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Viral vectors have a great potential for gene delivery, but manufacturing is a big challenge for the industry. The baculovirus-insect cell is one of the most scalable platforms to produce recombinant adeno-associated virus (rAAV) vectors. The standard procedure to generate recombinant baculovirus is based on Tn7 transposition which is time-consuming and suffers technical constraints. Moreover, baculoviral sequences adjacent to the AAV ITRs are preferentially encapsidated into the rAAV vector particles. This observation raises concerns about safety due to the presence of bacterial and antibiotic resistance coding sequences with a Tn7-mediated system for the construction of baculoviruses reagents. Here, a faster and safer method based on homologous recombination (HR) is investigated. First, the functionality of the inserted cassette and the absence of undesirable genes into HR-derived baculoviral genomes are confirmed. Strikingly, it is found that the exogenous cassette showed increased stability over passages when using the HR system. Finally, both materials generated high rAAV vector genome titers, with the advantage of the HR system being exempted from undesirable bacterial genes which provides an additional level of safety for its manufacturing. Overall, this study highlights the importance of the upstream process and starting biologic materials to generate safer rAAV biotherapeutic products.

1. Introduction

Gene therapy gives rise to hopes for a large spectrum of genetic diseases that are mainly untreatable using conventional pharmacology. Over the past decade, recombinant adeno-associated viruses (rAAV) have been the most studied viral vectors and were successfully used in clinical trials for in vivo gene transfer. Importantly, animal-based studies for Duchenne muscular dystrophy which is the most common lethal muscle genetic disease, suggest the need for systemic gene delivery to achieve efficient whole-body treatment. The production of high doses of rAAV vectors for infusion, within the range of 10^{14} vector genomes/kg, is a limiting step for clinical trials with a large population of patients.[1]

AAV vectors are commonly produced in HEK293 adherent mammalian cells by co-transfection of two or three plasmids containing i) the AAV genes, ii) the essential adenoviral helper genes that are supplemented in trans, and iii) the viral genome with a maximal size of 4.7 kb which is framed by inverted terminal repeats (ITRs)

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required for genome replication and encapsidation.[2] This worldwide method led to the AAV2 and AAV8 manufacturing reference-standard materials to provide common genome and particle quantification methods for the scientific community and regulatory agencies.[3,4] The expansion and transfection of adherent mammalian cells may limit the manufacturing of rAAV biotherapeutic products. To scale-up, the procedure, suspension mammalian cells with serum-free medium,[5] and insect cells/baculovirus system[6,7] have been developed during the last decade.

The baculovirus expression vector (BEV) platform has become an established manufacturing platform for the production of viral vaccines and gene therapy vectors. This platform offers many advantages for industrial applications such as manufacturing speed, cost efficiency, and scalability by using bioreactors of 20 000 L (Flublock, Protein Sciences).[8] The standard method to generate a recombinant baculovirus is based on site-specific Tn7 transposition of an exogenous DNA cassette from a plasmid donor to the *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) baculovirus DNA called "bacmid."[9,10] Recombinant baculoviruses are produced in *Spodoptera frugiperda* 9 (Sf9) insect cell line, a workhorse in many laboratories for baculoviral vector amplification. Its ability to grow in suspension to high yields in serum-free media without CO_2 is an advantage for large-scale cGMP manufacturing. Originally, the BEV system has been adapted for rAAV production by co-infecting insect cells with three recombinant baculoviruses (rep, cap, and rAAV genome) where AAV promoters were replaced by baculovirus ones to allow expression of AAV genes into insect cells.[11] Later, Kotin and collaborators developed a simplified dual baculoviruses system by combining the rep and cap helper functions into one unique construct.[12] More recently, a further simplification of the system (i.e., using only one BEV) has been proposed either by integrating rep and cap genes into Sf9 packaging cell lines[13] or directly by engineering the bacmid template to carry all elements required for rAAV production in a unique construct.[14,15]

The baculovirus/Sf9 platform has proven its efficiency for the manufacturing of rAAV viral vectors in particular through the market authorization of Glybera, a gene therapy product indicated for the treatment of lipoprotein lipase deficiency. However, the regulatory agencies requested an assessment of the product for residual baculovirus DNA to the sponsor (uniQure), as a process impurity due to a potential side effect.[16] For this purpose, we have developed a protocol based on high-throughput sequencing to identify and quantify residual DNAs, called the single-stranded virus sequencing, and we demonstrated that baculoviral DNA is the major source of DNA contamination in the final rAAV product with up to 2.1% of total NGS reads.[17] The gentamycin resistance gene coding sequence that has been used to select recombinant baculovirus after Tn7 transposition, was still detectable by qPCR in research-grade rAAV lots. It has also been reported in the literature that the BEV genome derived from the Tn7-bacmid system is subject to instability and spontaneous deletion of the exogenous DNA cassette, leading to the generation of defective interfering viruses (DIs) which accumulate at the expense of the intact ones thus limiting scale-up of batch production.[18] Altogether, these technical concerns prompted us to consider options to overcome these barriers and define new standards to produce baculoviral reagents for rAAV vector manufacturing.

2. Experimental Section

2.1. Plasmid Constructions

The donor plasmid pFastBac1 (pFB), supplied with Bac-to-Bac kit (ThermoFisher), contains two selective genes coding for ampicillin (AmpR) and gentamicin (GmR), the two left (L) and right (R) Tn7 sites surrounding a polylinker to clone the gene of interest. A first construct has been generated containing the cytomegalovirus (CMV) promoter, the enhanced green fluorescent protein reporter gene, and a human β-globin polyadenylation signal, all flanked by two flop-oriented ITRs of AAV serotype 2 derived from the pSub-201 plasmid (Figure S1, Supporting Information).[19] The ITR upstream of the CMV promoter lacks 15 bp in the external A region, and the ITR downstream of the polyA is truncated by 17 bp with respect to the wild-type ITR2. The second construct was kindly provided by R.M. Kotin (NIH, Bethesda, USA) and carries the coding genes "Rep2" and "Cap8" that follows the design described by Smith and colleagues allowing the production of rAAV vectors using a dual baculovirus system.[12] We additionally generated an optimized CapAnc80 construct[20] which was cloned between the polyhedrin baculoviral promoter and the herpes simplex virus thymidine kinase polyadenylation signal sequence (Figure S1, Supporting Information).

In parallel, these constructs were also generated with the donor plasmid pBac-1 supplied with BacMagic-2 kit (Merck Millipore), containing a selective gene coding for AmpR and a polylinker to clone the gene of interest surrounded by the two wild-type homologous baculoviral sequences lef2/orf603 and orf1629. The PAC-1 is digested with the "PmeI/AclI" or "PmeI/DrdI" restriction endonucleases before transfection in Sf9 cells. All plasmids were validated by Sanger sequencing and used for the generation of the recombinant baculoviruses.

2.2. Generation of Recombinant Baculovirus

S. frugiperda Sf9 insect cells (ThermoFisher) were grown at 27 °C in Sf-900 III SFM in 100 to 250 mL glass spinner flask (ThermoFisher).

