Advancements in Insect-Cell Baculovirus Expression Vector Platform for Production of Recombinant Adeno-Associated Virus-Based Gene Delivery Vectors

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Abstract

Despite rapid progress in the field, scalable high-yield production of 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 a scalable production of recombinant proteins with clinically approved products for human use. In this review, we provide a detailed overview on advancements in IC-BEVS for rAAV production. Since the first report of baculovirus induced production of rAAV in insect cells in 2002, this platform has undergone significant evolutions. The original three baculovirus system was improved to enhance expression stability and further streamlined to reduce the number of baculoviruses to two and eventually one. 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, 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 molecular design of vectors to enable higher cell-specific yield of functional AAV particles in combination with bioprocess intensification strategies may further contribute to addressing current and future manufacturing challenges.

1 Introduction

Since the establishment of the first successful continuous insect cell line in 1959[1], unending research and development led to the establishment of insect cells as a workhorse for the production of numerous recombinant proteins for research and clinical applications. The discovery and isolation of Autographa californica multinuclear polyhedrosis virus (AcMNPV) in 1971 was an important step in the realization of the broad potential of the insect cell-baculovirus expression vector system (IC-BEVS)[2]. In the following years, extensive studies related to the biology of the baculovirus, its infection kinetics, genome sequences, and structural variants were completed. The identification of the polyhedrin as a strong late promoter, which was non-essential for the baculovirus replication, was the breakthrough finding by Smith et al. in 1983 that first established the IC-BEVS. Smith et al. demonstrated the expression of human interferon-beta (INF-β) and subsequently interleukin-2 (IL-2), using IC-BEVS [3],[4]. Additionally, another strong late promoter, p10, was discovered and was used in protein expression studies[5].

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 were extensively used due to their susceptibility to baculovirus infection and good growth performances in adherent and thereafter in suspension cell cultures[6]–[10].

In the early 90's cell culture engineers took the lead in studying the insect cell growth kinetics and metabolism in serum-supplemented and serum-free culture medium. Rapidly, serum-free cell media with shear protec-

tive properties were developed to enable suspension cell cultures in shake flasks and bioreactors[11]–[14] demonstrating scalability and robustness of the IC-BEVS process for protein production.

Initially employed for the production of baculoviruses as biopesticides, the IC-BEVS quickly gained popularity for the expression of a broad spectrum of recombinant proteins, including enzymes, glycoproteins, recombinant viruses and vaccines [10], [15]. The IC-BEVS platform found applications in the production of veterinary vaccines such as Porcilis pesti, Circumvent PCV, CircoFLEX[16], and human vaccines such as Cervarix®[17], and Flublok®[9]. Regulatory approval of Cervarix®, a virus-like particle-based vaccine against cervical cancer, was a critical milestone as it was the first biologics produced in insect cell and approved for human use. The ability of the insect cells to grow in scalable serum-free suspension cultures, with a demonstrated history of commercial scale manufacturing and a regulatory portfolio, established the IC-BEVS as a platform of choice for research and development and industrial manufacturing of biologics.

Currently, AAV is 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 the IC-BEVS scalable production systems for AAV manufacturing. From the regulatory perspective, additionally to the approval of Glybera® by the European Medicines Agency in 2012[18], a more recent and significant milestone is the breakthrough designation by USFDA of Biomarin‘s Hemophilia A gene therapy candidate, a recombinant adeno associated virus 5 (rAAV5) gene delivery vector produced in insect cells using IC-BEVS[19]. This further contributed to align the IC-BEVS manufacturing process of AAV with the current standard of Good Manufacturing Practice (GMP).

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

2 AAV biology

The AAV belongs to the parvoviridae family: a family of single-stranded DNA viruses that have evolved over the years, expanding a spectrum of hosts[20]. The viruses of this family share some common structural and functional features such as conserved phospholipase A_2 (PLA_2)-like enzymatic domain and identical replication components and pathways [20], [21]. Based on the host specificity, the parvoviridae family is divided into two subfamilies: Parvovirinae and Densovirinae, which infect mammalian and invertebrate hosts, respectively (Figure 1A). The Parvovirinae subfamily is further divided into eight genera, including Dependovirus, into which AAV is classified. The insect cells are a natural host of Densovirinae viruses, and from a taxonomical standpoint, they should provide an adequate cellular environment for replication of viruses from closely related subfamily Parvovirinae, including AAV. This relation was first recognized and later found to be applicable by Urabe et al., where the group demonstrated the first IC-BEVS based rAAV production platform [22].

AAV virus particle comprises a vector genome: the functional element of the virus, and the capsid, a metastable entity that preserves it. The vector genome is a linear single-stranded DNA with a size of about ˜4.7kb and a major coding region consisting of the sequences for non-structural replicase proteins (Rep) and structural capsid proteins (Cap or VP). This coding region is flanked by an inverted terminal repeat sequences (ITR). The ITR is a segment of 145bp with a T-shaped hairpin-like structure formed via complementary base pairing of palindromic sequence of 125 bp. The remaining 20 bases stay unpaired. The ITR region also encompasses the origin of replication formed by the terminal resolution site and rep-binding element (RBE). The free 3‘ hydroxyl region acts as an initiation sequence for DNA replication.

AAV contains four non-structural Rep proteins (Rep 78, Rep 68, Rep 52, and Rep40). Rep78 and Rep52

are expressed under the transcriptional control of p5 and p19 promoters, respectively. The larger Rep78 and its spliced variant Rep68 are crucial for- and participate in vector genome integration, excision, rescue, and replication at various stages of AAV life cycle[23]–[25]. The smaller Rep proteins Rep52 and its spliced variant Rep40 play a critical role in the packaging of vector DNA in preformed capsids and regulation of replication by repressing p5 in the absence of a helper virus[26], [27]. The three structural proteins (VP1, VP2, and VP3) expressed from the right open reading frame (ORF) under p40 promoter form an icosahedron capsid that carries the viral genome and delivers it to the host cell traversing through post cell surface attachment. A representative genomic map of wtAAV2 [28] and its transcriptional profile are shown in Figure 1B.

3 Molecular Design of Baculovirus Expression Vectors and Cell Lines For Production of rAAV

In the last two decades, various molecular design approaches have been used to achieve efficient AAV production employing IC-BEVS platform. Building upon intrinsic advantages of this platform, the active efforts were directed towards achieving higher expression stability, improved 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.

