82×10^4 2.07×10^4	7.9×10^{10j} 7.8×10^{10j} 4.44×10^{10j} 8.75×10^{10j} 7.45×10^{10j} 5×10^{11j}	NA	NA	NA	AAV production employing BIIC strategy. Demonstration of AAV production process robustness at bioreactor scale regardless of the type of transgene
Chen[36] (2008) Three-Bac Two-Bac Bac-*In*-Rep-Cap	2 6 8 12	1:1:1 1:1:1 1:1:1 1:1:1 1:1	GFP GFP GFP GFP GFP	NA	5×10^{6b} 5×10^{6b} 5×10^{6b} 5×10^{6b} 5×10^{6b}	NA	1.37×10^{11j} 3.53×10^{10j} 9.65×10^{10j} 4.41×10^{10j} 1.19×10^{11j}	NA	NA	NA	Bac-Intron constructs of baculovirus vector Baculovirus with enhanced passage dependent stability Bac-*In*-Rep-Cap and Bac-*In*-ITR
Smith et al.[35] (2009) Two-Bac Bac-Rep-Cap	1 2	1:1 1:1	GFP GFP	200 mL 200 mL	2.4×10^{6b} 1.2×10^{6b}	NA	1.07×10^{13m} 6.5×10^{12m}	NA	5.08×10^{10mn} ($\pm 5.9 \times 10^{10}$) 1.9×10^{10mn}	NA: 200:1 NA: 350:1	Bac-Rep-Cap and Bac-ITR based two baculovirus vectors with improved stability
Aslanidi et al.[38] (2009) *rep2capX*One-Bac 1.0	2 1	3 3	GFP GFP	NA	NA	1.4×10^5 7.3×10^4	NA	NA	NA	NA	Inducible packaging Sf9 cell line for AAV production

(Continues)

TABLE 1 (Continued)

Author, year and production system	Serotype	Bac-MOIs (absolute values) Rep: Cap: ITR	Transgene	Culture volume	Cell density[a] [cells per mL]	VG per cell	VG mL^{-1}	IVP or TU per cell	IVP or TU per mL	Cp: VG: IVP(TU)	Specific notes
Mietzsch et al.[49] (2014) One-Bac 1.0	1-11 12	3 3	GFP GFP	NA	NA	~10^4-10^5[o] ~10^3[o]	NA	NA	NA	NA	Production of all AAV serotypes employing One-Bac system
Mietzsch et al.[50] (2015) One-Bac 2.0	5	5	GFP	NA	NA	1×10^5[o]	NA	NA	NA	NA	One-Bac system with VP ratio restoration to improve AAV5 vector functionality
Joshi et al.[43] (2019) One-Bac 3.0	5 5 5 5	3 3 3 3	GFP GFP GFP GFP	25 ml 25 mL 1L 3L	1.6×10^6[l] 12×10^6[l] 12×10^6[l] 12×10^6[l]	2.4×10^4 3×10^4 3.16×10^4 2.7×10^4	4×10^{10}[j] 2.7×10^{11}[j] 3.8×10^{11}[j] 2.6×10^{11}[j]	6±1.4 20±5 22±5 15±5	1.7×10^7[jp] (±0.3×10^7) 1.9×10^8[jp] (±0.4×10^8) 2.1×10^8[jp] 1.4×10^8[jp]	NA: 15,600:1 NA: 1400:1 9600[q]: 2400[r]:1 9400[q]: 2500[r]:1	Robust, High MOI, Fedbatch AAV5 production process. Process validation at bioreactor scale. The improved volumetric yield of genomic and functional AAV5 vector
Wu et al.[88] (2019) One-Bac 3.0+	2 8 9	3 3 3	GFP GFP GFP	NA	2×10^6 2×10^6 2×10^6	1.35×10^5 2.16×10^5 1.80×10^5	NA	NA	NA	NA	One-Bac based Rep2 expressing transformed Sf9 cell line. Offer more flexibility for the production of multiple AAV serotypes production

[a] Sf9 cells have been dominantly used for AAV production regardless of the production systems except for a single report by Meghrous et al. (2005) where Hi5 cells were used.
[b] Cell density at the time of infection.
[c] Value reported for fraction collected from the CsCl purification run.
[d] Determined by infecting HEK293 cells and subsequent analysis under a fluorescence microscope for GFP positive cells. Originally reported as transducing units (TU).
[e] AAV2 production in Hi5 cells.
[f] Determined via infecting HEK293 EBNA cells with AAV vector in the presence of a helper virus and subsequent analysis via flow cytometry for GFP positive cells.
[g] Total virus capsids (Cp) analyzed via ELISA.
[h] Value of rAAV5-hGFP produced using Rep52 of Serotype 1.
[i] Value of rAAV5-hGFP generated via AAV2 VP1 domain swapping (VP1 2/5) and Rep52 of serotype 1.
[j] Value reported for per milliliter of cell culture.
[k] Analyzed via infecting adherent HEK293 cells and subsequent analysis via flow cytometry.
[l] The peak cell density during the production run.
[m] Value reported per milliliter of AVB-Sepharose affinity-purified eluate fraction.
[n] Determined via infecting HEK293A cells via AAV in the absence of helper virus and subsequent analysis using a fluorescence microscope.
[o] Titer value reported from the analysis of a one-step AVB-Sepharose affinity purified sample.
[p] Analyzed via infecting suspension adapted HEK293 cells with AAV in the presence of helper virus and subsequent analysis by flow cytometry.
[q] Determined via analytical ultracentrifugation analysis of one-step AVB-Sepharose affinity purified sample and originally reported as enhanced transduction units (ETU).
[r] The value of the relative ratio is derived from ETU units determined for AVB affinity-purified samples.
Abbreviations: GFP, green fluorescence protein; hGFP, Humanized Green Fluorescence Protein; NA, not available in the published report; STB, stirred-tank bioreactor.

