Recombinant adeno-associated viral (rAAV) vectors have been used in more than 150 clinical trials with a good safety profile and significant clinical benefit in many genetic diseases. In addition, due to their ability to infect non-dividing and dividing cells and to serve as an efficient substrate for homologous recombination, rAAVs are being used as a tool for gene-editing approaches. However, manufacturing of these vectors at high quantities and fulfilling current good manufacturing practices (GMP) is still a challenge, and several technological platforms are competing for this niche. Herein, we will describe the most commonly used upstream methods to produce rAAVs, paying particular attention to the starting materials (input) used in each platform and which related impurities can be expected in final products (output). The most commonly found impurities in rAAV stocks include defective particles (i.e., AAV capsids that do contain the therapeutic gene or are not infectious), residual proteins from host cells and helper viruses (adenovirus, herpes simplex virus, or baculoviruses), and illegitimate DNA from plasmids, cells, or helper viruses that may be encapsidated into rAAV particles. Given the role that impurities may play in immunotoxicity, this article reviews the impurities inherently associated with each manufacturing platform.

Adeno-associated virus (AAV) is a very small (20–26 nm), icosahedral, and nonenveloped virus (Figure 1). AAV particles contain a single-stranded DNA genome consisting of approximately 4.7 kb. The genome contains three open reading frames (ORFs) encoding for replication proteins (Rep), capsid proteins (Caps), and the assembly activating protein (AAP), and is flanked by two inverted terminal repeats (ITRs). These 145-nt ITRs are partially paired, and they fold upon themselves to maximize base pairing and form a T-shaped hairpin structure. The AAV genome contains two viral promoters known as p5 and p19, which regulate the transcription of the four Rep proteins with apparent molecular masses of 78, 68, 52, and 40 kDa (Rep68 and Rep40 being the splice variants of Rep78 and Rep52, respectively). Expression of the cap gene is driven by the P40 promoter and regulated by alternative splicing and different translation initiation sites, resulting in three Caps (VP1, VP2, and VP3) that form an icosahedral capsid of ~3.9 kDa. The molecular ratio of these proteins (VP1:VP2:VP3) is approximately 1:1:10. The AAV genome also encodes for the AAP in an alternative ORF of the cap gene that plays a major role for capsid assembly.

The life cycle of AAV is dependent on the presence or absence of a helper virus, hence its name. In the absence of helper viruses, the wtAAV genome is able to latently persist as episomes or be integrated into the host genome. This site-specific integration occurs preferentially into a 4-kb region on chromosome 19 (q13.4), named AAVS1, and requires the Rep proteins. These latent forms may be activated and “rescued” upon subsequent helper virus infection by inducing active replication of the viral genome, Caps synthesis, and DNA packaging, with these steps taking place inside the nucleus. In 1984, Hermonat and Muzyczka published the first paper of a recombinant AAV (rAAV) vector that was capable of expressing foreign genes in mammalian cells. Since then, AAV vectors from serotype 2 (AAV2) have been used as a prototype for gene therapy, and the subsequent identification of more than 12 AAV serotypes and more than a hundred variants in human and nonhuman primate (NHP) populations extended its applications. For most of the serotypes, the ITR and rep gene from AAV2 can be kept constant, while the cap gene from different serotypes or isolates is used to derive “pseudotyped” vectors that only differ by the nature of their capsid. To date, the following methods have been established for the generation of rAAV vectors at large scale: (1) plasmid DNA transfection in mammalian cells, (2) Ad infection of stable mammalian cell lines, (3) infection of mammalian cells with recombinant herpes simplex viruses (rHSVs), and (4) infection of insect cells with recombinant baculoviruses. With the same objective to offer a cost-effective, safe, and scalable manufacturing process, other alternatives have been explored. For example, taking advantage that vaccinia virus replicates in the cytoplasm, another method based on dual infection of HeLa-S3 cells with one vaccinia vector and one Ad-AAV hybrid vector has been used for rAAV production. The advantage of this system is that rep-cap sequence and rAAV genome are sequestered in different sub-cellular compartments, which eliminates the contamination with replication-competent AAV (rcAAV). Following seminal works of A. Galli’s and R.O. Snyder’s groups, Barajas et al. have reported recently that rAAV2 infectious particles can be generated in Saccharomyces cerevisiae after transformation with four plasmids allowing the expression of rep78/52, the three AAV Caps, and AAP under...
In 2002, Kotin and collaborators demonstrated that the replication and rescue of the recombinant genome. This involved the use of Ad or HSV for efficient replication and rescue of the recombinant genome. In mammalian cell-based production systems, the assembly of rAAV vectors requires: (1) the recombinant vector genome composed of the gene of interest (GOI) and the regulation elements for the GOI vectors requires: (1) the recombinant vector genome composed of the gene of interest (GOI) and the regulation elements for the GOI, (2) the AAV expression in target cells (promoter, poly A, introns, etc.) flanked by AAV ITRs, (3) helper functions from Ad or HSV for efficient replication and rescue of the recombinant genome.

In 2002, Kotin and collaborators demonstrated that the replication and assembly of rAAV vectors can also occur in insect cells by expressing rep and cap genes via baculovirus vectors. Interestingly, when rAAV is assembled in insect cells there is no need to add auxiliary HSV or Ad genes, very likely because baculovirus is also providing helper functions, although the genes involved in this process have not been identified yet. Each aforementioned system is capable of generating AAV particles. However, the overall yields and quality of the vectors still greatly vary based on the method utilized. Depending on the biological raw materials used upstream (input), i.e., plasmids, recombinant viruses, and cells, the final product of each manufacturing platform will differ in particular in terms of impurities (output). Likewise, the quantity of rAAV vectors and product-related contaminants (non-infectious vectors, particles that do not contain the transgene, degraded capsids) may vary from one method to another.