3.1. Three Baculovirus (BV) System: Three-Bac

The Three-Bac system achieved the production of the functional recombinant AAV2 particles via co-infection of Spodoptera frugiperda (Sf9) cells with three recombinant AcMNPV vectors [22]. In this particular study, these baculovirus vectors were identified as Bac-VP (Cap), Bac-Rep, and Bac-ITR, which harbored the AAV2 *cap* gene, AAV2 *rep* gene, and ITR flanked, for example, by *gfp* gene sequence cassettes as a transgene, respectively (Figure 2A). In general, the insect cell promoters in combination with cellular machinery are crucial to drive the production of foreign proteins in insect cells. During the development of the Three-Bac system, the main challenges faced were related to the optimization of molecular expression cassettes to achieve proportionate expression of capsid subunits and replicase proteins[29]. The main challenge with replicase protein expression was due to the colinear ORFs in AAV genome wherein Rep52 and its p19 promoter sequences are in-frame with the entire Rep78 sequence[28]. This arrangement also led to the duplication of sequences and any modifications such as insertion of insect promoter in the Rep52 sequence to facilitate transcription in insect cell will result in an altered sequence of entire Rep78 and its functional characteristics. Therefore, Urabe et al[22]. separately arranged Rep78 and Rep52 expression cassettes in transcriptionally opposite orientation (Bac-Rep) under different insect promoters to achieve their desired expression level (Figure 2A). On the other hand, the challenge with the capsid proteins was achieving stoichiometric expression of three VP proteins in a prototypic ratio of 1:1:10 for VP1:VP2: VP3. Sufficient expression and incorporation of VP1 in AAV capsid is critical for efficient transduction[30], [31]. During AAV production in mammalian cells, the intron splicing and leaky scanning of translation initiation codon by ribosomal complex results in differential expression of three VP proteins in the desired proportion which was not observed in insect cell[22]. As a result, the Bac-VP expression cassette was designed with a modified *cap* sequence consisting of a weak VP1 translation initiation codon-ACG in combination with a complex of nine nucleotide sequence to achieve the stochastic ratio of VP proteins. The resulting AAV2 particles demonstrated biophysical and functional characteristics identical to that of mammalian-cells produced AAV2[22].

An important finding by Urabe et al[22]. was that unlike mammalian cells, AAV production in insect cells via BEVS does not require an additional helper virus such as Adenovirus or Herpes simplex virus (HSV), and the necessary helper function is provided by the baculovirus in a fashion possibly distinct yet functionally comparable to that of Adenovirus or HSV. It can also be inferred that the insect cell adequately provided the components and cellular machinery necessary for AAV protein production, capsid assembly, genome replication, and its packaging.

The original Three-Bac system, although extremely promising and flexible, found minimal application due to the operational complexity from the bioprocessing standpoint and the instability of all three-helper baculoviruses coding *rep* , *cap* , and transgene cassette. Continuously generating, characterizing, and validating three baculoviruses seed stocks during AAV production runs was a significant undertaking to assure process

consistency and poses a significant burden. Moreover, Kohlbrenner et al. reported the passage dependent loss of AAV protein expression in these BVs, notably the loss of Rep proteins. The loss of expression of Rep proteins, which are essential for genome replication and its encapsidation, resulted in an overall reduced yield of packaged and functional AAV particles[32]. Palindromic orientation with Rep78 and consequent excision of the entire Rep 52 sequence was believed to be the reason for Bac-Rep instability (Figure 2A). When isolated onto different baculoviruses, the stability of Rep78 and Rep52 was restored over the extended BV passage, however, requires quadruple BV co-infection further enhancing operational complexity[32].

3.2 Two Baculovirus system: Two-Bac

The next generation baculovirus constructs design was driven by two key objectives: (1) the increase in expression stability of BV constructs, and (2) the reduction in number of baculoviruses for process simplification (Figure 3). In 2008, Chen proposed a strategy to improve the baculovirus stability with the introduction of a synthetic intron in the coding region of Rep (Bac-Intron-Rep: Bac*In* Rep) and Cap (Bac-Intron-Cap: Bac*In* Cap) sequences[33]. The synthetic intron consisted of a splice acceptor and a donor sequence flanking polh promoter sequence, which, when placed in a specific position upstream to Rep52 or VP2 stably expressed Rep and Cap over the extended passage numbers. The placement of a synthetic intron in Bac*In* Rep facilitated the independent expression of Rep78 and Rep52 from two different mRNA transcripts with improved stability. Similarly, in Bac*In* Cap, the synthetic intron drove independent expression of VP1 and VP2/3 from two different mRNA transcripts, respectively offering not only stability but also desired stoichiometry of all three VP proteins. The system was further simplified by combining both expression cassettes onto a single baculovirus: Bac*In* RepCap now requiring only two baculovirus co-infection for AAV production (Figure 2A).

Addressing the stability issue of Bac-Rep construct, in 2009, Smith et al. reported a different strategy leveraging ribosomal leaky scanning mechanism for stable expression of Rep proteins[34]. Here, the*rep* gene sequence was altered which consisted of (1) a modified weak translational initiation codon for Rep78-CUG and (2) Modification of nine downstream AUG codons without altering the functionality of Rep proteins. This resulted in a non-duplication of Rep78 and Rep52 sequences and circumvented the root cause of the genetic instability of the original Bac-Rep construct. These modifications resulted in a weak Kozak sequence for Rep78, and subsequent leaky scanning of Rep78 and Rep52 codons. As a result, the stable expression of both Rep78 and Rep52 in a desired proportion was achieved from a single mRNA transcript. Next, to reduce the number of baculovirus vectors from three to two, the original VP expression cassette was combined and arranged in transcriptionally opposite direction with reference to Bac-Rep cassette (Figure 2A). This combination resulted in Bac-Rep2CapX recombinant baculovirus now requiring dual baculovirus co-infection for AAV production.

3.3 One Baculovirus System: One-Bac1.0

The One-Bac system reported by Aslanidi et al. was a further adaptation of the IC-BEVS platform for rAAV production[35]. This system consists of two components: (1) A baculovirus-inducible stably transformed packaging Sf9 cell line harboring the AAV2 *rep* and*cap* sequences under the transcriptional control of polh promoter and (2) a single recombinant baculovirus carrying AAV2 ITR flanked transgene/gene of interest sequences (Bac-ITR). The inducible cell line offers the advantage of regulated yet amplified expression of AAV proteins resulting in a higher yield of AAV, whereas the stable packaging cell line ensures consistent expression levels requiring only a single baculovirus infection.