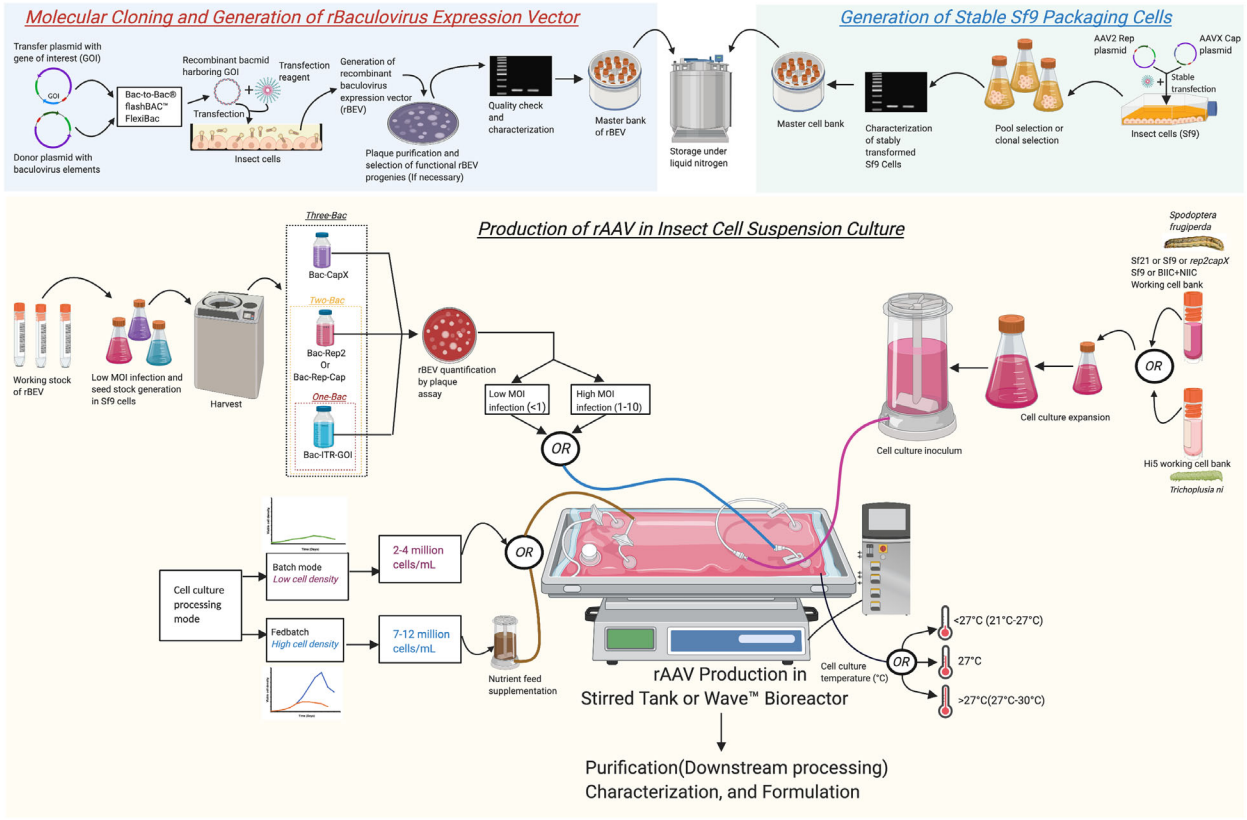

FIGURE 3 Overview of a process flow for rAAV production employing the IC-BEVS platform. AAV production using the IC-BEVS platform consists of two stages: 1. Generation of recombinant baculovirus expression vectors (rBEVs) and additionally *rep2capX* Sf9 packaging cells in the case of the One-Bac system (Top section), and 2. AAV production at the bioreactor scale in suspension culture of insect cells followed by purification and formulation (bottom part). The first part requires molecular cloning, transient expression, plaque purification, and characterization of rBEVs. Similarly, *the rep2capX* cells are generated via stable transfection of respective shuttle plasmid vectors and selection followed by characterization and master cell bank preparation. During the production stage, the rBVES and insect cells (Sf9 or Hi5) are sequentially expanded, as required for stirred tank or Wave™ production-scale bioreactors. At the production stage, the insect cells are infected with rBEVs at an appropriate multiplicity of infection (low, less than 1 or high, 1–-10) and at optimal cell density. Generally, under the batch mode of cultivation, the cells are infected at a low cell density of approximately 1–3 million cells per mL, whereas in the fed-batch or medium-exchange mode, the cells reach a higher peak cell density before infection, at 7–12 million cells per mL. The cultivation temperature for insect cells is generally set at 27°C, although one study reported AAV production at higher and lower temperatures, and their effect on yield and production kinetics. Post-infection, at 72–96 h, the culture is harvested, and cells are lysed to recover AAV. Next, the lysate is subjected to a multistep purification process followed by formulation in an appropriate buffer as a final step. The artwork in the figure was created using Biorender.com

4.1 | Multiplicity of infection of baculovirus: cell population and kinetics of baculovirus infection

The multiplicity of infection (MOI), defined as the number of active viral particles (BV in this context) infecting one cell, has been shown to influence the kinetics and extent of infection and protein expression, which ultimately affect the overall yield and composition of the product of interest.[58–61] In the Three-Bac system, the productive infection and consequent AAV generation necessitate the insect cells to receive all the essential genes (*rep*, *cap*, and *transgene*) delivered by three BEVs.[31]

Statistically, at an MOI of 1 for each of the three BEVs, while 95% of cells are infected with at least one BEV, only 22% of cells receive three BEV productive infections, leaving 78% of the cells unproductive in the primary BV infection.[62] This productive cell population is even lower at a higher MOI. Meghrous et al.[63] reported that at an optimal MOI of 5, less than 12% of cells are AAV-productive in the primary infection cycle. In two baculovirus systems (Two-Bac), the productive cell-population proportion ranges between 60% and 70%,[40] with a one baculovirus system, it can reach as high as 95% (based on the MOI used) in the first wave of infection.[62] The single baculovirus infection follows the Poisson distribution, and the relationship between the MOI and fraction of the productive cell population is a hyperbola.[62,64,65] It is believed that this increase in the productive cell population in Two-Bac may be responsible for the higher AAV yield.[40] However, this can be challenged given that the average cell-specific yield of AAV2 viral genomes (VG) achieved with Three-Bac (45,000 VG per cell)[31] is not significantly different from that of Two-Bac (78,000 VG per cell).[35] A somewhat higher titer with Two-Bac might be correlated to higher AAV Rep protein expression stability, which is necessary for vector genome rescue, replication, and encapsidation.[35,66,67] The lower productive cell population at the primary BEV infection can be circumvented via subsequent waves of infections where BEV progenies produced

after the primary infection round infect the remaining cell population, providing near 100% productive infection. This phenomenon is characteristic of asynchronous baculovirus infection caused by low MOI used in the primary infection round.[68] This process has been used for the production of enveloped virus-based vaccine candidates in insect cells and mammalian cells.[69,70]

When applied to AAV production using Three-Bac, Mena et al.[68] and Negrete et al.[71] reported that at both low and high MOIs, the overall volumetric yields of AAV are comparable; however, in the case of a low MOI process, the culture harvest time is delayed by an additional 24–48 h post-infection (~96 hpi) due to an apparent delay in the transition to the productive infection phase as compared to the standard high MOI process.[68] A full factorial DOE study conducted by Aucoin et al.[72] provided detailed insight into single-factor and multifactorial interaction effects of MOI of the three baculoviruses on AAV yields and the timing of expression for each AAV protein. This study suggested a self-controlling nature of the insect cell-baculovirus infection process, where differing MOIs or delaying the infection of one of the three baculoviruses did not lead to significant changes or improvements in overall AAV yield when reported in combination with the relative proportions of total, genome containing, and infective virus particles (Cp:VG:IVP).

Besides low BEV stock requirements, another advantage of the low MOI process relates to the lower production of non-functional defective interfering particles (DIP) of BV progenies after the primary infection.[73] Therefore, a low MOI is also recommended for BV/BEV production. It has been reported that during BV production, the co-synthesized DIP can lack up to 43% of the original BV genome,[73] the missing elements may be complemented by a high MOI infection facilitating DIP co-production. In contrast, a low MOI primary infection reduces this genetic complementation and probability of DIP progeny formation, hence minimizing DIP-mediated non-AAV-productive infections during the AAV production process.