Upstream Methods for AAV Vector Assembly (Input)

Transient Transfection of HEK293 Cells

The most established method for the production of rAAV vectors is the plasmid transfection of human embryonic HEK293 cells. Typically, HEK293 cells are simultaneously transfected by a vector plasmid (containing the GOI) and one or two helper plasmids, using calcium phosphate or polyethylenimine (PEI), a cationic polymer. HEK293 lines are usually propagated in DMEM completed with L-glutamine, 5%–10% of fetal bovine serum (FBS), and 1% penicillin-streptomycin, except for suspension HEK293 cells that are maintained in serum-free suspension F17, Exp293, or other manufacturer-specific media. For adherent cells, the percentage of FBS can be reduced during rAAV production in order to limit contamination by animal-derived components. Generally, the rAAV vectors are recovered 48–72 hr after plasmid transfection from the cell pellet and/or supernatant, depending on the serotype. The cell lysis step has a great impact on the amount and type of impurities found in the crude bulk, as well as on particles recovery, and thus will be described hereafter.

Infection of Insect Cells with Recombinant Baculovirus

As an alternative to the rAAV production in mammalian cells, the baculovirus-S9 platform has been notably established as a GMP-compatible and scalable system, generating as many vector genomes per cell as the mammalian cell-based methods, i.e., up to \( 2 \times 10^7 \) vector genomes (vg) per cell in crude harvests. The production of rAAV vectors by infection of insect cells with three recombinant baculoviruses was first described in 2002 by Kotin’s group. Subsequent improvements were then introduced, reducing the number of baculoviruses to two: a baculovirus expression vector (BEV)
allowing the synthesis of Rep78/52 and Caps, and a recombinant baculovirus carrying the GOI flanked by the AAV ITRs (Figure 2, input). Baculovirus carrying the GOI bracketed by AAV ITR (rHSV-AAV) and the second gene, the AAV p5 promoter. Production of rAAV vectors was first described by Clark et al., who created stable HeLa-derived producer cell lines transformed with a plasmid containing both rep-cap genes of AAV2 and an rAAV vector genome along with a drug selection marker. Several groups have also established packaging cell lines through transformation with the rep-cap genes of AAV2 in HeLa and A549 cells (Table 1). The high-level expression of the rep and cap genes induced by Ad infection and subsequent rAAV assembly was correlated with gene amplification and associated with the presence of a replication element within the AAV2 p5 promoter. Infectious rAAV vectors can be generated upon infection of these packaging cell lines with wild-type Ad type 5 and providing the rAAV genome by either plasmid transfection or after infection with a recombinant Ad/AAV hybrid virus (Figure 2, input). Possible replacement of Ad with HSV-1 as the helper virus was also demonstrated.

Establishment of producer cell lines was also reported in HEK293 cells. In this case, expression of the Rep proteins had to be tightly controlled by a Cre recombinase-inducible system where the p5 promoter is separated from the rep and cap coding sequences by transcription termination signals flanked by loxP sites, because Rep is known to inhibit cell proliferation by inducing cell-cycle arrest or apoptosis. Indeed, HEK293 cells constitutively express the Ad5 E1 gene that activates the AAV p5 promoter. Production of rAAV from these cells is then triggered by infection with a recombinant, E1-deleted, Ad5 virus expressing Cre recombinase.

Mammalian stable cell lines infected with Ad were actually the first system to provide a scalable upstream process for manufacturing of purified rAAV particles. Moreover, a HeLa-based stable cell line was used for production of the first AAV product administrated in humans in a clinical trial for the treatment of cystic fibrosis. The selection of producer cells for manufacturing of clinical-grade AAV vectors has been optimized using HeLaS3 cells, a

Table 1. Characteristics of rAAV Producer Cell Lines Related to Safety

| Cell Line   | Gene(s)          | Origin          | Methodology                                                                 |
|-------------|------------------|-----------------|-----------------------------------------------------------------------------|
| HEK293      | E4, E5, I2 genes | Human origin    | Allows synthesis of Rep/52 and Caps, and a recombinant baculovirus          |
| HEK293T     | SV40 T antigen   | Human origin    | Left arm of adenovirus 5 genome (I.3-kbp fragment of Ad5 including the 3' terminal repeat, the E1A/B gene, and the partial Ix and IVa2 genes sequences) |
| A549        | Human origin     | Non-transformed | Human papillomavirus type 18 partial genome (no expression of E4, E5, and I2 genes) |
| HekLa       | Human origin     | Transformed     | Human papillomavirus type 18 partial genome (no expression of E4, E5, and I2 genes) |
| BHK21       | Hamster origin   | Non-transformed | Subclone of the Sf21AE cells stemming from spontaneous immortalization of Spodoptera frugiperda ovarian cells, has been commonly adopted for the generation of recombinant baculovirus and the production of rAAV vectors (Table 1). Several serum-free media are adapted for Sf9 cell growth in suspension. The dual-baculovirus-Sf9 production system has many advantages over other production platforms regarding these safety issues: (1) the use of serum-free media; (2) despite the discovery of adventitious virus transcripts in Sf9 cells, most of the viruses infecting insects do not replicate actively in mammalian cells; and (3) no helper virus is required for rAAV production in insect cells besides baculovirus. More recently, stable Sf9 insect cell lines expressing Rep and Cap proteins have been developed requiring the injection of only one recombinant baculovirus for the production of infectious rAAV vectors at high yield. However, baculovirus-related impurities should not be considered as a trivial concern because this issue has been raised in the assessment report of Glybera, an AAV-based product for the treatment of lipoprotein lipase deficiency and the first gene therapy drug approved by the European Medicines Agency (EMA). Indeed, in this report, the EMA committee asked for a more comprehensive characterization of contaminants of baculovirus origin (residual DNA, proteins, infectious baculovirus particles).