The development of this system provided answers to two key questions, (1) how to devise a regulation switch for inducible expression of AAV proteins in insect cells? and (2) how to achieve sustained and higher expression of incorporated Rep and Cap proteins in the desired proportion? The initial unsuccessful attempt of generating stable cell line suggested an absolute requirement of baculovirus genomic elements in *cis* . One such element used in the final expression cassette was a modified homologous region 2 (hr2-0.9), derived from A. californica, which is inducible in the presence of immediate early (IE-1) transcriptional *trans* - regulator, herein provided by Bac-ITR baculovirus. Related to the second question, the optimal expression

level of Rep78/Rep52 is essential and has a direct effect on the overall yield. The lower expression ratio of Rep78:Rep52 is favored for better AAV yield[29]. In order to further improve the system, the rep-binding element (RBE) was included in the rep and cap expression cassette. This was based on the findings that the ITR and a complex of p5 and rep-binding element (RBE), alone or in combination, upregulate p19 promoter activity in mammalian cells[36], [37]. Further studies reported the evidence of partial activity p19 promoter in the insect cells[32], [38]. Combining these findings, the final version of *rep* and *cap* expression cassettes to generate a stable cell line consisted of hr2-0.9 and RBE regulatory elements derived from baculovirus and AAV2, respectively (Figure 2A). As a result, lower Rep78:Rep52 expression ratio and sustained higher expression of rep and cap proteins (Figure 2B) were obtained with up to ten times higher cell-specific yield of AAV2 when compared to the original Three-Bac system.

The modular construct design of this One-Bac was further explored for the production of AAV serotypes 1-12 [39]. The AAV production yield for all the serotypes was either comparable or higher than the original Three-Bac system. Notable though was the deficient VP1 expression and consequent reduction in transduction efficiency of some serotypes, specifically of AAV5.

3.4 Low VP1 Expression and Functional AAV yields in Insect Cells

In the mammalian cells, AAV's natural host, the combination of mRNA splicing of p40 promoter-driven transcript and leaky ribosomal scanning of VP2's weak translation of initiation codon-ACG result into the expression of three VP subunits in a standard ratio of 1:1:10 for VP1:VP2:VP3 from a mRNA transcript. The VP1 carries the sequence of PLA_2-like enzymatic domain and nuclear localization signal, both of which are critical due to their postulated role in AAV transduction[30], [40]. Therefore, the expression of VP1 in an appropriate proportion and its subsequent incorporation in the AAV capsid is crucial to generate functional viral particles. Due to the inherent difference of splicing mechanism in insect and mammalian cells, in the original Three-Bac system, authentic start codon AUG of VP1 was mutated to weak ACG codon facilitating leaky scanning via insect cell ribosomal machinery achieving expression of all three VP proteins in a proportion comparable to mammalian cells[22]. The Three-Bac though originally successful for AAV2, was found to be non-versatile for other serotypes such as AAV5 and AAV8[32]. AAV5 being the evolutionary most distant serotype[41], is of important clinical significance due to its relatively better immuno-privileged characteristics compared to other serotypes that are closely related to human origin serotype AAV2. The AAV5, when produced in insect cell, exhibited no or lesser transduction efficiency, which was found to be associated with insufficient VP1 expression[32], [39], [42]. The deficient VP1 expression was linked to the AAV5 VP1 sequence and the leaky scanning efficiency in insect cells. The efficiency of ribosomal scanning for recognizing AUG as a translation initiation codon relies on Kozak sequence: a sequence context surrounding AUG, importantly ACCAUG*G,* where G at +4 position is crucial (A of AUG being +1) in case of weak initiation codon such as ACG[43], [44]. In contrast to AAV2, the G of +4 position is U, in the case of AAV5, which is believed to be responsible for deficient expression and subsequent lesser incorporation of VP1 in AAV5 capsid[42]. Urabe et al. addressed this challenge by domain swapping where 86 amino acid long AAV5 VP1 sequence at specific positions was substituted with that of AAV2 VP1 sequence resulting in AAV5 chimeric capsid with infectivity comparable to that of AAV2[42]. Similarly, Kohlbrenner et al. performed the domain swapping of an entire AAV5 and AAV8 VP1 sequences with that of AAV2 VP1, resulting in increased VP1 proportion and PLA_2 activity with restoration of transduction efficiency[32]. In One-Bac2.0, the second generation One-Bac, the mutated VP1 initiation codon (ACG) was replaced with an authentic VP1 strong codon (AUG), which in combination with synthetic intron placement strategy as reported earlier by Chen[33], demonstrated stronger expression of all three VP proteins from two mRNA transcripts and restored original composition of VP1 and AAV5 functionality. The RBE negative version of this cell line ([?]RBE) also significantly reduced the cross packaging of *rep/cap* sequences and hence the proportion of replication competent viral particles confirming the role of RBE as a packaging signal[45]. Overall, these modifications improved the final quality of the AAV5 serotypes produced using One-Bac2.0. Further improved version was the third generation system:One-Bac3.0 where the canonical VP1 initiation codon AUG was restored and was located in serotype-specific strategically modified Kozak context sequence to achieve optimal expression level of all three VP proteins[46]. Here, the strong AUG codon was

surrounded by a weak Kozak sequence, which led to suboptimal translation initiation efficiency of 40%-45% compared to optimal mammalian Kozak sequence. As a result, reduced yet sufficient VP1 expression was achieved, and concomitant leaky scanning of following VP2/VP3 start codons restored their optimal expression ratio with respect to VP1. AAV5 and AAV9 serotypes produced using this system demonstrated in-vivo transduction efficiency better than or comparable to those produced in mammalian cells. Coupled with the [?]RBE strategy, this system produced vectors with only marginal encapsidation of foreign DNA sequences as reported with next-generation sequencing analysis[46].

Recently, in the Two-Bac system, the selection of optimal translation initiation codon and associated downstream nucleotide sequence which exhibited optimal stoichiometric expression of all three VP proteins of AAV5 with improved functionality of the resultant vector was reported [47].

4 Bioprocessing of rAAV Using The IC-BEVS Manufacturing Platform

Besides the molecular design of expression systems, the bioprocessing aspect of IC-BEVS is equally essential for successful manufacturing of AAV. In a typical IC-BEVS process, the cellular state depends on the cell-virus interaction and infection kinetics, cellular energetics and metabolism, and extracellular culture environment. The overall outcome is a result of multifactorial interactions under a dynamic and productive environment of the cell culture process. The following section will discuss the effect of these parameters on the overall production performances of rAAV vectors in insect cells.