4.2 | Cell density effect and mode of cell culture operation

The cell density effect, which refers to the loss of cell-specific yield of the product of interest above specific cell density (breakpoint) in the cell culture, has been widely reported in the literature, concerning AAV production.[43,63,68,74,75] This effect has been associated with metabolic limitation of the culture, especially in the productive infection phase, and has been successfully alleviated via supplementation of fresh growth medium (medium replacement strategy) or bolus supplementation of the nutrient cocktail under the fed-batch mode of operation.[63,75]

While commercially available serum-free growth media for insect cell culture such as SF-900-II or-III can support a peak cell density of up to 13 million cells during a standard growth curve (our unpublished data), they fail to sustain protein expression in baculovirus-infected high cell density cultures. When analyzed for AAV production using Three-Bac, the optimal cell density at the time of infection was reported to be approximately 1 million cells per mL in the EX-CELL 420 medium[63] or 3.5 million cells per mL in SFX serum-free medium.[76] Similarly, during AAV5 production in One-Bac3.0 Sf9 cells using Sf900-II or Sf900-III medium, the cell density breakpoint was approximately 2.0 million cells per mL.[43] Any combination of cell density at the time of infection and an MOI that resulted in a peak cell density above the breakpoint resulted in a drop in the cell-specific yield, limiting the AAV volumetric titer in a low-cell density culture. Such a low-cell density culture process operating under a batch mode of cultivation generally results in a lower volumetric yield of AAV, necessitating large-scale bioreactor production to meet the exceptionally high demand of AAV vector material (up to 10^{17} VGs) for late-phase clinical studies.[39]

A straightforward way to boost the overall volumetric titer is to infect and induce AAV production at higher cell densities while maintaining the cell-specific yield. The medium replacement strategy was used to attain increased AAV production at cell density as high as 7.5 million cells per mL in shake-flasks, and the process was further validated at the 3 and 20 L bioreactor scale at up to 2.5 million cells per mL cell density.[63] Similarly, in Sf21 cells, the fresh growth medium supplementation successfully alleviated nutrient limitation at 1 million cells per mL density offering increased cell-specific yield compared to controlled production runs.[77] The fed-batch mode of operation combined with low MOI infection of Three-Bac BEVs resulted in an almost 1-log increase in volumetric yield of functional AAV2 particles when the nutrient cocktail was provided at the time of infection and 24 h before and after infection (Table 1).[68] In this case, Sf9 cells were infected at ~5 million cells per mL at a low MOI of 0.1 (for each BV), which resulted in non-infected cells growing up to 48 hpi, attaining a peak cell density as high as ~10 million cells per mL followed by a sustained AAV production phase.

4.3 | Effect of temperature

The temperature modulation strategy is reported to affect protein expression yield and glycosylation in insect cells,[78–81] which grow best at temperatures between 27°C and 30°C. Aucoin et al.[82] reported higher functional AAV2 vector production in Sf9 cells at a higher temperature. Of different temperatures tested (21, 24, 27, 30, and 33°C), the highest yield of AAV functional particles was achieved at 30°C, which was 2.5 times and five times more than at 27°C and temperatures below that, respectively. Also, temperature modulation affected the onset of expression and kinetics of AAV protein production. The use of temperature-responsive promoters or other regulatory elements to achieve inducible production of AAV proteins at a controlled rate or expression levels remains open to further investigation.

4.4 | AAV production in small and large-scale stirred-tank bioreactors and Wave bioreactors

The attractive features of insect cell baculovirus platforms include the ability of insect cells to grow in suspension culture at a high cell

density, proven linear scalability, and a history of regulatory acceptance of the platform for the production of various biologics.[11,26] A commercial-scale fed-batch manufacturing process for Flublok in Sf9 cells was successfully scaled-up and demonstrated at 2500 L stirred-tank bioreactor (STB)[11] with targeted production at a more than 20,000 L scale. Multiple reports of AAV production in either conventional STBs (glass, stainless steel, or disposable vessel),[63,76,83] or Wave bioreactors[83] suggest comparable yields and quality of rAAV vector material (Table 1). In a controlled bioreactor environment maintained at optimal cell-culture conditions, the AAV production yield improved compared to a shake flask run, as demonstrated in a 20 L scale bioreactor production, which generated around 5×10^{12} AAV2 functional particles[63] (Table 1). Subsequent reports of AAV production using an improved Three-Bac system indicated AAV volumetric yield in the range of 5×10^{12}–3×10^{13} VG per L with a comparable consistency at various scales of Wave (up to 20 L) and STB (up to 40 L), respectively.[83] Even though the reported yield was somewhat lower, the bioreactor scale process performance, when expressed as the ratio of genomic:transducing units (VG:TU), was consistent in all production runs.

Although simple and straightforward at a smaller-scale, AAV production using Three-Bac at a large scale was challenging due to a multitude of reasons. First, a large amount of three different BEV stock, required to infect large-scale production cultures, necessitates the parallel production of these BEVs. During this BEV production step, BEVs may undergo high passage numbers, resulting in lower and variable expression stability and presenting a direct source of plausible process variability. Second, the parallel production, quantification, and characterization of three different baculoviral vectors remain cumbersome and resource-extensive steps. Moreover, if a high MOI process is selected (more than 3 MOI for each BEV), the total volume of three-BEV stock (1×10^8 pfu mL^{-1}) required may be as high as 20% v/v, leading to a substantial dilution of production culture volume at the time of infection. To address these challenges, an alternate approach to the proven low-MOI process[68] was reported by Cecchini et al.,[76] where the low MOI infection characteristics were mimicked and reproduced in situ by an expansion of a mixture of cryo-preserved baculovirus-infected insect cells (BIIC) and non-infected insect cells (NIIC). BIIC and NIIC, originally mixed in an 1:10,000 ratio, eliminated the need for exogenous baculovirus infection during production and offered consistent baculovirus infection kinetics and AAV expression yield. When assessed at different scales (shake flask to 200 L bioreactor) for different serotypes (AAV-6 and -9), a comparable yield of AAV serotypes was reported[76] (Table 1).

4.5 | AAV production process monitoring in bioreactors

In addition to the pH, temperature, and dissolved oxygen concentration sensors, various in-line process monitoring tools have been reported for AAV production. These tools include dielectric spectroscopy[84] and capacitance sensors for monitoring cell growth, productive infection, infection kinetics, and relative permittivity of insect cells.[43,68] Infrared sensors for measuring the carbon dioxide evolution rate of cell culture[68] and recently reported on-line digital holographic microscopy for AAV production yield and cell concentration measurement have also been documented.[85] These tools provide a practical approach for process optimization and process control, including making the decision on culture harvest time.

5 | RECENT ADVANCEMENTS IN THE AAV PRODUCTION PROCESS

As discussed in the previous section, the extensive documentation of AAV production employing Three-Bac (Table 1) provided the key learnings that laid the foundation of the current manufacturing systems and process using improved and follow-on IC-BEVS. Recently, there has been a shift towards a simpler and more straightforward Two-Bac system for AAV production, as it combines the flexibility of Three-Bac and the process simplicity of the less flexible One-Bac/Mono-Bac system to some extent. The two-baculovirus system has been reportedly used for large-scale AAV production by AAV vector manufacturers.[86,87]

Although Mono-Bac provides a higher volumetric titer,[37] it has not been extensively documented. Generating a stable baculoviral vector harboring all three gene sequences is technically challenging. Moreover, it is the least modular system compared to the Three-Bac or the Two-Bac systems, making it difficult to produce a library of AAV capsids in the early screening phase. Current standard protocols for AAV production involve Rep proteins and ITR packaging functions from AAV2, whereas the serotype and vector expression cassettes are selected based on in vivo/in vitro transduction studies conducted at the screening phase. Following this rationale, a modified version of the original Two-Bac, Duo-Bac was reported, which consists of Bac-Rep and Bac-CapX-GOI (gene of interest) baculoviruses.[48,87] Similarly, a modified version of One-Bac consisting of only the *rep*-expressing Sf9 cell line and Bac-CapX-GOI for AAVX (X = serotype) production has also been recently reported.[88]

A study by Joshi et al.[43] reported AAV5 production in high cell density fed-batch cultures using One-Bac3.0, with a final volumetric yield exceeding 2×10^{14} VG per L of cell culture. In contrast to the previously reported fed-batch process,[68] the culture at a 10 million cells per mL cell density was infected at a higher optimal MOI of 3, which resulted in the transition of the cell culture to the AAV-production phase from the growth phase within 24 hpi. The nutrient cocktail was supplied to maintain the cell culture in the mid-exponential phase before infection and support AAV production in the post-infection stage to alleviate nutrient limitation, resulting in a six-fold increase in VGs and an 18-fold increase in bioactive AAV5 particles as compared to the standard low-cell density batch process.[43]

In compliance with regulatory and GMP requirements, newer yeastolate-free and chemically defined serum-free media such as ExpiSF[89–91] or TheraPEAK-SfAAV[92] have been developed as alternatives to traditional yeastolate-based serum-free media such as Sf900-II.