The recombinant baculoviruses BEV rep2/cap8, BEV rep2/capAnc80, and BEV ITR-GFP were generated using both the Tn7-bacmid DNA derived from Bac-to-Bac (ThermoFisher) and the HR-bacmid DNA chiA$^-$/v-cath$^-$ derived from BacMagic-2 (Merck Millipore) or flashBAC GOLD (Oxford Expression Technologies).

Tn7 site-specific transposition of the cassette from donor plasmid pFB to the Tn7-bacmid backbone derived from bMON14272 was performed by transformation of 10 ng of the donor plasmid in *Escherichia coli* DH10Bac competent bacteria (Bac-to-Bac user guide, ThermoFisher). The recombinant Tn7-bacmid was validated for the presence of the cassette by PCR using primers M13-pUC-F: 5′-CCAGTCACGACGTTGTAAAACG and M13-pUC-R: 5′-AGCGGATAACAATTTCACACAGG from either

side of the insert and primers M13-pUC-F and BAC-G: 5′-AGCCACCTACTCCCAACATC targeting the selective gene sequence of the cassette, and then confirmed by Sanger sequencing. Then, 1×10^6 Sf9 insect cells are transfected with 1 µg of Tn7-bacmid DNA by adding 9 µL of Cellfectin-II reagent (ThermoFisher) in a final volume of 1 mL. The supernatant (P0) was harvested at 96 h post-transfection. Baculoviral clones are isolated by plaque assay with five clones amplified in a T25 flask (P1) followed by ten serial passages to validate the genetic stability of the cassette. When meeting specifications, that is, sequence identity and genetic stability, a unique clone is selected, and a larger stock is then generated by amplification of the recombinant baculovirus in Sf9 insect cells at a multiplicity of infection (MOI) of 2 infectious unit (IU) as determined by cell size assay (CSA) and seeded in spinner-flask to generate the master viral seed P2 and P3.

Homologous recombination (HR) involved transfecting 1×10^6 Sf9 insect cells with 500 ng of double digested (linear) pBac-1 donor plasmid, 100 ng of the HR-bacmid, and adding 5 µL of Cellfectin-II reagent in a final volume of 1 mL (BacMagic-2 user guide, Merck Millipore). As described for the Tn7 system, the supernatant (P0) was recovered 96 h post-transfection and five clones were amplified and characterized up to P2 stock.

2.3. Recombinant Baculovirus Isolation by Plaque Assay

Isolation of recombinant baculovirus by viral plaque assay was performed by seeding a 6-well plate with 1 mL of cell suspension at a concentration equal to 1×10^6 viable Sf9 cells mL^{-1}. After 30 min at 27 °C, attached cells were infected with serial dilutions of baculovirus. An initial dilution was realized from 50 µL of the baculovirus stock in 500 µL of Sf-900 III serum-free medium and sequential 1:3 dilutions were done in a 48-well plate adding 250 µL of the initial dilution in 500 µL of the medium. Typically, to quantify the lysis plaque for a baculovirus stock of $> 10^7$ plaque-forming units per mL, 200 µL of the dilutions 7.2×10^{-4}, 2.2×10^{-5}, and 6.5×10^{-5} were added in duplicate to each well. The plate was incubated for 3 h at 27 °C in a humidified chamber. To prepare plaquing overlay, 4 mL of 4% agarose gel (ThermoFisher) preheated at 70 °C was mixed with 12 mL de SF900 medium 1.3X (ThermoFisher), the inoculum was removed from each well from high to low dilution and replaced with 2 mL of the diluted agarose. After gel solidification, the plate was moved to a humidified chamber and incubated between 9 and 11 days at 27 °C. Isolated clones were individually amplified and characterized (P1).

2.4. Recombinant Baculovirus Titration by CSA

An easy and fast method for baculovirus titration, CSA, is based on measures of infected viable cell diameter.[21] Briefly, dilutions were prepared in a 48-well plate as following: 48 µL of the virus stock was added to 252 µL of Sf-900 III SFM, and seven additional 1:2 serial dilutions realized by adding 150 µL of the previous dilution to 150 µL of the medium. A volume of 875 µL of Sf9 cell suspension at 1.15×10^6 cells mL^{-1} was dispensed into each well of a 24-well low-attachment plate and completed with 125 µL of each dilution. An uninfected control and a concentrated control were included by adding 125 µL of medium and 125 µL of the undiluted virus stock, respectively. The plate was placed at 27 °C under shaking at 175 rpm for 17 to 20 h. After incubation, the cell diameter was measured for each dilution using a Vi-CELL counter (Beckman Coulter).

2.5. Characterization of Recombinant Baculovirus

The identity of the baculoviral genomes was verified by Sanger sequencing from PCR products of DNA extracts. The copy numbers of the rAAV genome and Rep/Cap cassettes were determined using a high pure viral nucleic acid kit (Roche Life Science) followed by qPCR using primer/probe sets described previously.[17]

To study the stability of baculovirus clones across passages, 2 mL of Sf9 cells were seeded per well at density 1×10^6 cells mL^{-1} in a 6-well plate. For each clone, cells were infected with the baculovirus supernatant of the previous passage (from P1 to P10) at MOI = 2 IU and incubated for 72 h at 27 °C in a humidified chamber. The cell pellet was recovered at each passage by low-speed centrifugation and AAV Rep and Cap protein expression verified by SDS-PAGE and western blotting. Five micrograms of total proteins extracted in RIPA buffer were loaded on a 10% Tris-Glycine mini-gel (ThermoFisher) and run at 1 mL/cell, 100 V for 2.5 h. Proteins were transferred onto PVDF membranes (ThermoFisher) for 7 min at 25 V, 1.3 A constant (Trans-Blot Turbo Transfer System, Bio-Rad). Overnight blocking membranes were incubated for 1 h at room temperature with the Rep303.9 antibody or the anti-AAV VP1/VP2/VP3 B1 antibody (Progen) at a dilution of 1:20 and 1:2000 for the detection of Rep and Cap proteins, respectively. Following washing membranes were incubated for 1 h at room temperature with the HRP-linked secondary antibody diluted at 1:2000 (Agilent Dako). After three brief washes, the chemiluminescent signal was revealed using the Western Pierce ECL substrate (ThermoFisher) and exposed with Amersham Hyperfilm ECL (Cytiva GE Life Sciences).

2.6. rAAV Production and Purification

Sf9 cells were infected at a density of 1×10^6 cells mL^{-1} with the combination of BEV rep2/cap8 or BEV rep2/capAnc80 and BEV ITR-GFP at MOI = 1 IU per baculovirus. Four days after infection, cells were lysed by the addition of 0.5% Triton X-100 (Sigma-Aldrich) for 2.5 h at 27 °C with agitation. The crude bulk was clarified by centrifugation for 15 min at $500 \times g$ and 20 °C, the supernatant filtered through a 0.2 µm polyethersulfone membrane (Merck Millipore). rAAV vectors were purified by immune affinity chromatography with a single POROS CaptureSelect AAV8 column (ThermoFisher) and formulated in Dulbecco's PBS (Lonza) containing 0.001% Poloxamer 188 (Merck Millipore).