4.1 Insect Cell Lines

Various insect-derived cell lines are currently being used for the manufacturing of recombinant proteins. As previously discussed Sf9 cells are predominantly used in AAV production process. Meghrous et al. studied the AAV production in Sf9 and Hi5 cells using Three-Bac system[48]. The study suggested that both cell lines offered comparable cell-specific yields of functional AAV particles. Due to better suitability of Sf9 for baculovirus stock generation over Hi5 cells[49] and no significant advantage of Hi5 over Sf9 in overall AAV yield, Sf9 cells were selected for further process development work (Table 1)[48]. Another study involving AAV production in Sf21 using a triple BV co-infection strategy also suggested better performance of Sf9 for AAV production[50].

4.2 Multiplicity of Infection (MOI) of Baculovirus Vectors

In the IC-BEVS, the MOI of the baculovirus has a critical impact on the rate of cell growth and viability, the production kinetics, the quantity and quality of the recombinant protein expressed, and the harvest time of the culture. The low and high MOI infections are characterized by their different rate of infection (synchronous Vs. asynchronous) and the associated difference in kinetics of protein expression. Expression of viral vector or virus-like particles, which typically involve co-infecting cell cultures with more than one rBV carrying the coding sequences of individual components adds another complexity to the production process [51], [52]. Altering the MOI of multiple baculoviruses and their time of infection often result in altered capsid compositions as reported by various groups[53], [54]. AAV production using triple baculovirus co-infection strategy is also an example of such a complex and dynamic system where primary work has relied on multicomponent system. Initial studies by Meghrous et al. demonstrated the effect of Bac-Rep on overall Sf9 growth behavior and AAV vector yield using Three-Bac system and its association with encapsidation of vector DNA, and the transducing particle units[48]. The Bac-Rep showed no notable effect on cell viability and growth behavior irrespective of MOI used. The study focused on the multidimensional interactive effect of multiple baculoviruses, each expressing an essential protein of rAAV, suggested a significant effect of Bac-Rep and Bac-Cap MOI on packaging efficiency and functional AAV particles. A full factorial study conducted by Aucoin et al. provided an in-depth analysis of single factor and multifactorial interaction effects of MOI of the three baculoviruses on AAV yields [55]. This study also suggested a self-sustaining nature of insect cell-baculovirus infection process, where differing MOI of one baculovirus did not lead to the drastic changes in overall AAV yield when expressed collectively as relative proportions of total, genome containing and functional particles.

4.3 Effect of Cell Density at Infection

The cell density over which the cell-specific yield of recombinant protein expression drops, is an important indicator for selecting the infection time and the cell density at infection for a productive batch. This has been referred to in many studies as the cell density effect or the time of infection. In the case of insect cell-baculovirus system, this critical indicator is a result of the overall nutrient availability, the cellular physiological state, and the ratio of cell population infected versus total viable cells in the culture determined by the MOI (i.e. synchronous Vs. asynchronous) during the production phase. Commercially available serum-free media such as Sf 900-II support a maximum cell density growth in batch mode of around 10 million cells/mL[56]. However, the same medium does not sustain protein expression in the post-infection phase at such a cell density. When analyzed for AAV production using a triple BV co-infection strategy at an overall MOI 5, the optimal cell density at the time of infection was reported to be 1 million cells/mL in the EX-CELL$^{(r)}$ 420 medium[48]. Below this cell density, the nutrient consumption was suboptimal, and above this cell density, the cell-specific yield dramatically dropped. In another study, Cecchini et al. reported that cell-specific yield of AAV production in Sf9 cells was maintained up to the cell density of 3.5-4 million cells/mL in SFX serum-free medium[57]. When analyzed for AAV5 production in One-Bac Sf9 cells using Sf900-II or Sf900-III medium by Joshi et al., the cell density breakpoint was found to be 1.7-2.0 million cells/mL under synchronous infection [58]. Any combination of cell density at the time of infection and MOI that results in an overall cell density above 2 million cells/mL resulted in a drop in the cell-specific yield.

4.4 Effect of Operation Mode

The 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 exceptionally high demand of the late clinical phase studies. When coupled with a sustained cell-specific yield, the high cell density culture process under fedbatch or perfusion mode of operation provide a mean to enhance the volumetric yield. Both modes have been successfully implemented to achieve a high cell density culture of Sf9 cells[58], [59]. The nutrient feeding strategy in a fedbatch process aims at maintaining the cells in the mid-exponential phase for a sustained time to achieve a high cell density before infection. When coupled with nutrient feeding in the post-infection phase, metabolic limitations are alleviated. Bedard et al. described an exemplary work where the nutrient feed formulation was designed to meet metabolic need of Sf9 cells to support the sustained expression of β-galactosidase following BV infection[60]. Building on this, Elias et al. reported β-galactosidase production at a cell density of 14 million cells/mL without reduction of the cell-specific yield[61]. Meghrous et al. reported that when applied at the time of infection for the triple BV co-infection system, a complete medium replacement strategy that practically mimics the perfusion at shake flask scale, resulted in a linear increase in volumetric yield at various cell densities at the time of infection without any loss of cell-specific yield of AAV[48]. When further scaled up in a 3L bioreactor, the infection of Sf9 cells at 2.5 million cells/mL in a fresh medium offered similar cell-specific yield and a proportionate increase in volumetric yield (Table 1). In a further study by Mena et al. the medium replacement strategy coupled with infection at 5 million cells/mL with high MOI of triple BVs (3 MOI of each) resulted in around three fold increase in genomic AAV particles in comparison to the control, low cell density batch process[59]. This increase in volumetric yield was proportionate to the increase in cell density, which demonstrated sustained cell-specific yield. Furthermore, when carefully tailored, the fedbatch strategy in combination with low MOI applied to AAV production in Sf9, resulted in almost 1 log increase in volumetric yield of functional AAV particles (Table 1)[59]. In this case, Sf9 cells were infected at 5 million cells/mL at an MOI of 0.1 (of each BVs), which resulted in cell density increase up to 9.6 million cells/mL at 48 hpi. The nutrient supplementation was provided at the time of infection, and 24 h before and after infection. A more recent study by Joshi et al. involving AAV5 production employing One-Bac under fedbatch mode of operation also suggested improved yield of genomic and functional particles of AAV5 (Table 1)[58]. The high cell density at the time of infection (˜10 million cells/mL) was achieved via bolus nutrient supplementation followed by high MOI infection, which resulted in an immediate transition of cell culture from the growth phase to the productive phase within 24hpi under synchronous infection of baculovirus. Coupled with an additional bolus feeding, the process exhibited sustained AAV production in post-infection phase[58].