6 | FUTURE PERSPECTIVES

6.1 | Insect cell and baculovirus engineering

Although insect cells have been in use for AAV production for more than 15 years, the first major question yet unanswered relates to the uncertainty of whether the maximum cellular protein production/processing capacity has been reached. One-Bac1.0 reported almost 1-log higher cell-specific AAV yield (~500,000 VG per cell)[38] compared to the original Three-Bac (~45,000 VG per cell),[31] which indicates that current AAV production protocols demonstrating routine cell-specific yield of up to ~100,000 VG per cell (Table 1) may be under-utilizing the insect cells AAV production capacity.

The second question is related to the efficiency of vector genome packaging in insect cells. In general, insect cells have been reported to produce more AAV empty capsids[43,56,93] compared to mammalian cell platforms,[94–96] which counteracts any success achieved with increased cell-specific AAV yield and necessitates active efforts towards engineering insect cells or BEVs to improve packaging efficiency and produce more functional particles. A head-to-head comparison of the mammalian cell versus insect cells for wild type AAV production may help identify any insect-cell specific limitations for producing packaged AAV particles because mammalian cells, the natural host of AAV, have been reported to produce nearly 100% genomic and functional particles of wtAAV in the presence of helper virus functions.[97] Moreover, in contrast to well-defined and well-characterized helper genes of adenovirus or HSV,[98,99] the baculovirus helper elements have not yet been fully identified, even though their helper function has been known for almost two decades.[31] Aslanidi et al.[38] reported the absolute requirement of baculovirus genomic elements such as homologous region (hr2-0.9) in *cis* as a part of the *rep* expression cassette for its rescue and replication in insect cells upon BV infection. Identifying similar helper elements to modulate AAV gene expression and subsequent genome-encapsidation efficiency of insect cells presents an opportunity for further research.

Another critical factor requiring significant consideration is the functionality of ITR, which is not yet fully understood,[100] especially in the context of insect cells. In contrast to traditional AAV2 Rep- ITR, the selection of optimal combinations of serotype-specific Rep- ITR also remains an open area of investigation to further support the findings of a single report by Urabe et al.[34] studying the effect of such a combination on genome packaging efficiency and the overall yield of AAV5.

The exclusive use of Sf9 cells over Hi5 for AAV production has not been supported with sufficient documentation despite proven commercial viability and regulatory acceptance of Hi5 cells for the production of a therapeutic biologic for use in humans.[11,26] Meghrous et al.[63] reported a somewhat higher cell-specific yield of AAV2 in Hi5 cells compared to Sf9. However because of the known ease of baculovirus production in Sf9 cells over Hi5 cells,[17] the former was selected for both AAV and BEV production, streamlining the overall process. With the demonstrated success of AAV production in the One-Bac Sf9 packaging cell line, the generation of a similar Hi5 cell line remains open to further evaluation. Additionally, in the context of a recent report regarding the effect of post-translational modifications (PTMs) of AAV capsids on vector transduction efficiency,[93] a side-by-side comparison of AAV produced in Sf9 and Hi5 cell lines might provide new insights into the vector quality attributes (PTMs, in vivo potency) imparted by different cell lines.

It should be noted that in recent years, commercially available Sf9 and *expres*SF+, and Hi5 cell lines have been found to produce rhabdovirus[101,102] and nodavirus,[103] respectively, wherein the latter was found to acquire latency in Hi5 cells in the absence of baculovirus superinfection. As such, rhabdovirus cannot replicate in human or monkey cell lines[101,104]; therefore, it is deemed harmless, whereas the host range of nodavirus is not yet clear. Importantly, the team from Glyobac recently established and characterized rhabdovirus-negative Sf9 cell line[102] and nodavirus-negative Hi5 cell line,[105] which showed the absence of corresponding adventitious viruses overextended cell passages making these cell lines preferentially more suitable for biologics manufacturing than the current Sf9 and Hi5 cell lines in use because of improved product safety. Notably, none of these new cell lines have been reportedly used for AAV production and remain open to further investigation.

AAV production using IC-BEVS is a transient phenomenon. Because of the lytic nature of baculovirus infection, AAVs are recovered by harvesting the cell culture within 96 hpi, preferably at viability more than 70%,[43] to minimize potential degradation of AAV capsids by cellular-proteases released as a result of cell apoptosis. At this time, AAV VG titer often reaches a plateau; however, this may not be the optimal harvest time since packaged virions are believed to undergo a so-called "capsid maturation" phase reflected as delayed onset in infective virus particles.[43,106] Extending the time of harvest while maintaining insect cells at higher viability offers a plausible solution to increase the overall yield of the functional AAV vectors. Expression of anti-apoptotic proteins such as vankyrins has been shown to maintain more than 90% viability at 96 hpi in both Sf9 and Hi5 cells.[107] Similarly, the anti-apoptotic *p35* gene expression also showed a 4-fold increase in GFP titer while maintaining the culture at more than 50% viability at approximately 120 hpi.[108] Incorporation of these advancements in the AAV production process and their effect on AAV yield could be of potential interest in the future.

The protease-driven degradation of secreted recombinant proteins produced via IC-BEVS has been known for a while, which prompted the use of a chitinase and cathepsin gene-deleted BEV.[109] Similarly, recent observations reported on the degradation of VP proteins of specific serotypes suggest further exploration in this area to identify other protein-degrading elements of the baculovirus genome.[52] Further identification and deletion of such detrimental or non-essential genes may facilitate the insertion of multiple copies of AAV gene expression cassettes in the baculoviral vector. In this way, more copies of AAV genes, compared to standard baculoviral vectors, can be delivered to insect cells without increasing the MOI, enabling a lower overall requirement of baculoviral vector stock and an economized approach to AAV bioprocessing.

6.2 | AAV bioprocessing

In addition to molecular and cellular engineering, improvements in high cell density production processes can also contribute to addressing the challenges of high-yield production by incorporating advancements in the field of biologics manufacturing. The strategies involved are not limited to the development of cellular proteomics- and metabolomics-based optimized insect cell medium formulation, nutrient cocktail, or AAV titer enhancer formulations. Moreover, a detailed understanding of the critical process parameters guided by the critical quality attributes of AAV serotype therapeutic products is crucial for a robust manufacturing process.

The high-cell density upstream process should be designed with consideration of downstream processing. AAV is an inherently intracellular product and the primary recovery step involves cell lysis and release of intracellular AAVs in lysis buffer.[110] In high cell density cultures, the removal of co-extracted cellular components (host cell genome, proteins, and other components) released upon cell lysis exerts a significant burden on downstream processing. It would be appealing to study if the AAV capsids can be modified, without altering the functionality, to direct AAV secretion in cell-culture supernatant to continuously recover them with minimum contamination of intracellular impurities.