Infection of Mammalian Cells with rHSV Vectors

Although less common than the other two methods described above, the AAV production using co-infection with rHSV is a very efficient method to generate a large amount of rAAV. In addition to high overall yields (up to 1.5 × 10⁷ vg/cell), the apparent increased quality of the rAAV stocks, as measured by an improved viral potency, makes this method very attractive. The principle of the method relies on the role of the HSV in AAV life cycle as a helper virus for replication in permissive cells. Therefore, the HSV virus can serve both as a helper and as a shuttle to deliver the necessary AAV functions that support AAV genome replication and packaging to the producing cells. Cells, typically the hamster BHK21 cell line (Table 1) or HEK293 and derivatives, are infected with two rHSVs, one carrying the GOI bracketed by AAV ITR (rHSV-AAV) and the second with the AAV rep and cap ORFs of the desired serotype (rHSV-repcap) (Figure 2, input). After 2–3 days, the cells and/or the media are collected and rAAV is purified over multiple purification steps to remove cellular impurities, HSV-derived contaminants, and unpackaged AAV DNA. Processes utilized to date to remove process-derived impurities from rAAV stocks will be described briefly below. To date, three clinical trials have been initiated for one metabolic disorder, alpha-1 antitrypsin (AAT), and two retinal diseases, achromatopsia and X-linked retinoschisis (XLS), supporting the suitability of the method for human use.

Mammalian Stable Cell Lines

The establishment of stable mammalian cells for the production of rAAV vectors was first described by Clark et al., who created stable HeLa-derived producer cell lines transformed with a plasmid containing both rep-cap genes of AAV2 and an rAAV vector genome along with a drug selection marker. Several groups have also established packaging cell lines through transformation with the rep-cap genes of AAV2 in HeLa and A549 cells (Table 1). The high-level expression of the rep and cap genes induced by Ad infection and subsequent rAAV assembly was correlated with gene amplification and associated with the presence of a replication element within the AAV2 p5 promoter. Infectious rAAV vectors can be generated upon infection of these packaging cell lines with wild-type Ad type 5 and providing the rAAV genome by either plasmid transfection or after infection with a recombinant Ad/AAV hybrid virus (Figure 2, input). Possible replacement of Ad with HSV-1 as the helper virus was also demonstrated.

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suspension adapted HeLa subclone, and a full manufacturing process including production in animal components-free medium, and purification was developed at the 250-L scale for a rAAV1 vector. The process was recently implemented to a 2,000-L commercial scale for rAAV1-SERCA2a, a gene therapy product intended for the treatment of heart failure, as reported in a press release (Celladon Corporation Conducts Initial Scale Up of Manufacturing Process for MYDICAR up to Commercial Scale, January 5, 2015). Thus, stable producer cells allow efficient and scalable rAAV vector production. In addition, wild-type Ad5 helper virus is genetically stable and can be easily produced at high titers. However, a time-consuming step is the establishment and screening of stable and high-producer clones. Indeed, this step requires selecting cells with (but not limited to) the following characteristics: (1) the integrated rep-cap and rAAV vector sequences remain stable over time without maintaining drug selection; (2) the AAV promoters remain silenced during cells sub-culturing and expansion; and (3) infection with Ad5 induces a high level of rep and cap gene expression and amplification, as well as rAAV genome rescue and replication. Thus, screening of tens to thousands of clones may be required to get a high producer cell line for a specific vector, which has probably limited the wide adoption of this system. Another critical aspect of this technology is the requirement for a downstream process allowing efficient clearance of the Ad5 helper virus, which will be discussed below.

Characterization of rAAV Stocks and Related Impurities (Output)

In vivo data have validated the safety of all of the above-mentioned methods for the production of pre-clinical and clinical-grade rAAV. However, the safety and efficiency profiles of the AAV-based drugs depend on the upstream and downstream steps that ensure complete removal of process-derived impurities and on the development of robust and precise assays for the detection and quantification of these impurities (Table 2). Here, we will mostly focus on impurities that are inherently associated with each of the production methods and steps utilized to reduce, eliminate, and quantify them (Figure 2, output). In addition, we will try to compare data across the

![Figure 2. Schematic Representation of Raw Materials (Input) and Product-Related Impurities (Output) in Each System Used for the Production of rAAV Vectors](image-url)
manufacturing platforms. However, because very limited data exist with direct side-by-side comparisons, the overall interpretation should be concluded with caution. Finally, specifications are not always established for rAAV quality tests, and the determination of acceptable levels of these impurities has to be done based on risk assessment studies.

### Nucleic Acids

Recombinant rAAV capsids are known to internalize not only the recombinant genomes, but also illegitimate DNA during their production in mammalian and insect cells. To limit the genotoxic risk of residual DNA in rAAV stocks, the US Food and Drug Administration (FDA) recommends a level of residual cellular DNA below 10 ng per parenteral dose and a median DNA fragment size of less than 200 bp. Supplementary quality controls regarding DNA contaminants are also asked for gene therapy product, in particular qPCR quantification of potential hazardous sequences, such as antibiotic-resistance genes and E1 and E4 adenoviral genes. For the transient transfection method, less than 100 ng of residual plasmid DNA, as determined by antibiotic-resistance gene-targeted qPCR, is advised per $1 \times 10^{12}$ vg in the purified bulk product. However, without associated risk assessment studies, it would be recommended to inquire about the proportion of each potential DNA contaminant in rAAV stocks taking advantage of novel sequencing technologies.