4.5 Effect of Temperature

The cell cultivation temperature is crucial for optimal cellular metabolism, protein production, and processing. The temperature modulation strategy is often applied to alter the cell metabolism, production of glycoprotein variants, and their processing. The insect cells are reported to best grow at temperature between 27°C to 30°C[62]. In addition, no heat shock protein expression has been reported at a temperature below 37°C[63]. However, there are reports available with varying conclusions on protein expression yield at various temperatures[64]–[67]. Some reports suggest better protein processing at temperature lower than 27°C (20°C -24°C) without any effect on the overall yield. Aucoin et al. reported AAV production in Sf9 cells using a triple BV co-infection strategy at various temperatures[68]. Across different temperature range tested (°C:21, 24, 27, 30 and 33), the highest yield of AAV functional particle was achieved at 30°C which was 2.5X and 5X more than at 27°C and temperatures below it, respectively. At the temperature below 27°C, the cells show reduced growth rate and lower protein expression; however, the cell viability was maintained in the post-infection phase. There was no significant decrease in overall maximum cell density achieved at various temperatures. Interestingly, the temperature above 27°C showed a significant effect on the kinetics of AAV helper protein expression.

4.6 High Cell Density-High MOI Production Process: A Case Study of Three-Bac

When various proteins are co-expressed via multiple baculoviruses, the kinetic of expression of these proteins have an important effect on the overall quantitative and qualitative yield of AAV. The optimal MOI (MOI of 5 of each) of three baculovirus vectors for AAV production was very high[55]. However, infection of Sf9 cells at such a high MOI in a high cell density process poses an additional burden on baculovirus stock preparation. Generally, the baculovirus stock with a concentration of 1-3x10^8 pfu/mL requires addition of a large volume to achieve a high MOI, which often results in a substantial dilution of the culture. For example, in an AAV production process involving infection at 3-4 million cells/mL, the baculovirus co-infection at individual MOI of 5 requires around 17%-20% v/v of each of three baculoviruses stock (1x10^8 pfu/mL) which results in an overall dilution of the culture by around 50%-60% v/v which is not desirable. The direct solution to this is to use either concentrated stock of baculovirus, preparations of which has its challenges, or low MOI process[59], which results in asynchronous infection. An alternative solution was reported by Cecchini et al., where the researchers proposed the strategy that mimics low MOI infection culture behavior and involves baculovirus-infected insect cell (BIIC)[57]. Here, the Sf9 cells were infected at low MOI of each of three baculoviruses, which results in an asynchronous infection and infection of the entire cell population within 48 hpi. Following this, the BIIC at 48 hpi were mixed with non-infected insect cells (NIIC) in a ratio of 1:10000 and frozen under liquid nitrogen. When thawed, the cells undergo a secondary wave of baculovirus infection from the progeny generated from BIIC and infect the rest of NIIC in the culture within 48-72 hours post vial thaw. This strategy eliminated the baculovirus infection step in the actual culture and offered consistency in baculovirus infection kinetics, and protein expression since the relative proportion of BIIC and NIIC is kept constant in the frozen cell bank. After the vial thaw, the culture is volumetrically expanded by the addition of fresh medium until the desired working volume in a bioreactor is achieved. When assessed at different scales (shake flask to 200L bioreactor) for different serotypes (AAV -6 and -9), a comparable yield of AAV serotypes was reported (Table 1) [57].

4.7 AAV Production Yield in STB and Wave Bioreactors

The favorable features of insect cell baculovirus platform are the ability of insect cells to grow in suspension culture at a high cell density, a proven linear scalability, and a history of regulatory acceptance of the platform for the production of various biologics[9], [17]. Various reports of AAV production in either conventional stirred-tank bioreactors (STB) (glass, stainless steel, or disposable vessel)[48], [58], [69] or Wave bioreactors[69] suggest comparable yields and quality of rAAV vector material (Table 1). The very first report of AAV production employing a Three-Bac system in a 20L bioreactor by Meghrous et al. suggested AAV material generation with around 3-fold improvement in cell-specific yield of functional AAV particles in a controlled environment of bioreactor compared to shake flask[48]. This production run generated around 5x10^{12} AAV transducing units (Table 1). Subsequent reports of AAV production using modified Three-Bac

system in Wave and stirred tank bioreactors suggest that AAV volumetric yield was in the range of $5x10^9$ – $3x10^{10}$ VG/mL with a comparable consistency at various scales of Wave and STB, respectively[69]. The lower volumetric yield of AAV in Wave bioreactor was attributed to poor oxygen mass transfer control in the culture. However, the vector quality, as indicated by the ratio of genomic: transducing units, was consistent in all production runs. Table 1 summarizes the AAV yield at various scales, and in different IC-BEVS production reported. A schematic overview of AAV production process using IC-BEVS platform is depicted in Figure 3.

5 Conclusion and Future Perspectives

From a molecular design standpoint, the Three-Bac system offers the greatest flexibility by providing many possible combinations of expression of VP proteins, replicase protein, and the gene of interest independently. It is also a modular tool for pseudotyping various AAV capsids[45],[32] allowing the production of mosaic AAV capsids with different combinations of one or more VP proteins from other serotypes. The Two-Bac system somewhat restricts this flexibility, especially in a case where *rep* and *cap* sequences are combined and inserted in a single baculovirus construct. The One-Bac system, which involves Rep2CapX packaging cell line and a Bac-GOI, offers restricted flexibility. The generation of the stable packaging cell line represents a strategy, though effective and simple from a processing standpoint, often complex and resource intensive. An alternative and more flexible variant to existing One-Bac system reported is the combination of RepX (X=Serotype dependent Rep) insect cell line and Bac-CapY-ITR-GOI BV (Y=Serotype of interest) for AAV serotypes production[70]. Overall, the modular Three-Bac system provides the necessary flexibility at the early stage of development that involves screening of multiple serotypes. However, at larger production scales, it is the less attractive system due to inherent manufacturing challenges, as discussed in previous sections. On the other hand, One-Bac, though less flexible and potentially difficult to implement at an early stage of development, offers a robust yet simple manufacturing process for well-established clinically relevant serotypes such as AAV5 or AAV9.

A recently added tool offers a number of advantages, the Mono-Bac[71] or Bac-RepX/CapY/ITR (Figure 2A) combines all the AAV elements in a single baculovirus construct. Upon infection with this single rBV vector, the insect cells produce AAV. Yet the Mono-Bac system has not been extensively used.