2.7. rAAV Characterization

For real-time PCR analyses, 3 µL of each purified rAAV stock was pretreated or not with 20 U of DNase I (Roche) in a total volume of 200 µL of DNase reaction buffer for 45 min

at 37 °C before DNA extraction with the High Pure viral nucleic acid kit (Roche Life Science). The AAV vector genome (vg) copy number was determined by a qPCR assay targeting the free ITR sequence "itr."[22] Baculoviral DNA contaminations were quantified by targeting the baculoviral DNA polymerase sequence "bac"[17] or the resistance gene gentamycin sequence "genta" using primers Genta-F: 5′-AGCCCGCATGGATTTGAC, Genta-R: 5′-GGGCATCATTCGCACATGTA, and Genta-Pr: 5′-TGGTCAGGGCCGAGC.

For rAAV vector characterization, SDS-PAGE and western blotting were realized (as described above) for the detection of AAV Cap proteins using 2.5 to 5×10^9 vg. Vector purity was assessed by SDS-PAGE and silver staining (PlusOne silver stain kit; GE Healthcare Life Sciences) of 2×10^{10} vg of each rAAV stock. Capsid titers in the cleared lysate at harvest were quantified by ELISA AAV8 kit (Progen). Capsid titer in purified samples was assessed by SDS-PAGE and Coomassie Blue staining (Imperial Protein stain, Thermofisher) using 1×10^{10} to 1×10^{11} vg of an internal control rAAV2/2 CAG GFP vector devoid of empty particles as reference. Briefly, rAAV standard (five dilutions) and samples (two dilutions each) were diluted in water, followed by heat denaturation for 5 min in a boiling water bath. After cooling, standard and samples were transferred onto Mini-gels WedgeWell Tris-Glycine 10% (Thermofisher) for electrophoresis. Quantification was performed with ICY software (v.1.9.7.0).

2.8. Quantifications and Statistical Analyses

Statistical analysis was applied in presence of at least $n = 3$ biologic replicates, and only descriptive statistics are reported. Data were expressed as mean ± SD, where a one-tailed nonparametric Mann-Whitney test was performed to compare two independent groups. In all the analyses, the significance threshold was set at 0.05. Differences were considered statistically significant at $*p < 0.05$. Analyses were performed using GraphPad Prism v8.4.1.

3. Results

3.1. HR- and Tn7-Mediated Systems Operate with a BAC Integrated into Baculoviral DNA

The generation of BEVs by Tn7 transposition in bacteria is a well-described method in the literature and commercialized as Bac-to-Bac system. The circular baculoviral DNA derived from the AcMNPV has been engineered to insert a bacterial artificial chromosome (BAC) into the polyhedrin locus[23] that contains the kanamycin selective gene, the mini-F bacterial replicon, and a mini-attTn7 site inserted into the LacZα region (Figure 1A).[10] This shuttle vector named "bacmid" can easily be modified and amplified using conventional *E. coli* bacterial transformation in which a helper plasmid (pMON7124) provides the functions required in trans for Tn7 transposition. The bacmid with DNA insert is selected under GmR antibiotic pressure (Figure 1B). An alternative system to Tn7 transposition which is based on HR has emerged.[24] The HR-bacmid used for the generation of BEV has been commercialized as BacMagic or flashBAC. This system does not require the laborious and time-consuming bacterial step for cloning into the baculovirus backbone. Nonetheless, the parental bacmid contains a chloramphenicol resistance gene to allow its amplification and selection in bacteria. By directly co-transfecting insect cell line with the defective parental bacmid DNA (orf1629$^-$) and a donor plasmid containing the Rep2/Cap8 or rAAV genome cassette flanked by homologous sequences lef2 and non-deleted orf1629, HR events induce bacmid knock-out of the bacterial replicon from the targeted polyhedrin locus and knock-in of the cassette that simultaneously restores the orf1629 entire sequence allowing BEV genome replication and the generation of viable recombinant baculoviruses progeny named P0 seed stock (Figure 1B). Importantly, commercial sources for the HR system also offer different bacmid versions that are deleted in some "accessory" genes. In this study, HR-derived BEVs are devoid of genes coding for proteases that are non-essential for baculovirus in vitro replication such as chitinase (Ac126, chiA) and cathepsin (Ac127, v-cath) (Figure 1A).[25] Indeed, AAV capsids 1, 3, 6, 7, 8, and rh10, have been shown to be susceptible to the baculovirus cathepsin and VP1/VP2 cleavage of the AAV capsid serotype 8 has been documented to impair in vivo infectivity.[26] The sequences of both capsids, AAV8 and AAVanc80, used in this study contain the major predicted cathepsin cleavage site.

3.2. HR System Is an Easy, Fast, and Safe System to Generate Recombinant Baculovirus

The HR system was first assessed for the generation of recombinant baculoviruses intended for rAAV vector production, by co-transfecting Sf9 insect cells with the HR-bacmid and the donor plasmid pBac-Rep/Cap carrying AAV2 rep and AAV8 cap ORFs. The donor plasmid has been previously digested (linear) or not (circular) with restriction enzymes on both sides of the cassette. We hypothesized that a linear donor plasmid would be more prone to achieve efficient HR, as reported.[27] We collected, amplified, and characterized five individual clones for each condition. The circular pBac led to heterogeneous clones with low infectivity ($<5.0 \times 10^8$ IU mL^{-1}). Inversely, the linear donor plasmid produced clones with high infectivity with a mean of $9.5 \pm 1.1 \times 10^8$ IU mL^{-1} (**Figure 2**A). The chloramphenicol coding sequence was still detectable by PCR in the cleared supernatant for the circular plasmid meaning that the parental bacmid contaminates the P1 baculovirus stock, contrary to the linear plasmid condition (data not shown). The genetic stability of the recombinant baculovirus was assessed for each clone by qPCR targeting the AAV Rep sequence (insert) and the baculovirus DNA polymerase gene (baculovirus backbone). The results are represented as a ratio bac/rep (Figure 2B). This ratio backbone/insert was close to 1 in all clones produced with the linear plasmid, validating that all five baculoviral clones carried the exogenous DNA cassette Rep/Cap. We also evaluated whether this cassette expresses AAV proteins in Sf9 cells by Western blot analysis for each clone. The expression of Rep78, Rep52, and VP1, VP2, VP3 proteins were detected at P1, P5, and P10 only for baculoviruses derived from the linear pBac plasmid (Figure 2C) and was stable over the ten serial passages. In contrast, no signal was detected for clones derived from circular pBac plasmid. Altogether, these observations suggest that it is preferable to use a linear donor template to favor HR efficiency.