### Table 2. Current Testing and Specifications for AAV-Based Products Purity and Safety

| Test                        | Method                          | Specification                                      |
|-----------------------------|---------------------------------|----------------------------------------------------|
| **Safety**                  |                                 |                                                    |
| endotoxin                   | LAL assay (EP 2.6.14, USP85)    | $<2$ EU/injected dose$^a$                          |
| sterility/bacterio- and fungi-static activity | sterility tests (EP 2.6.1, USP71) | negative$^b$                                      |
| replication-competent AAV   | serial infection on permissive cells/rep or cap qPCR | $<1$ rcAAV in $1 \times 10^8$ vg                  |
| **Purity**                  |                                 |                                                    |
| general purity              | SDS-PAGE/silver staining        | identify proteins other than VP (or VP degradation products) |
| protein                     | SDS-PAGE/Coomassie blue         | $\geq 80\%$                                        |
| residual BSA$^c$             | ELISA                           | $\leq 50$ ng/mL$^d$                                |
| residual production reagent$^e$ | HPLC, MS, ELISA…                   | report results                                     |
| host cell proteins          | ELISA, MS                       | report results                                     |
| residual cell DNA           | qPCR, HTS                        | report results                                     |
| residual DNA from raw material$^f$ | qPCR, HTS                        | report results                                     |
| empty/intermediate particles | ratio vg (qPCR)/vp (ELISA)       | report results                                     |
|                            | electron microscopy, AUC        |                                                    |
| **Quality**                 |                                 |                                                    |
| total protein               | Bradford protein assay, BCA, NanoOrange | report results                                    |
| appearance                  | visual inspection               | clear to slightly hazy, free of foreign materials |
| pH                          | potentiometry                   | pH 6.5–8                                           |
| osmolality                  | osmometry                       | product specific, report results (250–350 mOsm/kg) |
| aggregation                 | DLS                             | report results                                     |

AUC, analytical ultracentrifugation; BCA, bicinchoninic acid protein assay; DLS, dynamic light scattering; EP, endotoxin units; HPLC, high-performance liquid chromatography; HTS, high-throughput sequencing; LAL, limulus amebocyte lysate; MS, mass spectrometry; USP, U.S. Pharmacopoeia.

Adapted from Wright, Wright and Zelenai, Wright, Snyder et al., and Robert et al.

$^a$US Food and Drug Administration or European Pharmacopoeia specifications; other specifications are recommended.

$^b$Only for processes using fetal bovine serum.

$^c$Depending on the purification process (Benzonase, detergent, BSA, cesium, PEG, affinity ligands).

$^d$Plasmid, baculovirus, adenovirus, or HSV DNA.

$^e$Plasmid, baculovirus, adenovirus, or HSV DNA.

$^f$Plasmid, baculovirus, adenovirus, or HSV DNA.
may induce nicks in DNA, and cap expression could trigger immune response. The main cause of rAAV formation was attributed to recombination events that could arise between homologous sequences in ITR and adjacent sequences and the rep/cap cassette. Fortunately, these were avoided by replacing the p5 promoter with heterologous promoters, or removing RBE adjacent to rep-cap sequences. Splitting of the rep and cap sequences in two separate cassettes and engineering of an oversized cap sequence by addition of an intrinsic sequence were also proposed to limit expression could trigger immune response. The main cause of rAAV formation was attributed to recombination events that could arise between homologous sequences in ITR and adjacent sequences and the rep/cap cassette. Fortunately, these were avoided by replacing the p5 promoter with heterologous promoters or removing RBE adjacent to rep-cap sequences. 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DNA has been described to be higher for scAAV with up to 26% of the amp sequence detected in scAAV-gfp stocks.15

Antibiotic sequences were also found in rAAV particles produced in mammalian stable cell lines through Ad infection, whether the clone was generated by rAAV infection or by plasmid transfection. Indeed, amp copies represented 3%–6% of the total rAAV2-lacZ vg generated in stable cell lines.69 Consistently, puromycin-resistance DNA was also found in rAAV2, rAAV8, and rAAV-rh8R lots produced in HeLaS3-derived producer cell lines at a level of 0.2%–1%.56,20 In these cases, antibiotic resistance could come from the rep-cap plasmid or the plasmid harboring rAAV genome that were used to stably integrate the corresponding sequences in the cell genome.69

**Helper Virus Sequences.** In the dual-baculovirus/Sf9 platform, we demonstrated that the baculoviral DNA can account for up to 2.1% of rAAV8 vector genomes content, with an increased proportion for the sequences adjacent to ITRs to be encapsidated, such as the gentamycin-resistance gene in the Bac-to-Bac backbone.68 Thus, undesirable packaging of vector plasmid sequences is strongly thought to be ITR dependant.

To date, there are no formal data aimed at determining specifically the percentage of viral or genomic DNA encapsidated in AAV vectors when using the HSV system. However, total residual HSV DNA detection in AAV stocks has been described in detail. Because the genome of HSV is very large (~150 kb), multiple primer sets must be selected across the genome to ensure proper detection of possible variants. They often include a primer set of ICP27 to detect ICP27 replicating DNA in AAV stocks has been described in detail. Because the genome of HSV is very large (~150 kb), multiple primer sets must be selected across the genome to ensure proper detection of possible variants. They often include a primer set of ICP27 to detect ICP27 replicating DNA, which is usually performed by measurement of total proteins concentration. Concentration of host cell proteins in the purified vector stocks is proportional to the ITR and insertion site.