Although insect cells are being used for AAV production for more than fifteen years, the first major question, yet unanswered, relates to the uncertainty whether the maximum cellular protein production/processing capacity has been reached. The second question is the efficiency of vector genome packaging in insect cells. Recent reports suggested that AAV serotypes produced in insect cells show more empty particles and consequently less viral genome containing particles when compared side-by-side to mammalian cell productions [72] -submitted]. This necessitates active efforts towards engineering insect cells to produce more genomic and functional particles. The production of wild type AAV serotypes may provide a model for such study since nature has perfected the process of generating 100% genomic and functional particles in the presence of helper virus function in mammalian cells, the natural host of AAV[73]. Moreover, another important factor, requiring significant consideration is the selection of appropriate combination of ITR and Rep proteins for their optimal functionality in genome replication and its packaging into pre-formed capsids. Typical production protocols of different AAV serotypes production in insect cells involves rep and ITR sequences from AAV2 and the cap sequence of choice. The selection of appropriate combinations of serotype-specific rep/ITR remains an open area of investigation to further support the findings of a single report studying its effect on genome packaging efficiency and genomic particle yield of AAV5[42]. In addition to molecular and cellular engineering, the improvement in high cell density production can also contribute to address the challenges of high-yield productions taking advantage of advancements in the field of biologics manufacturing. This involves and not limited to, optimization of medium formulation, design and optimization of nutrient feed for insect cells, and detailed understanding of the critical process parameters guided by the critical quality attributes of AAV serotype therapeutic products. However, increasing the cell density in a production process beyond a point may turn counterproductive at a downstream process stage. Because AAV is an inherently intracellular product, the primary recovery step involves the cell lysis and

release of AAV in lysis buffer. In the high cell density culture process, the cellular components (host cells genome and proteins) are co-extracted with AAV in the lysate during the harvest and clarification process. Their removal exerts a significant burden on downstream processing. It would be appealing to study if the AAV capsid sequences can be modified, without altering the functionality, to direct its secretion in the extracellular environment as is the case with secretory proteins such as monoclonal antibodies. This way, a high cell density-high volumetric yield process can be derived devoid of the presence of major interfering cellular components. Such modification can also be adopted for a perfusion production process with an ultra-high cell density culture, which will ultimately lead to a very high volumetric yield of AAV and reduce the production scale. In conclusion, comforted by the increased regulatory acceptance, ease of scale-up, and recent advancements in production technologies, insect cell baculovirus system is 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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7 Conflict of interest

The authors declare no financial or commercial conflict of interest

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Table 1. Summary of AAV Production in IC-BEVS Platform

Author and year	Production system	Serotype	Transgene	Culture volume	Cell density (cells/mL)	VG/cell	VG/mL	IVP or TU/ cell	IVP or TU/mL	Cp/mL	Cp: VG: IVP or TU	S r
Urabe et al[22]. (2002)	Three-Bac	2	GFP	200mL	$2x10^{6}$ [a]	$4.5x10^{4}\pm 0.32x10^{4}$ [b]	NA	NA	NA	NA	NA: 1344:1[c] ±283	F r c r p c i c
Meghrous et al[48]. (2005)	Three-Bac	2	GFP	3L 20L	$3.08x10^{6}$ [a] $1.7x10^{6}$ [a]	NA	NA	132 253	$4.09x10^{8}$ [b] [d] $4.46x10^{8}$ [b] [d]	$6.7x10^{11}$ [b] [e] $3.71x10^{11}$ [b] [e]	1640: NA: 1 830: NA: 1	F M c r t a c s t A p c p s b i b
Urabe et al [42]. (2006)	Three-Bac	5	hGFP	NA	NA	$5.6x10^{4}\pm 0.32x10^{4}$ [f] $7.67x10^{4}\pm 2.12x10^{4}$ [g]	NA	NA	NA	NA	NA	F C r c V I r s p p c f t A v p

Author and year	Production system	Serotype	Transgene	Culture volume	Cell density (cells/mL)	VG/cell	VG/mL	IVP or TU/ cell	IVP or TU/mL	Cp/mL	Cp: VG: IVP or TU	S n
Aucoin et al[55]. (2006)	Three-Bac	2	GFP	20mL	NA	NA	$2x10^{10}$ $2x10^{9}$	NA	$5x10^{7}$ [d] $1.7x10^{8}$ [d]	NA	3400:400:1 3000:12:1	
Nagrete and Kotin[69] (2007)	Three-Bac	2	GFP	200mL 5L-Wave 20L-Wave 10L-STB 40L-STB	$2x10^{6}$ [a]	NA	˜$3x10^{10}$ [b] ˜ $9x10^{9}$ [b] ˜$5x10^{9}$ [b] ˜$2x10^{10}$ [b] ˜$4.33x10^{10}$ [b] ($\pm 4.1x10^{10}$)	NA	˜$1x10^{9}$ [h] ˜$5x10^{8}$ [h] ˜$3x10^{8}$ [h] ˜$5x10^{8}$ [h] ˜$7.5x10^{8}$ [h] ($\pm 2.5x10^{8}$)	NA	NA: 30:1 NA: 18:1 NA: 16:1 NA: 40:1 NA: 20:1	
Aucoin et al [68]. (2007)	Three-Bac	2	GFP	NA	NA	˜12000	NA	200[d]	$4x10^{8}$	NA	4600:79:1	
Mena et al[59]. (2010)	Three-Bac	2	GFP	20mL 3L	$9.43x10^{6}$ [i] $9.5x10^{6}$ [i]	NA	$1.17x10^{11}$ [b] $2.2x10^{11}$ [b]	NA	$2.9x10^{9}$ [b d] ($\pm 0.32x10^{9}$) $2.35x10^{9}$ [b d]	NA	NA: 40:1 NA: 94:1	