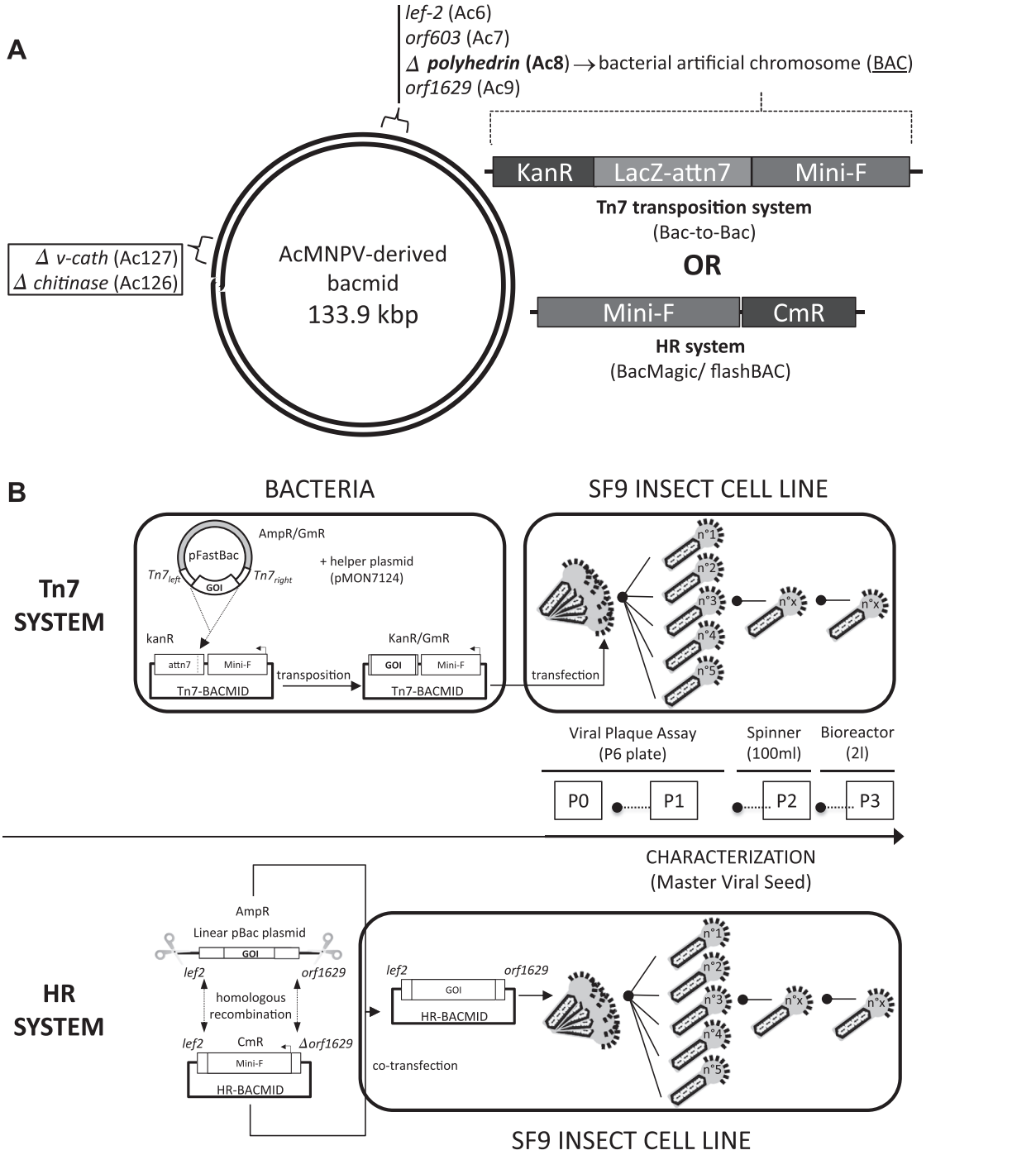


Figure 1. Gene transfer systems for recombinant baculoviruses. (A) Tn7- and HR-bacmid systems are derived from wild-type circular baculoviral genome AcMNPV by insertion of a bacterial artificial chromosome (BAC) in place of the structural polyhedrin gene (Ac8) as described previously.[13,28] Commercial HR systems are optionally deleted for accessory genes, i.e. chitinase (Ac126) and/or v-cathepsin (Ac127). (B) Tn7 system is handled in bacteria where both pFB donor plasmid and Tn7-bacmid DNA are maintained and amplified through selective genes. pMON7124 helper plasmid promotes Tn7 transposition by expressing transposition proteins (TnsABCD) in trans leading to cassette transfer (Rep/Cap or rAAV genome) from pFB to Tn7-bacmid DNA by attTn7 site recognition. The presence of the expression cassette into the Tn7-bacmid DNA is validated by PCR, purified and transfected into Sf9 insect cell line, where the first BEV progeny is generated (P0). Five viral clones are isolated by plaque assay and amplified (P1). A stable clone meeting specification is amplified to generate a master viral seed (P2 or P3). In HR system, pBac donor plasmid and HR-bacmid DNA are co-transfected in Sf9 insect cell line, without the need of a bacterial step. Homologous recombination occurs naturally by homology arms recognition of lef2 and non-deleted orf1629 sequences from pBac to HR-bacmid DNA. The cassette is integrated into the HR-bacmid in place of the existing BAC sequence that is finally removed from the baculoviral genome. Moreover, the orf1629 sequence is fully reconstituted leading to viable BEV progeny generation (P0). Finally, recombinant baculovirus is processed similarly to Tn7 system.

3.3. HR System Allows Better Genetic Stability of the Exogenous DNA Cassette over Serial Passages

We investigated the genomic stability of recombinant baculovirus generated by HR and Tn7 systems. For this purpose, we produced with both systems i) a baculovirus vector carrying a GFP reporter gene flanked by the AAV2 ITRs and ii) two baculoviruses Rep/Cap, one allowing the expression of AAV8 capsid and the other the expression of AAVanc80 capsid variant. AAVanc80 is an ancestral AAV capsid reconstructed in silico allowing higher transduction efficiency in vivo.[28] For each construct, five clones were selected, and the genomic stability assessed side-by-side over ten serial passages in Sf9 cells. The copy numbers of AAV ITR (itr), Rep sequence (rep), or baculovirus DNA polymerase gene (bac) were assayed by qPCR at passages P1, P4, P7, and P10. HR system showed extended genomic stability of the exogenous DNA cassette with a consistent ratio bac/itr or bac/rep (**Figure 3**A – left). The baculoviruses generated with the Tn7 system were more heterogeneous and tended to lose the insert from passage P7 (Figure 3A – right). Finally, the expression of the Rep and Cap proteins required for the rAAV vector production was assessed by Western blotting. Positive signals for VP1, VP2, VP3 proteins were observed up to passage P8 for each HR-derived clone, with a slight decrease in capsid protein expression at P10 (Figure 3B – top). However, a drastic drop in capsid protein expression was observed already from passage P6 for the Tn7-derived clones (Figure 3B – bottom). Altogether, these observations suggest that the HR system robustly prevents the loss of exogenous DNA cassettes upon passaging.