Another futuristic approach is the development of a producer insect cell line that constitutively integrates and inducibly expresses all the genes necessary, including baculovirus helper functions, AAV proteins, and AAV vector production, using an alternative inducible system(s) not requiring baculovirus superinfection.[111–113] The Sf9 genome was published recently[114] and CRISPR-mediated knockout of genes expressing viral restriction factors in insect cells or targeted integration of AAV genes in the transcriptionally active regions of the insect cell genome are attractive strategies for producer cell-line generation. These strategies, combined with high-viability insect-cell cultures as discussed above, can be used to design a semi-continuous perfusion process that facilitates ultra-high cell density. Such a process may offer a significantly higher volumetric yield of AAV enabling a large amount of vector generation, even at a small-scale cGMP facility with reduced frequency of production runs.

7 | SUMMARY AND CONCLUSION

Supported by sustained efforts in baculovirus and insect-cell engineering, the IC-BEVS platform has undergone significant improvements to produce AAV vectors with better yield, quality, and process simplicity. Although the key understanding of AAV production in insect cells was based on the original Three-Bac system, more advanced and simplified versions, including Two-Bac and One-Bac, are being adopted for large-scale AAV production, accommodating and continuously improving the existing Three-Bac manufacturing process. Moreover, the emergence of novel process monitoring technologies and continuous improvements in process intensification strategies with demonstrated success in other eukaryotic expression platforms (e.g., CHO cell line) also presents a futuristic direction for AAV manufacturing. In conclusion, due to increased regulatory acceptance, ease of scale-up, and recent advancements in production technologies, insect cell baculovirus systems are being more broadly adopted for the production of multiple AAV serotypes (Table 1). As a result, more insect cell-produced AAV vectors can be expected in clinical trials in the future.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Pranav R.H. Joshi: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Software; Visualization; Writing-original draft; Writing-review & editing. Alina Venereo-Sanchez: Data curation; Formal analysis; Writing-original draft; Writing-review & editing. Parminder S. Chahal: Methodology; Formal analysis; Writting-review & editing. Amine A. Kamen: Conceptualization; Funding acquisition; Methodology; Project administration; Resources; Supervision; Validation; Writing-review & editing.

DATA AVAILABILITY STATEMENT

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ORCID

Amine A. Kamen https://orcid.org/0000-0001-9110-8815

REFERENCES

1. Gaw, Z. Y., Liu, N. T., & Zia, T. U. (1959). Tissue culture methods for cultivation of virus grasserie. *Acta. Virol.*, *3*, 55–60.
2. Vail, P. V., Sutter, G., Jay, D. L., & Gough, D. (1971). Reciprocal infectivity of nuclear polyhedrosis viruses of the cabbage looper and alfalfa looper. *J. Invertebr. Pathol.*, *17*(3), 383–388.
3. Kool, M., & Vlak, J. M. (1993). The structural and functional organization of the Autographa californica nuclear polyhedrosis virus genome. *Arch. Virol.* , *130*(1-2), 1–16.
4. Summers, M. D. (2006). Milestones leading to the genetic engineering of baculoviruses as expression vector systems and viral pesticides. *Adv. Virus. Res.*, *68*, 3–73.
5. Smith, G. E., Summers, M. D., & Fraser, M. J. (1983). Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Mol. Cell. Biol.*, *3*(12), 2156–2165.
6. Smith, G. E., Ju, G., Ericson, B. L., Moschera, J., Lahm, H. W., Chizzonite, R., & Summers, M. D. (1985). Modification and secretion of human interleukin 2 produced in insect cells by a baculovirus expression vector. *Proc. Natl. Acad. Sci. USA*, *82*(24), 8404–8408.
7. Kuzio, J., Rohel, D. Z., Curry, C. J., Krebs, A., Carstens, E. B., & Faulkner, P. (1984). Nucleotide sequence of the p10 polypeptide gene of Autographa californica nuclear polyhedrosis virus. *Virology*, *139*(2), 414–418.
8. Kirnbauer, R., Booy, F., Cheng, N., Lowy, D. R., & Schiller, J. T. (1992). Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc. Natl. Acad. Sci. USA*, *89*(24), 12180–12184.