In the Ad-infected mammalian producer cells, packaging of Ad5 DNA in AAV capsids has not been reported in the literature. Because the Ad genome is covalently linked to the terminal protein (used as a primer for DNA replication), illegitimate packaging of Ad DNA is not expected to occur at a high level, but next generation sequencing technologies, with higher sensitivity, should be used to further investigate these events. Encapsulation of viral oncogenes integrated in the producer cells genome is also of concern. Thus, the presence of Ad E1 and HPV E6-E7 gene sequences is systematically investigated in AAV produced in HEK293 cells and HeLa-based cell lines, respectively, although they are consistently found below the detection limit.56,64,66

**How Can We Limit the Risk Related to DNA Impurities?** Different purification strategies have been implemented to minimize residual DNA depending on their origin and their susceptibility to DNase treatment. The addition of a nuclease treatment step after clarification can be active on free DNA species if digestion conditions are met (pH, Mg²⁺, and salt concentrations). For DNA that is protected by the helper virus capsids, specific removal of Ad, baculovirus, or herpes virus particles is required. However, we have demonstrated that the vast majority of contaminating DNA in purified rAAV stocks is protected by the AAV capsid and is thus resistant to drastic DNase treatment.64,68

For vector plasmids, the bacterial selection marker should be carefully chosen in order to avoid spread of resistance against antibiotics used in humans. For example, the FDA recommends not using β-lactamase. Another strategy was developed to avoid the transfer of plasmid-derived sequences into the target tissue, namely, MCs. Schnödt and co-workers15 recently took advantage of this technology to produce ssAAV and scAAV in HEK293 cells in comparison with the traditional dual-plasmid transfection. The replacement of the helper plasmid by a helper MC had no effect on the amp gene sequences encapsidation level, confirming that prokaryotic impurities in ssAAV stocks come mostly from the vector plasmid, whereas changing the vector plasmid by an MC reduced the amp copy number by 2 logs. This effect is particularly remarkable for scAAV vectors where the use of MCs diminished the percentage of amp contaminants from 26.1% to 0.2% (vector MC + helper plasmid) or to an undetectable level (vector MC + helper MC). In conclusion, MC technology can be considered as a substantial improvement in rAAV vectors production in HEK293 cells, although it may significantly increase the cost of raw materials.

Another approach to decrease DNA contaminants in rAAV particles is to redesign the vector and helper plasmids. First, it has been suggested that increasing the vector plasmid backbone size over the rAAV packaging capacity (>5 kb) can decrease significantly ITR-mediated reverse packaging and subsequently plasmid-derived contaminants (7.6-fold decrease between a 2.6- and a 7.0-kb backbone for an rAAV genome of 4.3–4.8 kb).55,76 Furthermore, we and others have demonstrated that sequences close to ITRs are more prone to encapsidation.66,77 In conclusion, as advised by Gray and Zolotukhin,78 it is more secure to clone the potential hazardous sequences of the vector plasmid, such as antibiotic-resistance genes, far from the ITRs and to insert large fragments of stutter DNA (>5 kb) in between.

**Proteins** Residual proteins found in purified vector stocks originate from helper viruses, producer cell lines, possible adventitious viruses, culture medium, and purification process. In particular, the clearance of some proteins throughout the purification process needs to be documented, which is usually performed by measurement of total proteins concentration. Concentration of host cell proteins in the purified vector is measured using cell-line-specific ELISA kit (Host Cell Protein ELISA kits; Cygnus Technologies). Protein contaminants can also be identified using specific antibodies or mass spectrometry-based methods. Indeed, about 20 cellular proteins were identified by GelC-MS (gel electrophoresis liquid chromatography-mass spectrometry) in rAAV stocks produced in HEK293 cells.79,80 Among...
them, SET proteins were found associated to the full capsids in rAAV2 lots purified by CsCl gradient ultracentrifugation, independently of the serotype (2, 5, 6, 8, 9), but they were not detected or detected at lower level in IEX- or iodixanol-purified rAAV2 batches, respectively. This indicates that protein contamination in rAAV final stocks depends not only on the production system, but also on the purification method. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS), Satkunanathan et al. found 44 cellular proteins that were associated to AAV2, AAV5, or AAV8 particles produced in HEK293T cells and purified by AVB immunoaffinity chromatography. Strikingly, nucleolin and nucleophosmin were found in the three above-mentioned studies. These two nucleolar proteins have been previously described to bind specifically AAV2 capsids in vitro and may be involved in AAV particles assembly. Actually, most of the identified proteins have already been described as co-factors that restrict or enhance AAV genome replication and capsid assembly or trafficking. However, another study has identified a protein a priori unrelated to AAV life cycle in rAAV6 vectors produced in HEK293 cells and purified through CsCl gradients. This protein, galectin-3 binding protein (G3BP), is naturally secreted in body fluids and was previously found to interact with the capsid of some AAV serotypes (1, 5, and 6) incubated in human serum. Interestingly, G3BP expressed in HEK293 cells was co-purified with AAV6 particles collected from the culture supernatant, but not with particles purified from the cell pellet. Indeed, the hyperglycosylated secreted form of G3BP was found to bind AAV6 capsids, whereas the hypoglycosylated intracellular form was not. As a consequence, gene transfer of a SEAP reporter gene was found less efficient in mice when using rAAV6 vectors purified from the culture supernatant, which highlights the importance of carefully analyzing contaminants when designing a manufacturing process.

In rAAV vectors produced in stable cell lines, contamination with Ad Caps was reported when purification was performed through CsCl gradients. Efficient removal of Ad capsid polypeptides is critical because they are highly immunogenic. This can be achieved by purification through IEX using appropriate separation conditions and the use of affinity chromatography to specifically capture rAAV would also be a suitable option.

Similarly, residual HSV proteins or peptides present in the final AAV stocks could potentially elicit toxic or immune reactions in the host. Their detection is performed by in-house-developed ELISAs because there is no commercial HSV-1 ELISA currently available (N.C., unpublished data). However, the assay accuracy is greatly challenged by the lack of standard for fully quantified HSV-1 preparations that are used to establish the standard curve. The purification methods, the matrices, and the accessibility of various HSV-1 epitopes all can affect the sensitivity and reliability of the assay, and render direct comparison between groups difficult. Typically, in-house-produced and chromatography-purified HSV-1 preparations have been used as protein standards (N.C., unpublished data). Pre-clinical stocks tested showed values ranging from 17.2 and 120 ng/mL of HSV proteins or less than 16 ng per dose. No major response was detected in injected animals, but anti-HSV antibodies were detected at the highest doses. In the AAT clinical trial, the AAV1 vector lot contained 834 ng/mL and the highest human dose tested contained approximately 72 µg of HSV proteins. Overall, published data showed that residual HSV proteins are detectable in final rAAV stocks, but extensive pre-clinical toxicology studies showed that these levels were tolerated in animals and no reactions in humans have been reported so far.