3.4. Baculoviruses Derived from HR or Tn7 Systems Allow Similar Production Yields of Recombinant AAV Viral Vectors

We performed rAAV production by co-infection[12] with recombinant baculoviruses derived either from the HR or Tn7 system. To this end, Sf9 cells were seeded at 1×10^6 cells mL^{-1} and infected with a BEV ITR-GFP and a BEV Rep2/Cap8 or BEV Rep2/CapAnc80 at MOI = 1 IU each, as determined by CSA assay. Insect cells were harvested 96 h post-infection to maximize the production of rAAV using low MOI.[6] We assessed rAAV production yields in viral genomes by qPCR at harvest, in the cleared lysate, and after immunoaffinity purification. BEVs derived from HR and Tn7 systems yielded similar levels of rAAV particles when normalized per 125 mL of total culture volume regardless of capsid variant, although rAAV2/8 showed better productivity compared to rAAV2/anc80 (**Figure 4**A). We investigated the production of AAV viral particles (vp) with both systems in the harvested samples using an AAV8 ELISA commercial kit. The HR-derived BEVs tended to produce more AAV vp while the number of vg was similar to the Tn7 system, suggesting the presence of slightly more empty capsids for HR (Figure 4B). Indeed, the ratio vg/vp was 0.12 versus 0.20 (HR and Tn7, respectively). To further validate this observation, we also quantified vp in purified rAAV samples by semi-quantitative SDS-PAGE and observed a ratio vg/vp of 0.10 for the HR system versus 0.22 for the Tn7 system (Figure 4C), in agreement with ELISA titers at harvest. Furthermore, we investigated the infectivity of rAAV stocks by infectious titer assay (ICA) and by calculating the ratio of vg/IU. No difference in infectivity was observed between AAV vectors by the ICA method (Figure 4D), which evaluates rAAV particle

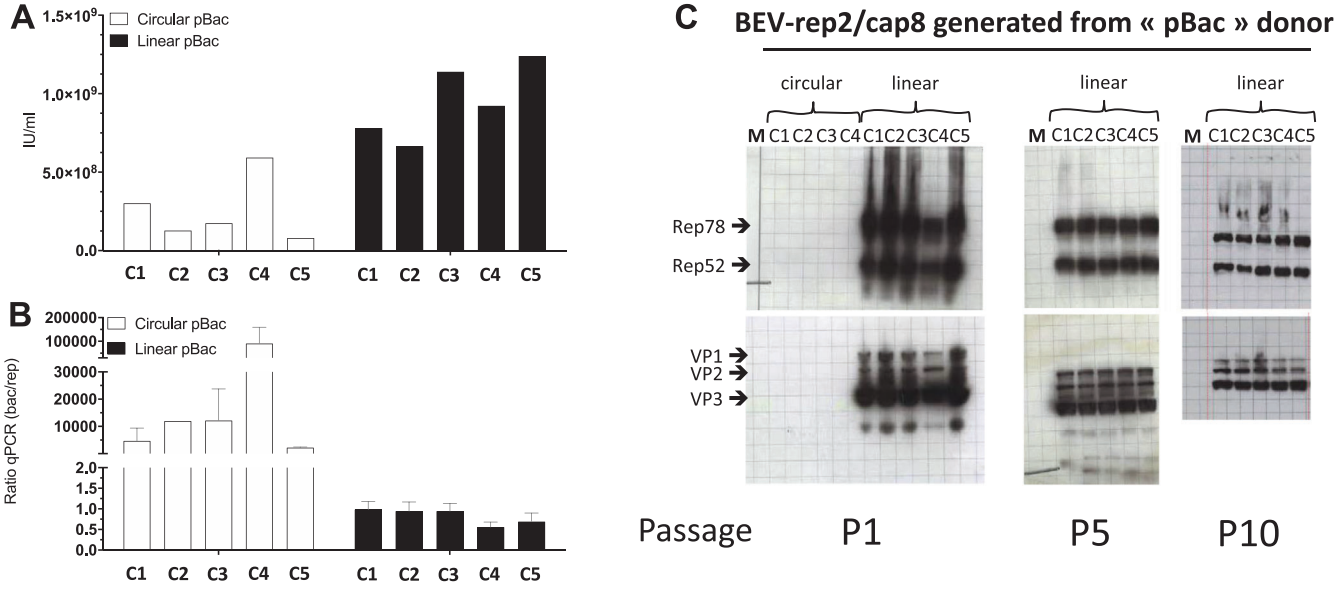
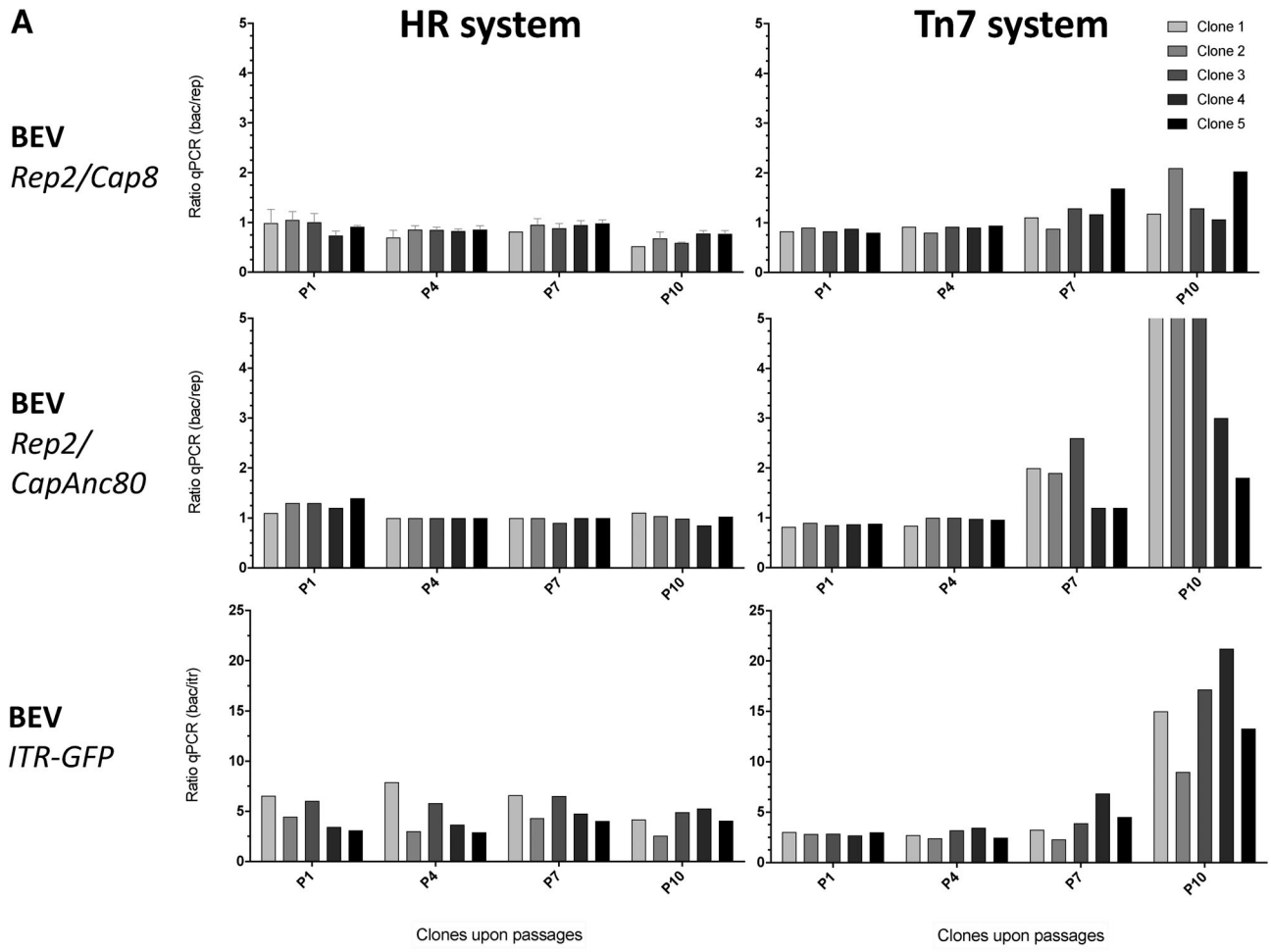