9. Wickham, T. J., Davis, T., Granados, R. R., Shuler, M. L., & Wood, H. A. (1992). Screening of insect cell lines for the production of recombinant proteins and infectious virus in the baculovirus expression system. *Biotechnol. Prog.*, *8*(5), 391–396.
10. Felberbaum, R. S. (2015). The baculovirus expression vector system: A commercial manufacturing platform for viral vaccines and gene therapy vectors. *Biotechnol. J.*, *10*(5), 702–714.
11. Buckland, B., Boulanger, R., Fino, M., Srivastava, I., Holtz, K., Khramtsov, N., & Cox, M. M. (2014). Technology transfer and scale-up of the Flublok® recombinant hemagglutinin (HA) influenza vaccine manufacturing process. *Vaccine*, *32*(42), 5496–5502.
12. van Oers, M. M., Pijlman, G. P., & Vlak, J. M. (2015). Thirty years of baculovirus–insect cell protein expression: From dark horse to mainstream technology. *J. Gen. Virol.*, *96*(1), 6–23.
13. Bruinzeel, W., Yon, J., Giovannelli, S., & Masure, S. (2002). Recombinant insect cell expression and purification of human β-secretase (BACE-1) for X-ray crystallography. *Protein. Expr. Purif.*, *26*(1), 139–148.
14. Granados, R. R., Li, G., & G. W Blissard. (2007). Insect cell culture and biotechnology. *Virol. Sin.*, *22*(2), 83–93.
15. Krammer, F., Nakowitsch, S., Messner, P., Palmberger, D., Ferko, B., & Grabherr, R. (2010). Swine-origin pandemic H1N1 influenza virus-like particles produced in insect cells induce hemagglutination inhibiting antibodies in BALB/c mice. *Biotechnol. J.*, *5*(1), 17–23.
16. Rendić, D., Wilson, I. B., & Paschinger, K. (2008). The glycosylation capacity of insect cells. *Croat. Chem. Acta*, *81*(1), 7–21.
17. Wilde, M., Klausberger, M., Palmberger, D., Ernst, W., & Grabherr, R. (2014). Tnao38, high five and Sf9-evaluation of host–virus interactions in three different insect cell lines: Baculovirus production and recombinant protein expression. *Biotechnol. Lett.*, *36*(4), 743–749.
18. Cervarix. (2019). Human papillomavirus vaccine Type 16 and 18 (Recombinant, AS04 adjuvanted), *Suspension for injection*, *Active immunizing agent*, ATC code: J07BMO2. GlaxoSmithKline Inc.
19. Assessment Report. Glybera. *European Medicines Agency*. EMA/882900/2011. Committee for Medicinal Products for Human Use. 2012.
20. Kamen, A. A., Bédard, C., Tom, R., Perret, S., & Jardin, B. (1996). On-line monitoring of respiration in recombinant-baculovirus infected and uninfected insect cell bioreactor cultures. *Biotechnol. Bioengin.*, *50*(1), 36–48.
21. Kamen, A. A., Tom, R. L., Caron, A. W., Chavarie, C., Massie, B., & Archambault, J. (1991). Culture of insect cells in helical ribbon impeller bioreactor. *Biotechnol. Bioeng.*, *38*(6), 619–628.
22. Kamen, A. A., Chavarie, C., Andre, G., & Archambault, J. (1992). Design parameters and performance of a surface baffled helical ribbon impeller bioreactor for the culture of shear sensitive cells. *Chem. Eng. Sci.*, *47*(9-11), 2375–2380.
23. Maranga, L., Cunha, A., Clemente, J., Cruz, P., & Carrondo, M. J. (2004). Scale-up of virus-like particles production: Effects of sparging, agitation and bioreactor scale on cell growth, infection kinetics and productivity. *J. Biotechnol.*, *107*(1), 55–64.
24. Mena, J. A., & Kamen, A. A. (2011). Insect cell technology is a versatile and robust vaccine manufacturing platform. *Expert. Rev. Vaccines*, *10*(7), 1063–1081.
25. Desrosiers, R., Clark, E., Tremblay, D., Tremblay, R., & Polson, D. (2009). Use of a one-dose subunit vaccine to prevent losses associated with porcine circovirus type 2. *J. Swine. Health. Prod.*, *17*(3), 148–154.
26. Harper, D. M. (2008). Impact of vaccination with Cervarix™ on subsequent HPV-16/18 infection and cervical disease in women 15–25 years of age. *Gynecol. Oncol.*, *110*(3), S11–S17.
27. Ylä-Herttuala, S., (2012). Endgame: Glybera finally recommended for approval as the first gene therapy drug in the European union. *Mol. Ther.*, *20*(10), 1831–1832.
28. AAV5-hFVIII-SQ (BMN 270). Annex III A. BioMarin Pharmaceutical Inc.; 2017.
29. Kailasan, S., Agbandje-McKenna, M., & Parrish, C. R. (2015). Parvovirus family conundrum: What makes a killer? *Annu. Rev. Virol.*, *2*, 425–450.
30. Ruffing, M., Zentgraf, H., & Kleinschmidt, J. A. (1992). Assembly of viruslike particles by recombinant structural proteins of adeno-associated virus type 2 in insect cells. *J. Virol.*, *66*(12), 6922–6930.
31. Urabe, M., Ding, C., & Kotin, R. M. (2002). Insect cells as a factory to produce adeno-associated virus type 2 vectors. *Hum. Gene. Ther.*, *13*(16), 1935–1943.
32. Srivastava, A., Lusby, E. W., & Berns, K. I. (1983). Nucleotide sequence and organization of the adeno-associated virus 2 genome. *J. Virol.*, *45*(2), 555–564.
33. Kohlbrenner, E., Aslanidi, G., Nash, K., Shklyaev, S., Campbell-Thompson, M., Byrne, B. J., & Zolotukhin, S. (2005). Successful production of pseudotyped rAAV vectors using a modified baculovirus expression system. *Mol. Ther.*, *12*(6), 1217–1225.
34. Urabe, M., Nakakura, T., Xin, K. Q., Obara, Y., Mizukami, H., Kume, A., & Ozawa, K. (2006). Scalable generation of high-titer recombinant adeno-associated virus type 5 in insect cells. *J. Virol.*, *80*(4), 1874–1885.
35. Smith, R. H., Levy, J. R., & Kotin, R. M. (2009). A simplified baculovirus-AAV expression vector system coupled with one-step affinity purification yields high-titer rAAV stocks from insect cells. *Mol. Ther.*, *17*(11), 1888–1896.
36. Chen, H. (2008). Intron splicing–mediated expression of AAV Rep and Cap genes and production of AAV vectors in insect cells. *Mol. Ther.*, *16*(5), 924–930.
37. Galibert, L., Merten, O.-W., & Jacob (Genethon), A. (2018). US 2018/0305717 A1.
38. Aslanidi, G., Lamb, K., & Zolotukhin, S. (2009). An inducible system for highly efficient production of recombinant adeno-associated virus (rAAV) vectors in insect Sf9 cells. *Proc.Natl. Acad. Sci. USA*, *106*(13), 5059–5064.
39. Galibert, L., & Merten, O. W. (2011). Latest developments in the large-scale production of adeno-associated virus vectors in insect cells toward the treatment of neuromuscular diseases. *J. Inverterb. Pathol.*, *107*, S80-S93.
40. Merten, O. W. (2016). AAV vector production: State of the art developments and remaining challenges. *Cell. Gene. Ther. Insights*, *2*(5), 521–551.
41. Johannes, W. T., Hermens, M. C., S. J. K. Haast, Biesmans, D. J., & Bakker, A. C. (2017). (uiQure IP B.V.) US 2017/0152525 A1.
42. Savy, A., Kaikkonen, M. U., Léger, A., Dickx, Y., Galibert, L., & Merten, O. W. (2018). Genetics instability of wtAAV2 genome and AAV promoter activities in the Baculovirus/Sf9 cells system. *PLoS One*, *13*(7), e0199866.
43. Joshi, P. R., Cervera, L., Ahmed, I., Kondratov, O., Zolotukhin, S., Schrag, J., &Kamen, A. A. (2019). Achieving high-yield production of functional AAV5 gene delivery vectors via fedbatch in an insect cell-one baculovirus system. *Mol. Ther. Methods. Clin. Dev.*, *13*, 279–289.
44. Girod, A., Wobus, C. E., Zádori, Z., Ried, M., Leike, K., Tijssen, P., & Hallek, M. (2002). The VP1 capsid protein of adeno-associated virus type 2 is carrying a phospholipase A2 domain required for virus infectivity. *J. Gen. Virol.*, *83*(5), 973–978.
45. Grieger, J. C., Snowdy, S., & Samulski, R. J. (2006). Separate basic region motifs within the adeno-associated virus capsid proteins are essential for infectivity and assembly. *J. Virol.*, *80*(11), 5199–5210.
46. Kozak, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell*, *44*(2), 283–292.
47. Kozak, M. (1999). Initiation of translation in prokaryotes and eukaryotes. *Gene*, *234*(2), 187–208.