Author and year	Production system	Serotype	Transgene	Culture volume	Cell density (cells/mL)	VG/cell	VG/mL	IVP or TU/ cell	IVP or TU/mL	Cp/mL	Cp: VG: IVP or TU	S n
Liu et al [50]. (2010)	Three-Bac	2	GFP	50mL	$1x10^6$	NA	NA	240	$2.4x10^8$	NA	NA	A p d t i S c i f b c
Cecchini et al[57]. (2011)	Three-Bac	9 8 6	GFP	10L	$3.55x10^6$ [i]	$2.24x10^4$	$7.9x10^{10}$ [b]	NA	NA	NA	NA	A p d e p I s e I s t A p d p r n b a s r l t t t
			U7smOPT	20L	$3.23x10^6$ [i]	$2.41x10^4$	$7.8x10^{10}$ [b]					
			GFP	100L	$3.88x10^6$ [i]	$1.48x10^4$	$4.44x10^{10}$ [b]					
			U7smOPT	200L	$4.29x10^6$ [i]	$2x10^4$	$8.75x10^{10}$ [b]					
			PLS	20L	($\pm 1x10^6$)	($\pm 0.3x10^4$)	($\pm 4.04x10^{10}$)					
					$4.41x10^6$ [i]	$1.82x10^4$	$7.45x10^{10}$ [b]					
					$3.44x10^6$ [i]	$2.07x10^4$	$5x10^{11}$ [b]					

Author and year	Production system	Serotype	Transgene	Culture volume	Cell density (cells/mL)	VG/cell	VG/mL	IVP or TU/ cell	IVP or TU/mL	Cp/mL	Cp: VG: IVP or TU	S r
Chen[33]. (2008)	Three-Bac Two Bac BacInRep/Cap	2 6 8 1 2	GFP GFP GFP GFP GFP	NA	5x10^{6} [a] 5x10^{6} [a] 5x10^{6} [a] 5x10^{6} [a] 5x10^{6} [a]	NA	1.37x10^{11} [b] (±0.34x10^{11}) 3.53x10^{10} [b] 9.65x10^{10} [b] 4.41x10^{10} [b] 2.2x10^{11}	NA	NA	NA	NA	I I c s c u v I u w e h p s c c s i I I a I I
Smith et al. [34]. (2009)	Two-Bac BacRep2CapX	1 2	GFP GFP	200mL 200mL	2.4x10^{6} [a] 1.2x10^{6} [a]	NA	1.07x10^{13} [j] (±0.46x10^{13}) 6.5x10^{12} [j]	NA	5.08x10^{10} [j k] (±5.9x10^{10}) 1.9x10^{10} [j k]	NA	NA: 200:1 NA: 350:1	I I a I I b t b u v w i p s
Aslanidi et al[35]. (2009)	One-Bac 1.0	2 1	GFP GFP	NA	NA	1.4x10^{5} 7.3x10^{4}	NA	NA	NA	NA	NA	I p i c l A p

Author and year	Production system	Serotype	Transgene	Culture volume	Cell density (cells/mL)	VG/cell	VG/mL	IVP or TU/ cell	IVP or TU/mL	Cp/mL	Cp: VG: IVP or TU	S r
Mietszch et al[39]. (2014)	One-Bac 1.0	1-11 12	GFP GFP	NA	NA	~10^4-10^5 l ~10^3 l	NA	NA	NA	NA	NA	H c A s e p C H s
Mietszch et al[45]. (2015)	One-Bac 2.0	5	GFP	NA	NA	1x10^5 l	NA	NA	NA	NA	NA	C H s t w V r r t t i p A v t f

Author and year	Production system	Serotype	Transgene	Culture volume	Cell density (cells/mL)	VG/cell	VG/mL	IVP or TU/ cell	IVP or TU/mL	Cp/mL	Cp: VG: IVP or TU	S n
Joshi et al [58]. (2019)	One-Bac 3.0	5	GFP	25mL 3L	$12x10^{6}$ [i] $12x10^{6}$ [i]	$3x10^{4}\pm 0.4x10^{4}$ $2.7x10^{4}$	$2.7x10^{11}$ [b] ($\pm 0.37x10^{11}$) $3.8x10^{11}$ [b] ($\pm 0.53x10^{11}$)	20±5 22±5	$1.9x10^{8}$ [b m] ($\pm 0.43x10^{8}$) $2.1x10^{8}$ [b m] ($\pm 0.48x10^{8}$)	ND	9600[n]: 2400:1 9400[n]: 2500:1	F F M F b A p c p c F v t b a s I p v n y g n a f t A v

Author and year	Production system	Serotype	Transgene	Culture volume	Cell density (cells/mL)	VG/cell	VG/mL	IVP or TU/ cell	IVP or TU/mL	Cp/mL	Cp: VG: IVP or TU	S n
Yang et al [70]. (2019)	One-Bac 3.0+	2 8 9	GFP GFP GFP	NA	$2x10^6$ $2x10^6$ $2x10^6$	$1.35x10^5$ $\pm0.46x10^5$ 2.16x105 $\pm0.53x10^5$ $1.80x10^5$ $\pm0.39x10^5$	NA	NA	NA	NA	NA	C E b E e p i t f S l C n f i t p c c t A s p

[a] Cell density at the time of infection

[b] Value reported for per milliliter of cell culture

[c] Determined by infecting HEK293 cells and subsequent analysis under a fluorescence microscope for GFP positive cells. Originally reported as

transducing units (TU).

[d] 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

[e] Total virus capsids (Cp) analyzed via ELISA

[f] Value of rAAV5-hGFP using Rep52 of Serotype 1.

[g] Value of rAAV5-hGFP generated via AAV2 VP1 domain swapping (VP1 2/5) and Rep52 of serotype 1

[h] Analyzed via infecting adherent HEK293 cells and subsequent analysis via flow cytometry

[i] The peak cell density during production run

[j] Value reported per milliliter of AVB-Sepharose affinity-purified eluate fraction

[k] Determined via infecting HEK293A cells via AAV in the absence of helper virus and subsequent analysis using a fluorescence microscope

[l] Titer value reported from the analysis of one-step AVB-Sepharose Affinity purified sample

[m] Analyzed via infecting suspension adapted HEK293 cells with AAV in the presence of helper virus and subsequent analysis by flow cytometry

[n] Determined via analytical ultracentrifugation analysis of one-step AVB-Sepharose Affinity purified sample and originally reported as enhanced

transduction units (ETU)

NA: Not available in the published report

ND: Not determined

GFP: Green Fluorescence Protein

hGFP: Humanized Green Fluorescence Protein

STB: Stirred-tank bioreactor

Figure legends

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 Densovirinae subfamily, it shares common structural and functional features with viruses from this subfamily, which mainly infect invertebrates hosts such as insects. (B) wtAAV2 genome map and protein expression profile. The numbers represent the nucleotide position. The viral genome is flanked by two ITRs, one on each end. Two promoters in the Left ORF drive the expression four functional 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. The intron splicing generates four Rep proteins of different sizes from two mRNA transcripts, whereas the leaky scanning of weak translational codons results in three VP proteins production in a stochastic ratio of 1:1:10. The diamond shape end represents the N-terminal of the peptide. Two polyadenylation (PA) signal sequences are shown via orange line.