Figure 2. Generation of baculoviral reagents with HR system. Characterization of five BEV-Rep2/Cap8 P1 clones (from C1 to C5) generated with HR bacmid system and derived from circular or double digested (= linear) "pBac" donor plasmid transfection. (A) Infectious titers for each clone using the cell size assay (CSA) method (n = 1). (B) Viral genome qPCR quantification using specific bac and rep amplicons. A ratio (bac/rep) is calculated and plotted to compare clones altogether (n = 2). Mean ± SD. (C) Rep (Rep78 and Rep52) and Cap proteins (VP1, VP2 and VP3) western blot analysis of BEV rep2/cap8 derived from HR after serial steps of clone amplification (P1, P5 and P10).



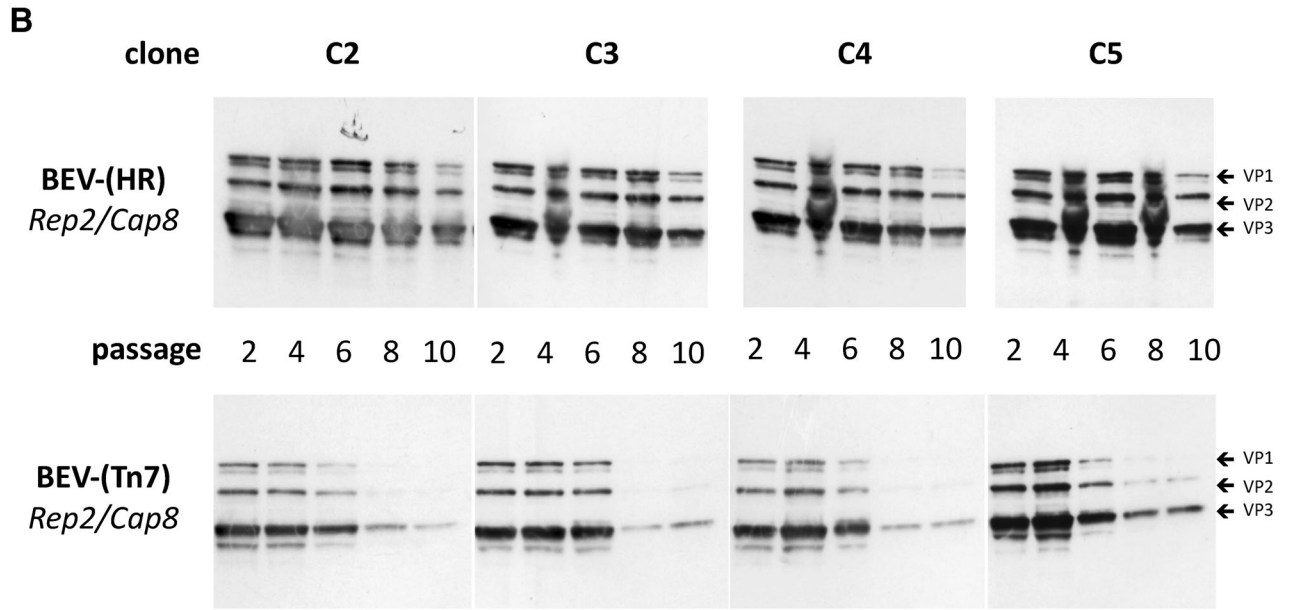


Figure 3. Side by side characterization of baculoviral reagents derived from HR or Tn7 systems. (A) Characterization of five BEV clones derived from the HR- or Tn7 system where genome stability is quantified by qPCR using specific amplicons along serial passages of amplification. The ratio bac/rep and bac/itr are calculated and plotted to compare clones (n = 1 or 2). Mean ± SD. (B) Cap proteins (VP1, VP2 and VP3) western blot analysis of BEV rep2/cap8 derived from HR and Tn7 systems after serial steps of clone amplification (P2 to P10).