**Infectious Helper Viruses**

**Residual Baculovirus.** Baculoviruses can infect mammalian cells, and thus are used as viral vectors for gene transfer. Furthermore, 12 baculoviral genes, including the immediate early 1 gene, have been shown to be transcribed in HeLa and BHK cells after Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) infection by DNA microarray and RT-PCR analysis. Although there is no evidence of their functionality, baculoviral DNA and proteins could be immunogenic. Indeed, contamination with baculovirus capsids is likely to activate an innate immune response as shown in vaccine preparations, which may negatively impact gene transfer efficiency. AAV vectors are generally recovered from insect cells by chemical lysis. Triton X-100, added at a concentration of 0.5% v/v at harvest, has the dual advantage of being efficient for cell membrane and baculovirus envelope disruption. This step inactivates most of the baculoviruses, but does not alter the integrity of the nucleocapsids. Of approximately 50 nm in diameter and 300 nm in length, they are generally subsequently removed during the purification process, in particular at the filtration steps. For example, Planova 35-nm cutoff nanofilters have been proven to specifically clear infectious baculovirus spiked in rAAV vector stocks by 6 logs (below the 1.5 log10 limit of quantification of the 50% tissue culture infective dose [TCID50] test). Despite the fact that baculovirus contamination was a major concern of the Glybera report assessment, no standard assay is available to detect residual infectious baculovirus in rAAV batches. Nonetheless, baculovirus can transduce mammalian cells but are not able to replicate, and there is no evidence that baculoviruses are detrimental to human health, unless intravenously injected at very high doses (>109 IU). Furthermore, many recombinant products and vaccines are produced with BEV in insect cells.

**Residual HSV.** The major risk associated with the HSV method is the potential presence of residual HSV from the inoculum used to infect the cells, as well as replication-competent variants that may occur during the rHSV stock production. HSVs are engineered from a replication-deficient variant of HSV type 1 (d27.1) that is lacking the ORF encoding for the viral ICP27 protein (infected cell protein 27). In the absence of ICP27, the HSV genome cannot replicate unless ICP27 is provided in trans, like in the V27 cells, a Vero cell derivative stably transformed to express ICP27. V27 is the only cell line currently used for large-scale manufacturing of rHSV stocks used for AAV production. HSV stocks are prepared by infecting monolayers or V27 in flasks, or alternatively, in suspension using micro-carriers, harvested after 3–4 days and concentrated. HSV stocks, used as raw materials in AAV clinical production, contain
serum and V27 cell-derived impurities (DNA, proteins). Further, rcHSV or ICP27 revertants may arise during amplification on V27 by homologous recombination. There are two assays utilized to determine the presence of rHSV and rHSV in HSV and ultimately in AAV stocks. HSV stocks are assayed both on V27 cells, to determine the plaque-forming unit (PFU) titer of rHSV, and on Vero cells, which do not contain ICP27, to assess the presence of rHSV. Upon replication in the cell nucleus, rHSV or rcHSV induces a cytopathic effect and infected cells form plaques. Detection of residual HSV should be established to ensure the lowest detection limit possible. Currently, detection limits as low as 10–20 PFUs/mL have been described. Processes designed to purify AAV from an HSV-containing matrix typically contain at least one inactivation step. The two main methods described are: (1) use of powerful detergent such as Triton X-100 at concentrations as high as 1% v/v, added at collection of the harvest, and (2) low pH-induced flocculation of cellular and HSV proteins from denatured viral capsids. The first method has been thoroughly validated by viral clearance studies, demonstrating up to 6 log10 reduction of the rHSV input. The second has been tested at pre-clinical stage and has shown to be successful in reducing the rHSV input by several logs with a detection limit of 100 PFUs/mL. Virus inactivation by detergent is a widely accepted neutralization step and is likely more suitable for large-scale manufacturing. However, both methods present the challenge that typically the detergent-containing or low-pH matrices can be toxic or inhibit this cell-based assay, affecting the overall assay sensitivity. Ye and colleagues demonstrated an overall HSV clearance of up to 14 log10 when treatment with Triton was combined with multi-step chromatography for an AAV1 drug product. It was calculated that the largest human dose administered in the AAT trial would theoretically contain less than 2.74 × 105 PFUs of rHSV, and that rcHSVs were not detectable. Low levels of rcHSV have been shown in rHSV stocks, reported less than 1 PFU per 3 × 107 rHSV PFUs. It is worth noting that at the present time, guidance on the acceptable amounts of residual HSV in AAV drug products have yet to be issued by the regulatory agencies. Alternatively, or additionally, a PCR-based assay for ICP27 could be utilized for the detection of rHSV and/or rcHSV, with the major limitation that it will not indicate whether the particle is infectious. Serial passaging may reveal whether the detected signal is amplified over time, to determine whether the particles are replication competent and/or infectious.

Still, several small- to large-scale animal studies have been completed by various groups, using various AAV serotypes and production and purification methods, and a wide range of administered doses ranging from as low as 5 × 1016 vg/kg to an excess of 2 × 1014 vg/kg in mice and non-human primates. Pre-clinical evaluation by Investigational New Drug (IND)-enabling toxicology studies concluded that no life-threatening toxicity or immune reaction were observed, and the doses were all well tolerated. In addition, the highest dose administered to a human patient is 6 × 1012 vg/kg, or approximately 4.3 × 109 vg total dose. No life-threatening reaction or major side effect has been reported, and the trial has been completed. However, ongoing studies evaluating higher range doses for systemic disorders will tell more about the safety of AAV vectors produced with rHSVs (N.C., unpublished data).