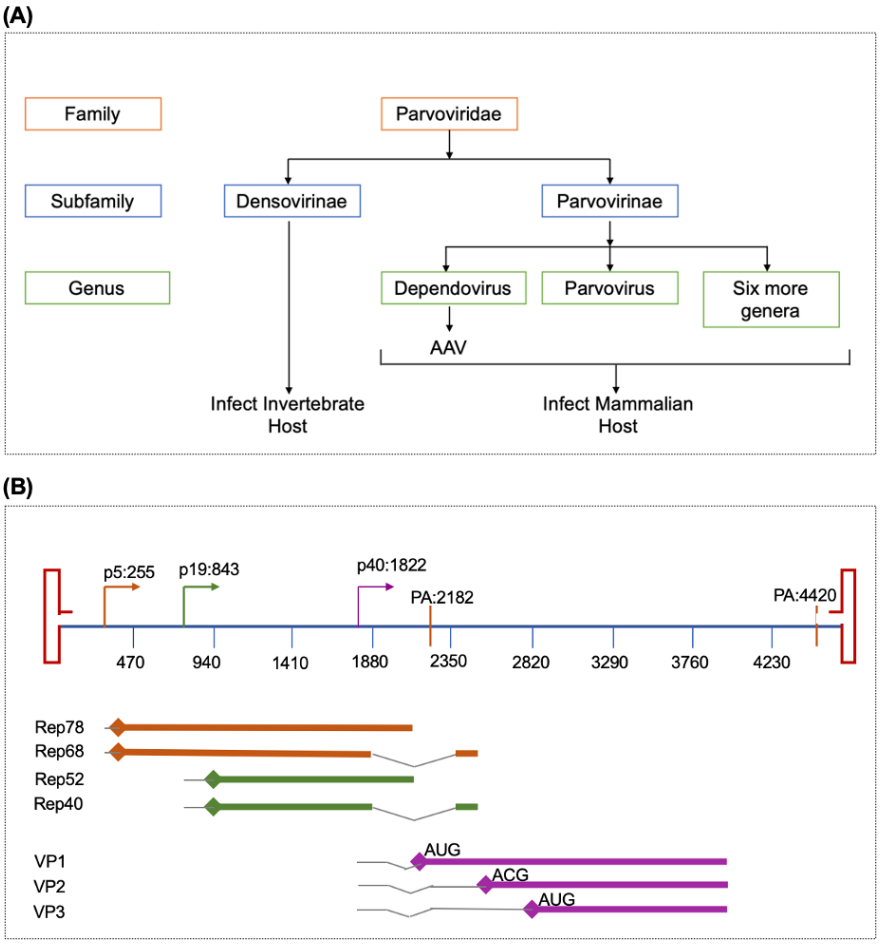
Figure 2. AAV Insect Cell Baculovirus Expression Systems for AAV Production and Mechanism of Inducible Expression in One-Bac.

(A) Four major systems for rAAV production using IC-BEVS. The initial Three-Bac system consisted of three rBV vectors, each carrying specific nucleotide sequence. Further study with this system identified critical shortcomings, related explicitly to expression stability of rBVs and mechanism behind it. The second-generation systems (Two-Bac) exhibited better expression stability of rBVs over the extended passage numbers and required only two rBV co-infection for AAV production. Third-generation systems such as One-Bac or Mono-Bac further simplified the manufacturing process requiring only single rBV co-infection. The One-Bac consisted of a Rep2CapX packaging cell line and a Bac-GOI, whereas in Mono-Bac, a single baculovirus carries all the necessary gene (Bac-ITR-GOI-Rep2-CapX) sequences. (B) The postulated mechanism of induction and amplification of *rep/cap* genes in One-Bac. Bac-ITR infection provides IE-1, which activates hr2-0.9 (1) and induces the 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 lower Rep78: Rep52 ratio. (The artwork in the figure was created with Biorender.com)

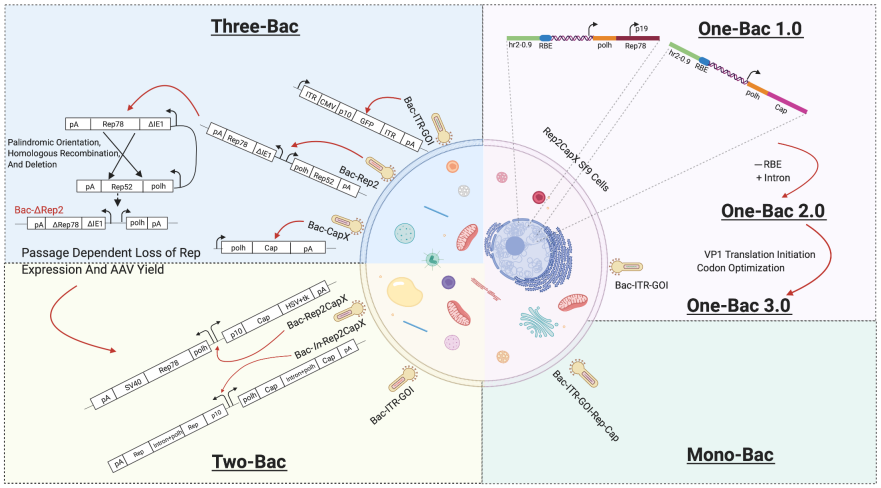
Figure 3. An Overview of Process-Flow for rAAV Production Employing IC-BEVS Platform

The 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 case of One-Bac system (Top section), and 2. AAV production at 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 rBVs. Similarly, the Rep2CapX cells are generated

via stable transfection of respective plasmid vector and selection followed by characterization and master cell bank preparation. During the production stage, the rBVES and insect cells are (Sf9 or Hi5) sequentially expanded in quantity required for stirred tank or Wave production scale bioreactor. At the production stage, the insect cells are infected with rBEVS at an appropriate multiplicity of infection (Low:<1 or High:1-10) and at optimal cell density. Generally, under the batch mode of cultivation, the cells are infected at low cell density around 2-4 million cells/mL, whereas under fedbatch or perfusion mode, the cells reach higher peak cell density before infection:7-12 million cells/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 kinetic. Post infection, at 72-96 hours, the culture is harvested, and cells are lysed to recover AAV. Next, the lysate is subjected to multistep purification process followed by formulation in appropriate buffer as a final step. (The artwork in the figure was created with Biorender.com)



(A)



(B)