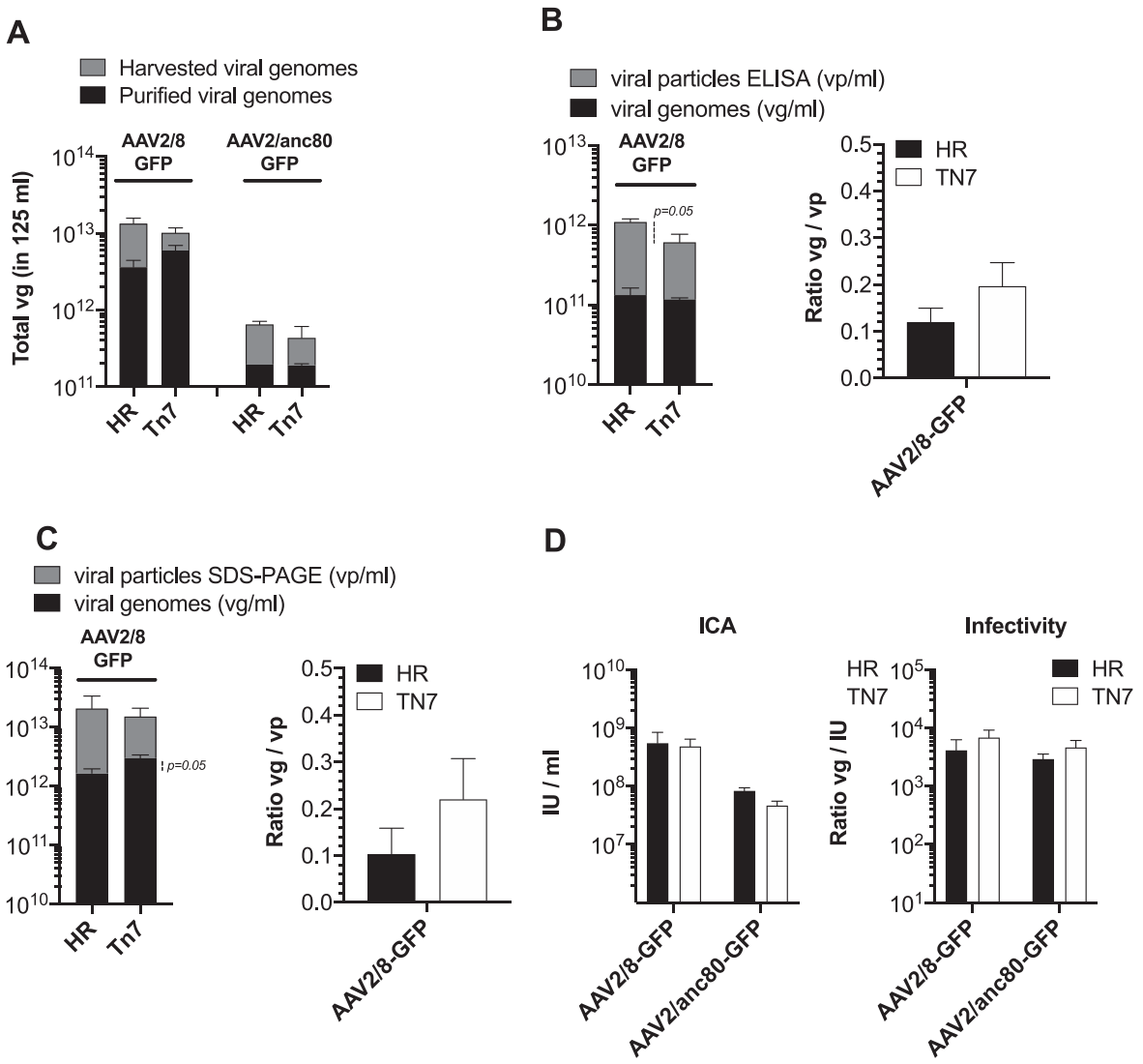


Figure 4. rAAV productions using HR or Tn7-derived baculoviral reagents. (A) Total AAV vector genomes (vg) quantified by qPCR at harvest or after immunoaffinity purification for AAV2/8-GFP (n = 3) or AAV2/anc80-GFP (n = 2) preparations. Titers were normalized for a standard production volume equal to 125 ml. (B) Left: Quantification of total AAV2/8-GFP viral particles (vp) by ELISA assay or total viral genomes (vg) by qPCR in cleared lysate (n = 3); Right: ratio AAV2/8-GFP vector genome (vg) / total AAV viral particles (vp) in cleared lysate (n = 3). (C) Left: Quantification of total AAV2/8-GFP viral particles (vp) by semi-quantitative SDS-PAGE assay or total viral genomes (vg) by qPCR after purification (n = 3); Right: ratio AAV2/8-GFP vector genome (vg) / total AAV viral particles (vp) after purification (n = 3). (D) Left: quantification of infectious units (IU) of AAV particles by ICA assay in purified AAV2/8-GFP (n = 3) and AAV2/anc80-GFP (n = 2) batches; Right: representation of infectivity expressed as a ratio "vg/IU" for the indicated constructs. Mean ± SD.

efficiency to enter HeLaC32 cells and to replicate in the presence of Rep proteins and adenovirus.[29] Altogether, these observations suggest that HR and Tn7 systems lead to comparable rAAV yields.

3.5. Advantages of HR System over Tn7 System in Term of rAAV Vector Product Safety

We investigated the amount of product-related DNA impurities present in the final product in the two production systems, and designed qPCR assays to specifically amplify baculoviral and bacterial DNA sequences in rAAV vector stocks after immunoaffinity purification. Baculovirus residual DNA was quantified by qPCR targeting the baculovirus DNA polymerase gene (named "bac" qPCR assay) located at the opposite of the Rep/Cap or rAAV genome insertion site.[17] We detected > 10^8 copies of baculoviral DNA polymerase sequence normalized per 10^{12} AAV vg and these values were similar for HR and Tn7 systems. The proportion of baculovirus DNA that was encapsidated in rAAV particles (+ DNase) is equivalent in both systems (**Figure 5**A). Moreover, we detected >10^9 copies of gentamycin (named "genta" qPCR assay) out of 10^{12} AAV vg in the Tn7-derived rAAV batches, while this value is null (or near LOD) in HR-derived rAAV batches (Figure 5B,C). The 1-log difference

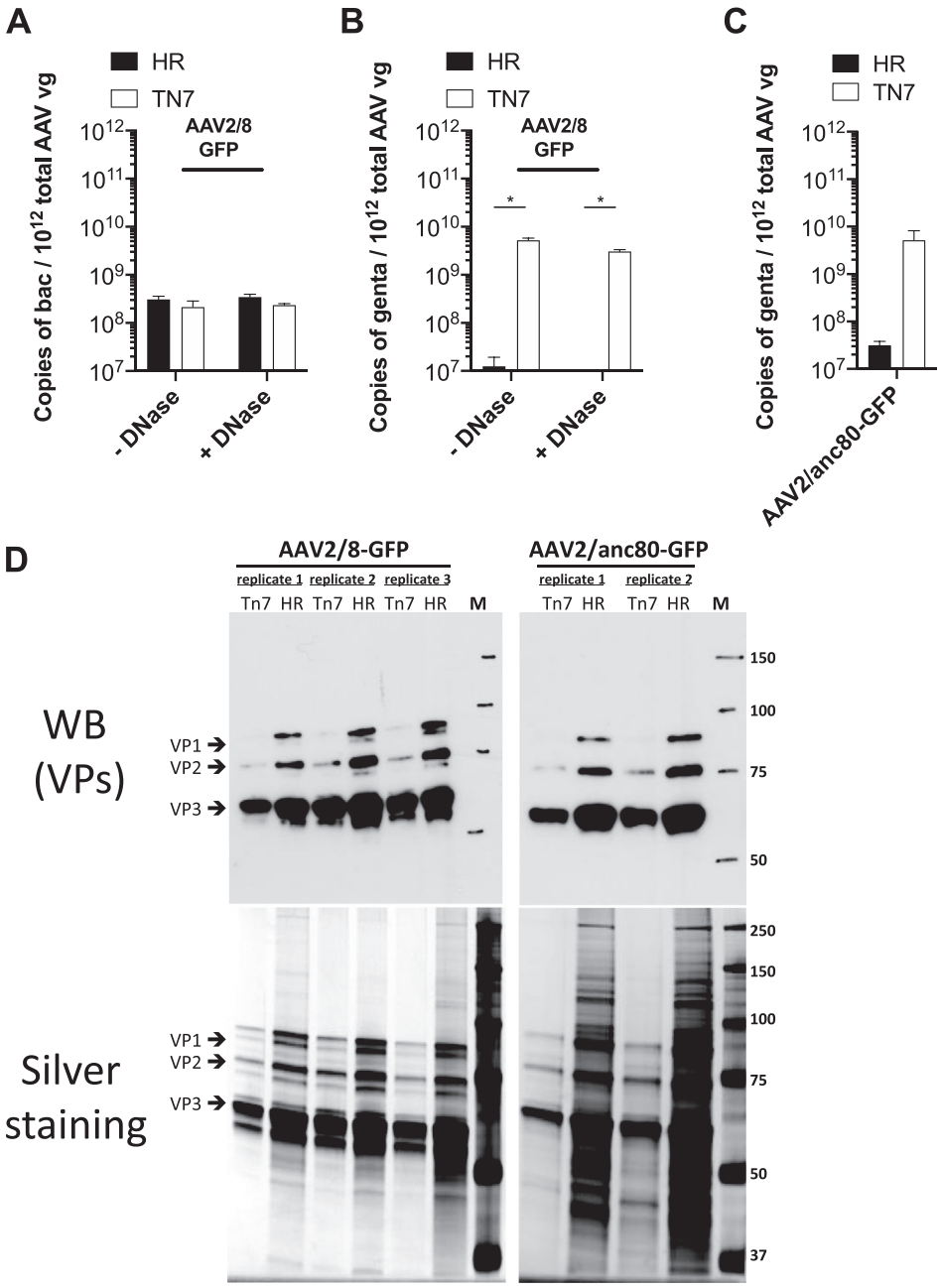


Figure 5. Characterization of final rAAV product derived from HR or Tn7 baculoviral reagents. (A) Total of baculovirus DNA polymerase (bac) copies quantified by qPCR in purified AAV2/8-GFP (n = 3) with or without DNase treatment and normalized to 1012 total viral genome (vg). (B) Total gentamycin copies quantified by qPCR in purified AAV2/8-GFP (n = 3) with or without DNase treatment and normalized to 1012 total viral genome (vg). (C) Total gentamycin copies quantified by qPCR in purified AAV2/anc80-GFP (n = 2) without DNase treatment and normalized to 1012 total viral genome (vg). Mean ± SD. (D) Top: western blot analysis of Cap proteins (VP1, VP2 and VP3) for different replicates of purified AAV preparations carrying indicated constructs; Bottom: silver staining analysis of total proteins and purity for different replicates of purified AAV preparations carrying indicated constructs.