Residual Ad. In the case of mammalian stable cell lines, the main issue is the use of wild-type adenovirus. As a main difference to HSV, inactivation of Ad is usually achieved by physical treatments. The most commonly used method is heat inactivation at a temperature where Ad capsid is disrupted and AAV capsid is not, usually between 51°C and 56°C, but other methods such as high pressure have been proposed. For rAAV1 vector manufacturing in HeLa producer cells, 10-min incubation at 52°C was shown to reduce Ad5 infectious titer by ~6 log10 with no impact on AAV infectivity. For large-scale clinical-grade rAAV1 production, a complete Ad clearance was ensured by a complex process including specific steps such as: (1) IEX to capture Ad in the cleared lysate, (2) AAV capture step followed by Ad heat inactivation, and (3) further AAV purification step followed by nano-filtration and polishing.

Another approach to reduce contamination with AdS is to use mutant Ads. In particular, the use of Ad with mutation in the E2b gene affecting the Ad DNA polymerase (ts149) or the pre-terminal protein (sub100r and del308ΔpTP) resulted in similar rAAV production yields compared with wild-type Ads. However, production of these mutant helper viruses is more laborious because they replicate only at low temperature (i.e., 32°C for ts149 and sub100r) or in trans-complementing cells (HEK293-pTP for del308ΔpTP). The use of single-round replication helper with the Ad protease deleted (AdΔPS) was also investigated but resulted in less efficient rAAV production.

AAV Defective Particles

The systems described above have been continuously improved, now allowing the production of approximatively 1 × 1012 vector genomes per cell. These different platforms coexist in vector core facilities, each with their pro and cons. However, rare studies compared them side by side in terms of vector safety and efficiency. One important quality attribute is the proportion of defective particles in rAAV stocks (Figure 2, output). For example, the quantity of empty particles has not been systematically investigated (or at least reported), and indeed, they can represent the majority of the viral particles in rAAV batches. This was the case for the clinical-grade scAAV2/8-FIX lot injected in patients with hemophilia B, which contained not more than 20% of full particles despite size-exclusion and anion-exchange chromatography purification steps.

The most comprehensive study investigating the rAAV packaging rate was realized from clarified cell lysates in HEK293 cells. Vector genome and physical particles titration were determined by dot blot and ELISA, respectively, revealing that only 1%–18% of rAAV2 particles contain the transgene in cleared lysate. Using three plasmids, other groups consistently reported that 6% and 18% of rAAV2 total particles contained the FIX and SEAP transgene, respectively, at harvest as determined by qPCR and ELISA assay. Qu et al. highlighted the lot-to-lot disparity of the p/vg ratio,
ranging from 3 to 30 when the triple-transfection production method was employed.108

Few studies have addressed this issue in the other platforms. The production of rAAV2–FIX vectors in A549–derived producer cell line was shown to generate 8%–13% of full particles at harvest.115 A recent study compared purified rAAV2 vectors produced in stable cell lines or in transfected HEK293 cells using analytical ultracentrifugation (AUC).109 The results indicated that rAAV produced in stable cell lines contained lower amounts of empty particles. The proportion of full (vector genome-containing) particles was up to 74% for a 3.4 kb-vector and 40%–45% for a 4.2 kb-vector, whereas the same vectors produced by triple-transfection contained only 8% and 10%–12% of full particles, respectively.

In parallel, using rHSV vectors, it seems that the packaging rate is in the same order of magnitude than other systems. Indeed, Kang et al.189 quantified 8% of the total assembled rAAV2 particles that incorporated the gfp transgene in crude lysate. In insect cells, Amine Kamen’s group102,103 demonstrated that the baculovirus infection conditions commonly used for S9 cells infection, i.e., equal amount of the three baculoviruses, lead to a percentage of full virions ranging from 0.4% to 0.9%, as determined by real-time PCR and ELISA at harvest. In the more recent dual-baculovirus infection system, 7%–30% of total assembled particles harbor the rAAV genome in rAAV2 and rAAV8 (M.P.-B., unpublished data). However, many parameters are involved in the full-to-empty ratio fluctuation (rAAV genome length and sequence, serotype, helper virus, purification method, measurement technique, etc.), rendering it difficult to convey a clear message regarding the packaging efficiency in each production system.

**Impurities Related to the Purification Process**

**Cell Lysis and Clarification**

AAV particles are not only intracellular and can be released to the supernatant depending on the serotype and culture conditions.132 AAV particles are highly resistant to extreme conditions of pH, detergent, and temperature. Thus, several methods based on chemical or mechanical lysis are available to retrieve rAAV vectors from the cells. Rudimentary mechanical technique to liberate rAAV vectors from the cells is freeze/thaw cycles followed by a low-speed centrifugation clarification step. However, this technique is not appropriate for large-scale purification. On the contrary, microfluidization is an efficient and scalable process that consists in the passage of producer cells through a small-diameter fluid path under high pressure. It also shears cellular DNA and reduces crude lysate viscosity, facilitating clarification. The advantage of physical methods is the lack of additional chemical reagents. Efficient cell lysis can also be performed using detergent such as Triton X-100,113 whereas Benzonase treatment is usually applied after cell lysis to reduce viscosity and remove free nucleic acids. Following cell lysis, the insoluble cell components are removed by low-speed centrifugation for small-scale production, and polyethylene glycol (PEG) may be used to concentrate rAAV particles. For large-volume cultures, a filtration step is performed by depth filtration or tangential flow filtration (TFF) to prevent clogging. If chemical reagents are used, such as Triton and PEG, they should be quantified in the final product as a process-related impurity. These chemicals are usually quantified by high-performance liquid chromatography (HPLC) methods. The same is true for nucleases that can be measured by commercial ELISA kits.