48. Bosma, B., F. du Plessis, Ehlert, E., Nijmeijer, B., de Haan, M., Petry, H., & Lubelski, J. (2018). Optimization of viral protein ratios for production of rAAV serotype 5 in the baculovirus system. *Gene. Ther.*, *25*(6), 415–424.
49. Mietzsch, M., Grasse, S., Zurawski, C., Weger, S., Bennett, A., Agbandje-McKenna, M., & Heilbronn, R. (2014). OneBac: Platform for scalable and high-titer production of adeno-associated virus serotype 1–12 vectors for gene therapy. *Hum. Gene Ther.*, *25*(3), 212–222.
50. Mietzsch, M., Casteleyn, V., Weger, S., Zolotukhin, S., & Heilbronn, R. (2015). OneBac 2.0: Sf9 cell lines for production of AAV5 vectors with enhanced infectivity and minimal encapsidation of foreign DNA. *Hum. Gene Ther.*, *26*(10), 688-697.
51. Kondratov, O., Marsic, D., Crosson, S. M., Mendez-Gomez, H. R., Moskalenko, O., Mietzsch, M., & Zolotukhin, S. (2017). Direct head-to-head evaluation of recombinant adeno-associated viral vectors manufactured in human versus insect cells. *Mol. Ther.*, *25*(12), 2661–2675.
52. Galibert, L., Savy, A., Dickx, Y., Bonnin, D., Bertin, B., Mushimiyimana, I., & Merten, O. W. (2018). Origins of truncated supplementary capsid proteins in rAAV8 vectors produced with the baculovirus system. *PLoS One*, *13*(11), e0207414.
53. Cecchini, S., Slack, J., & Maranga, L. (2020). Engineering of 5′ UTR to control the expression and incorporation level of VP1 during rAAV vector production using a baculovirus system. ASGCT 23rd Annual Meeting.
54. Bryant, L. M., Christopher, D. M., Giles, A. R., Hinderer, C., Rodriguez, J. L., Smith, J. B., & Wilson, J. M. (2013). Lessons learned from the clinical development and market authorization of Glybera. *Hum. Gene Ther. Clin. Dev.*, *24*(2), 55–64.
55. Wright, J. F. (2008). Manufacturing and characterizing AAV-based vectors for use in clinical studies. *Gene. Ther.*, *15*(11), 840–848.
56. Savy, A., Dickx, Y., Nauwynck, L., Bonnin, D., Merten, O. W., & Galibert, L. (2017). Impact of inverted terminal repeat integrity on rAAV8 production using the baculovirus/Sf9 cells system. *Hum. Gene. Ther. Methods*, *28*(5), 277–289.
57. Jacob, A., Brun, L., Gil, P. J., Ménard, L., Bouzelha, M., Broucque, F., & François, A. (2020). Homologous recombination offers advantages over transposition-based systems to generate recombinant baculovirus for adeno-associated viral vector production. *Biotechnol. J.*, https://doi.org/10.1002/biot.202000014.
58. Hu, Y. C., & Bentley, W. E. (2001). Effect of MOI ratio on the composition and yield of chimeric infectious bursal disease virus-like particles by baculovirus co-infection: Deterministic predictions and experimental results. *Biotechnol. Bioeng.*, *75*(1), 104–119.
59. Jiang, B., Barniak, V., Smith, R. P., Sharma, R., Corsaro, B., Hu, B., & Madore, H. P. (1998). Synthesis of rotavirus-like particles in insect cells: Comparative and quantitative analysis. *Biotechnol. Bioeng.*, *60*(3), 369–374.
60. Palomares, L. A., López, S., & Ramírez, O. T. (2002). Strategies for manipulating the relative concentration of recombinant rotavirus structural proteins during simultaneous production by insect cells. *Biotechnol. Bioeng.*, *78*(6), 635–644.
61. Mena, J. A., Ramírez, O. T., & Palomares, L. A. (2006). Intracellular distribution of rotavirus structural proteins and virus-like particles expressed in the insect cell-baculovirus system. *J. Biotechnol.*, *122*(4), 443–452.
62. Rosinski, M., Reid, S., & Nielsen, L. K. (2002). Kinetics of baculovirus replication and release using real-time quantitative polymerase chain reaction. *Biotechnol. Bioeng.*, *77*(4), 476–480.
63. Meghrous, J., Aucoin, M. G., Jacob, D., Chahal, P. S., Arcand, N., & Kamen, A. A. (2005). Production of recombinant adeno-associated viral vectors using a baculovirus/insect cell suspension culture system: From shake flasks to a 20-L bioreactor. *Biotechnol. Prog.*, *21*, 154–160.
64. Mena, J. A., Ramírez, O. T., & Palomares, L. A. (2007). Population kinetics during simultaneous infection of insect cells with two different recombinant baculoviruses for the production of rotavirus-like particles. *BMC. Biotechnol.*, *7*(1), 1–11.
65. Kotin, R. M., & Snyder, R. O. (2017). Manufacturing clinical grade recombinant adeno-associated virus using invertebrate cell lines. *Hum. Gene Ther.*, *28*(4), 350–360.
66. King, J. A., Dubielzig, R., Grimm, D., & Kleinschmidt, J. A. (2001). DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsids. *EMBO J.*, *20*(12), 3282–3291.
67. Collaco, R. F., Kalman-Maltese, V., Smith, A. D., Dignam, J. D., & Trempe, J. P. (2003). A biochemical characterization of the adeno-associated virus Rep40 helicase. *J. Biol.*, *278*(36), 34011–34017.
68. Mena, J. A., Aucoin, M. G., Montes, J., Chahal, P. S., & Kamen, A. A. (2010). Improving adeno-associated vector yield in high density insect cell cultures. *J. Gene. Med.*, *12*(2), 157–167.
69. Thompson, C. M., Petiot, E., Mullick, A., Aucoin, M. G., Henry, O., & Kamen, A. A. (2015). Critical assessment of influenza VLP production in Sf9 and HEK293 expression systems. *BMC Biotechnol.*, *15*(1), 31.
70. Kiesslich, S., J. P. V. C. Losa, Gélinas, J. F., & Kamen, A. A. (2020). Serum-free production of rVSV-ZEBOV in Vero cells: Microcarrier bioreactor versus scale-X™ hydro fixed-bed. *J. Biotechnol.*, *310*, 32–39.
71. Negrete, A., Yang, L. C., Mendez, A. F., Levy, J. R., & 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*(11), 938–948.
72. Aucoin, M. G., Perrier, M., & Kamen, A. A. (2006). Production of adeno-associated viral vectors in insect cells using triple infection: Optimization of baculovirus concentration ratios. *Biotechnol. Bioeng.*, *95*(6), 1081–1092.
73. Kool, M., Voncken, J. W., F. L. J. Van Lier, Tramper, J., & Vlak, J. M. (1991). Detection and analysis of Autographa californica nuclear polyhedrosis virus mutants with defective interfering properties. *Virology*, *183*(2), 739–746.
74. Bernal, V., Carinhas, N., Yokomizo, A. Y., Carrondo, M. J., & Alves, P. M. (2009). Cell density effect in the baculovirus-insect cells system: A quantitative analysis of energetic metabolism. *Biotechnol. Bioeng.*, *104*(1), 162–180.
75. Elias, C. B., Zeiser, A., Bédard, C., & Kamen, A. A. (2000). Enhanced growth of sf-9 cells to a maximum density of 5.2×10^7 cells per mL and production of β-galactosidase at high cell density by fed batch culture. *Biotechnol. Bioeng.*, *68*(4), 381–388.
76. Cecchini, S., Virag, T., & Kotin, R. M. (2011). Reproducible high yields of recombinant adeno-associated virus produced using invertebrate cells in 0.02-to 200-liter cultures. *Hum. Gene. Ther.*, *22*(8), 1021–1030.
77. Liu, Y. K., Yang, C. J., Liu, C. L., Shen, C. R., & Shiau, L. D. (2010). Using a fed-batch culture strategy to enhance rAAV production in the baculovirus/insect cell system. *J. Biosci. Bioeng.*, *110*(2), 187–193.
78. Cain, K. D., Byrne, K. M., Brassfield, A. L., LaPatra, S. E., & Ristow, S. S. (1999). Temperature dependent characteristics of a recombinant infectious hematopoietic necrosis virus glycoprotein produced in insect cells. *Dis Aquat. Organ.*, *36*(1), 1–10.
79. Donaldson, M., Wood, H. A., Kulakosky, P. C., & Shuler, M. L. (1999). Glycosylation of a recombinant protein in the Tn5B1-4 insect cell line: Influence of ammonia, time of harvest, temperature, and dissolved oxygen. *Biotechnol. Bioeng.*, *63*(3), 255–262.
80. Hara, T., Nonaka, K., Kawaguchi, H., Ogata, S., & Etou, N. (1993). Effects of temperature on Escherichia coli β-galactosidase expression in baculovirus-insect cell system. *Biosci. Biotechnol. Biochem.*, *57*(6), 996–997.
81. Reuveny, S., Kim, Y. J., Kemp, C. W., & Shiloach, J. (1993). Effect of temperature and oxygen on cell growth and recombinant protein production in insect cell cultures. *Appl. Microbiol. Biotechnol.*, *38*(5), 619–623.