observed between "genta" and "bac" copies in the case of the Tn7 system is likely due to proximity with ITR sequences and preferential encapsidation into AAV particles.[17] Finally, the identity and purity of rAAV vectors were determined by western blot and SDS-PAGE silver staining (Figure 5D). The profile of the proteins detected by SDS-PAGE was comparable in all cases, but the capsid signal was more intense in rAAV stocks produced with the HR system when loading the same amount of vg per well, and the same was true for western blot analysis. This result correlates with an increase of total capsids in HR conditions as measured by ELISA and SDS-PAGE (Figure 4B,C). Altogether, these observations suggest that the HR system definitively reduces the risk of bacterial sequence encapsidation in the final rAAV biotherapeutic product.

4. Discussion

Our study compares two systems to generate recombinant baculovirus vectors in the context of rAAV production. Baculovirus backbone can be engineered by exploiting both HR or Tn7 transposition, the latter being the most widely used system. First, we show that recombinant baculovirus derived from the Tn7 system tends to lose the exogenous expression cassette after a few rounds of viral amplification while the HR system preserves baculoviral genome stability upon serial passages. Second, we show that BEVs generated with the HR system preserve rAAV yields and infectivity comparable to the standard Tn7 system. Importantly, the HR system is devoid of any bacterial DNA sequences in the final rAAV biotherapeutic product.

As reported previously,[18] recombinant baculovirus generated by Tn7 transposition risks a substantial loss of the expression cassette during multiple amplifications. The mechanism underlying this genetic instability is not fully understood, although the presence of residual bacterial/transposase elements could be detrimental for baculovirus genome replication and favor DIs accumulation over passages. Our data demonstrate that the HR system resulted in remarkable genetic stability of the inserted cassette (Rep/Cap and rAAV genome), possibly due to removal of the BAC sequence from parental bacmid after HR, though we cannot rule out a possible role of chitinase and/or cathepsin in this process. Some limitations of the HR system have also been identified: i) the low frequency of natural HR events that occur in Sf9 insect cell could lead to the generation of fewer recombinant baculovirus clones and ii) the risk of generating DIs due to cross-complementation of ORF1629 essential protein from viable recombinant baculovirus. Here, we show that circular donor plasmid induces a very low HR frequency and leads to DIs accumulation that is not carrying the exogenous DNA cassette. We solved this issue by using a double digested (linear) pBac donor plasmid that maximizes the on-target cassette integration while leading to a similar yield of baculoviral clones as the Tn7 system. This observation suggests the need for the HR system to proceed with one further round of viral plaque isolation to definitively eliminate DIs and generate a pure baculoviral reagent before amplification and generation of the master viral seed. While the standard Tn7 system is limited to a few passages, we show that the HR system may allow an extended number of rounds without exogenous DNA cassette loss. This is a major advantage for biotherapeutic product manufacturing at an industrial scale requiring the use of multiple rounds of baculovirus reagent amplifications.

Our study also shows that HR and Tn7-derived BEVs generated similar amounts of rAAV vectors, even though the total capsid titer seems to be slightly higher with the HR system. This observation was confirmed both at harvest time and after immunoaffinity purification when normalizing by the vector genome copy number. Further investigations are necessary to determine whether the higher proportion of empty AAV capsids is due to the absence of chitinase and cathepsin proteases in the HR system or other genetic elements.

Importantly, we show that final rAAV products generated with HR-derived BEV are exempt from encapsidated bacterial sequences including antibiotic resistance genes, such as gentamycin. With Tn7-derived BEVs, the gentamycin sequence is encapsidated in rAAV at a 1-log higher efficiency compared to the baculoviral DNA polymerase sequence, likely due to its proximal location to the AAV ITRs. Consequently, none of these methods can avoid the encapsidation of baculoviral sequences into rAAV particles.[17]

In summary, we conducted a side-by-side comparison of different systems to generate recombinant baculovirus vectors and conclude that both methods are equally efficient to generate high yields of rAAV vectors. HR-derived BEVs showed higher genetic stability upon passaging with lower amounts of nucleic acid impurities (i.e., bacterial sequences). This has significant advantages for large-scale manufacturing of rAAV biotherapeutics that meet the safety requirements of regulatory agencies.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

V.B., C.R., M.P.-B, L.H.V., A.F., and E.A. are inventors on a patent describing the Anc80 baculovirus expression vector. E.A. is a consultant for AAV gene therapy companies and inventor of several patents related to AAV gene therapy licensed to biopharma companies. L.H.V. is an inventor on several patents related to AAV gene therapy, including AncAAV variants, AAV9, and method patents, which are licensed to several biopharma companies. L.H.V. further receives funding from Lonza/Houston, Selecta Biosciences, and Solid Biosciences, licensors to AncAAV technology. L.H.V. is a consultant to Nightstar, Selecta, Akouos, and Exonics and a founder of Akouos. L.H.V. has a financial interest in TDTx, a company developing AAV gene therapies; he is an inventor of technology related to AAV gene therapy, a founder of the company, and also serves on its board of directors. L.H.V.'s interests were reviewed and are managed by MEE and Partners HealthCare following their conflict of interest policies.

Author Contributions

M.P.-B. and E.A. share senior authorship. J.A.: Conceptualization-Equal, Data curation-Equal, Formal analysis-Equal, Investigation-Equal, Methodology-Lead, Validation-Equal, Visualization-Equal, Writing-original draft-Equal, Writing-review & editing-Equal. L.B.: Conceptualization, data curation, formal analysis, investigation, methodology, and validation. P.J.G.: Conceptualization, data curation, formal analysis, investigation, methodology, and validation. M.B.: Methodology. A.R.: Methodology. L.H.V.: Conceptualization, formal analysis, methodology, resources, validation, and writing-review and editing. O.A.: Funding acquisition and supervision. A.F.: Conceptualization, formal analysis, methodology, and writing-review and editing. V.B.: Conceptualization, formal analysis, methodology, and writing-review and editing. E.A.: Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, supervision, validation, visualization, writing-original draft, and writing-review and editing.

Data Availability Statement

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Keywords

baculovirus expression vector, gene therapy, homologous recombination, manufacturing, rAAV vectors, Tn7 transposition

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