82. Aucoin, M. G., Perrier, M., & Kamen, A. A. (2007). Improving AAV vector yield in insect cells by modulating the temperature after infection. *Biotechnol. Bioeng.*, *97*(6), 1501–1509.
83. Negrete, A., & Kotin, R. M. (2007). Production of recombinant adeno-associated vectors using two bioreactor configurations at different scales. *J. Virol. Methods*, *145*(2), 155–161.
84. Negrete, A., Esteban, G., & Kotin, R. M. (2007). Dielectric Spectroscopy monitoring for optimum harvest time determination during large-scale production of recombinant adeno-associated vectors. *Appl. Microbiol. Biotechnol.*, *76*(6), 761–772.
85. Pais, D. A., Galrão, P. R., Kryzhanska, A., Barbau, J., Isidro, I. A., & Alves, P. M. (2020). Holographic imaging of insect cell cultures: Online non-invasive monitoring of adeno-associated virus production and cell concentration. *Processes*, *8*(4), 487.
86. Bosma, B., F. du Plessis, Sanders, B. S., van Dveventer, A., & Lubelski, J. (2020). Generation of a DuoBac expression system for improved AAV production. ASGCT 23rd Annual Meeting.
87. Horowitz, E. D. (2020). Biophysical and in-vitro comparability analysis of an AAV vector produced by the baculovirus/Sf9 system and HEK triple transfection system. ASGCT 23rd Annual Meeting.
88. Wu, Y., Mei, T., Jiang, L., Han, Z., Dong, R., Yang, T., & Xu, F. (2019). Development of versatile and flexible Sf9 packaging cell line-dependent OneBac system for large-scale recombinant adeno-associated virus production. *Hum. Gene. Ther. Methods.*, *30*(5), 172–183.
89. Pesic, M., Ahamed, T., Golinska, M., & Lubelski, J. (2020). Production of recombinant AAV vectors in chemically defined media. ASGCT 23rd Annual Meeting.
90. Thompson, K., Bundy, M., Irvin, K., Yocheva, M., Barnes, M., van den Berg, A., & Zmuda, J. (2020). Recombinant adeno-associated virus (AAV) production in the ExpiSf™ expression system. ASGCT 23rd Annual Meeting.
91. Kurasawa, J., Park, A., Sowers, C., Halpin, R., Tovchigrechko, A., Dobson, C., … Ikeda, Y. (2020). Chemically-defined, high-density insect cell-based expression system for scalable AAV vector production. ASGCT 23rd Annual Meeting.
92. Jacques, J., Jones, A., Xing, D., Low, K., & Bossie, A. (2020). Evaluation of AAV production in insect cells using chemically defined media. ASGCT 23rd Annual Meeting.
93. Rumachik, N. G., Malaker, S. A., Poweleit, N., Maynard, L. H., Adams, C. M., Leib, R. D., & Sinn, P. (2020). Methods Matter–Standard Production Platforms for Recombinant AAV Produce Chemically and Functionally Distinct Vectors. *Mol. Ther. Methods. Clin. Dev.*, *18*, 98–118.
94. Grieger, J. C., Soltys, S. M., & Samulski, R. J. (2016). Production of recombinant adeno-associated virus vectors using suspension HEK293 cells and continuous harvest of vector from the culture media for GMP FIX and FLT1 clinical vector. *Mol. Ther.*, *24*(2), 287–297.
95. Martin, J., Frederick, A., Luo, Y., Jackson, R., Joubert, M., Sol, B., & Vincent, K. (2013). Generation and characterization of adeno-associated virus producer cell lines for research and preclinical vector production. *Hum. Gene. Ther. Methods*, *24*(4), 253–269.
96. Thorne, B. A., Takeya, R. K., & Peluso, R. W. (2009). Manufacturing recombinant adeno-associated viral vectors from producer cell clones. *Hum.Gene. Ther.*, *20*(7), 707–714.
97. Zeltner, N., Kohlbrenner, E., Clément, N., Weber, T., & Linden, R. M. (2010). Near-perfect infectivity of wild-type AAV as benchmark for infectivity of recombinant AAV vectors. *Gene. Ther.*, *17*(7), 872–879.
98. Geoffroy, M. C., & Salvetti, A. (2005). Helper functions required for wild type and recombinant adeno-associated virus growth. *Curr. Gene Ther.*, *5*(3), 265–271.
99. Meier, A. F., Fraefel, C., & Seyffert, M. (2020). The interplay between adeno-associated virus and its helper viruses. *Viruses*, *12*(6), 662.
100. Tai, P. W. (2020). ITRs: The terminal frontier. *Hum. Gene Ther.*, *31*(3-4), 143–144.
101. Ma, H., Galvin, T. A., Glasner, D. R., Shaheduzzaman, S., & Khan, A. S. (2014). Identification of a novel rhabdovirus in Spodoptera frugiperda cell lines. *J. Virol.*, *88*(12), 6576–6585.
102. Maghodia, A. B., Geisler, C., & Jarvis, D. L. (2016). Characterization of an Sf-rhabdovirus-negative Spodoptera frugiperda cell line as an alternative host for recombinant protein production in the baculovirus-insect cell system. *Protein Express. Purif.*, *122*, 45–55.
103. Li, T. C., Scotti, P. D., Miyamura, T., & Takeda, N. (2007). Latent infection of a new alphanodavirus in an insect cell line. *J. Virol.*, *81*(20), 10890–10896.
104. Maghodia, A. B., & Jarvis, D. L. (2017). Infectivity of Sf-rhabdovirus variants in insect and mammalian cell lines. *Virology*, *512*, 234–245.
105. Maghodia, A. B., Geisler, C., & Jarvis, D. L. (2020). A new nodavirus-negative Trichoplusia ni cell line for baculovirus-mediated protein production. *Biotechnol. Bioeng.*, *117*(11), 3248–3264.
106. Cecchini, S., Negrete, A., & Kotin, R. M. (2008). Toward exascale production of recombinant adeno-associated virus for gene transfer applications. *Gene. Ther.*, *15*(11), 823–830.
107. Steele, K. H., Stone, B. J., Franklin, K. M., Fath-Goodin, A., Zhang, X., Jiang, H., & Geisler, C. (2017). Improving the baculovirus expression vector system with vankyrin-enhanced technology. *Biotechnol. Prog.*, *33*(6), 1496–1507.
108. Gómez-Sebastián, S., López-Vidal, J., & Escribano, J. M. (2014). Significant productivity improvement of the baculovirus expression vector system by engineering a novel expression cassette. *Plos One*, *9*(5), e96562.
109. Kaba, S. A., Salcedo, A. M., Wafula, P. O., Vlak, J. M., & van Oers, M. M. (2004). Development of a chitinase and v-cathepsin negative bacmid for improved integrity of secreted recombinant proteins. *J. Virol. Methods*, *122*(1), 113–118.
110. Chahal, P. S., Aucoin, M. G., & Kamen, A. (2007). Primary recovery and chromatographic purification of adeno-associated virus type 2 produced by baculovirus/insect cell system. *J. Virol. Methods*, *139*(1), 61–70.
111. Hegedus, D. D., Pfeifer, T. A., Hendry, J., Theilmann, D. A., & Grigliatti, T. A. (1998). A series of broad host range shuttle vectors for constitutive and inducible expression of heterologous proteins in insect cell lines. *Gene*, *207*(2), 241–249.
112. Pfeifer, T. A. (1998). Expression of heterologous proteins in stable insect cell culture. *Curr. Opin. Biotechnol.*, *9*(5), 518–521.
113. Kempf, J., Snook, L. A., Vonesch, J. L., Dahms, T. E., Pattus, F., & Massotte, D. (2002). Expression of the human μ opioid receptor in a stable Sf9 cell line. *J. Biotechnol.*, *95*(2), 181–187.
114. Nandakumar, S., Ma, H., & Khan, A. S. (2017). Whole-genome sequence of the Spodoptera frugiperda Sf9 insect cell line. *Genome. Announc.*, *5*(34), e00829-17.

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