**Purification by Ultracentrifugation**

For research-grade batches, rAAV vectors are traditionally and usually purified by density gradient ultracentrifugation, e.g., CsCl isopycnic gradients14 or iodixanol step gradients.115 Interestingly, the CsCl method allows the separation of the particles containing the genome from empty particles by exploiting its specific buoyant density. The conventional purification protocol included two rounds of CsCl gradient centrifugation followed by dialysis against the appropriate buffer in order to remove cytotoxic CsCl.14 In parallel, AAV vectors purified on discontinuous iodixanol gradients also show a high degree of purity but harbor more empty particles (~20%) as compared with CsCl-purified vectors (<1%).80 Two advantages of iodixanol-based protocols over CsCl gradients are that the ultracentrifugation is shorter (2 versus 24 hr) and that iodixanol is not toxic for humans because it is often used as radiocontrast agent. The major drawback of the density gradients methods is that they are not suitable, in the first instance, for direct purification from a large volume of cleared lysate. Nevertheless, a CsCl continuous gradient centrifugation can be included in a large-scale purification scheme after a previous step of concentration, in particular for empty particles removal.

**Purification of rAAV Vectors Using Affinity Columns**

Heparin columns were initially used to purify rAAV2 vectors.52,115–117 Then, heparin-affinity chromatography has been successfully demonstrated to purify rAAV3 and rAAV6 vectors.52 Based on the same principle, mucus, a sialic-acid-rich protein, was used for rAAV1,116 rAAV4, rAAV5, and rAAV6 vector purification because these serotypes can bind to the cell surface via O-linked and N-linked sialic acid moieties.

In parallel, several antibodies that recognize different AAV capsid epitopes have been immobilized on a solid support matrix or surface. For example, A20 monoclonal antibody recognizes specifically assembled AAV particles of serotypes 2 and 3.2 It was immobilized on a HiTrap-Sepharose to efficiently purify rAAV2 particles.14 The most popular immunoaffinity column is the AVB Sepharose High Performance medium (GE Healthcare Life Sciences) that is adapted for purification of a broad spectrum of AAV serotypes (although at different efficiency depending on the serotype).20,24,118 The ligand of AVB column is a 14-kDa single-chain Llama antibody produced in yeast. The binding epitope of this antibody has been recently identified in the regions 326–331 and 656–672 of VP proteins, near the 5-fold symmetry axis.118 The AVB purification of AAV1, AAV2, AAV3B, AAV5, and AAVrh.10 is very efficient with a recovery ranging between 70% and 90%, whereas AAV8 and AAV9 poorly bind to this matrix. A brand-new immunoaffinity matrix has just been commercialized by Thermo Fisher Scientific, the POROS CaptureSelect AAVX Affinity Matrix. It
has been designed for the purification of AAV particles of various serotypes (AAV1–8 and AAVrh10) and will probably replace POROS CaptureSelect AAV8 and AAV9 affinity resins that are currently being used only for those serotypes. This new immunoaffinity matrix seems very promising and versatile as it is intended for the purification of different rAAV serotypes, according to the manufacturer. In contrast with density gradients, immunoaffinity chromatography is suitable for large-scale production, but unfortunately does not discriminate between empty and full particles. In terms of impurities, it is well recognized that affinity columns based on antibodies suffer from leaching, i.e., antibodies detaching from the support and found in the final product. Indeed, to quantify the residual ligands, both GE and Thermo Fisher commercialize its respective ELISA kits.

Vector Purification on IEX

The principle of IEX is the recovery of particles from the clarified cell lysate based on their net surface charge. The surface charge of AAV can be positive or negative depending on the environmental pH.

Several chromatography supports can be used as membrane absorbers (Mustang S), stationary monoliths (CIM), or particle-based adsorbents (Sepharose, POROS). For better purity, the purification protocol can be consolidated by a second column based on IEX, hydrophobic interaction chromatography (HIC), or size-exclusion chromatography (SEC) principle. Despite the small difference between empty and full particles isoelectric points (6.3 and 5.9, respectively), it has been shown that IEX can separate empty capsids from full genome-containing particles by optimizing elution conditions. Furthermore, the IEX-based processes are reproducible and can be automated and adapted to large-scale production in order to fulfill industrial manufacturing conditions. However, substantial development of IEX protocols is usually needed for the following reasons: (1) IEX is very sensitive to changes in the upstream conditions for vector stability and to the application. AAV particles have been found to be remarkably stable at high temperature (up to 60°C for most of the serotypes) and in a pH ranging from 5.5 to 8.5. Despite the high resistance of AAV capsids to extreme conditions, other problems can occur such as particles aggregation. This can be a real issue for highly concentrated rAAV stocks, notably for rAAV2. It has been shown that particles aggregation can be minimized by increasing the ionic strength of the solution, preferably with divalent ions salts (~200 mM magnesium sulfate). Non-ionic surfactant can also be added at low concentration to avoid this phenomenon, such as Pluronic F68 poloxamer that was added at a volume concentration of 0.001% in the final product used for a hemophilia B clinical trial. Even if Pluronic F68 has been proven to increase in process recovery of rAAV2, rAAV4, rAAV5, rAAV8, and rAAV9, no effect was observed on rAAV2-purified product.

Conclusions

The market authorization of Glybera (developed by uniQure using S9 cells) and the recent recommendation of the FDA to approve Luxturna, an AAV2 vector for the treatment of Leber congenital amaurosis (developed by Spark Therapeutics using HEK293 cells), demonstrate the maturity of AAV-based gene therapy drugs. Moreover, hundreds of pre-clinical and clinical studies conducted thus far have all provided supporting data for the use of AAV in the clinical setting, independently of the manufacturing method. Despite significant improvements in upstream and downstream processes, AAV stocks still contain significant amounts of impurities that should be minimized as much as possible during production, and quantified and identified accurately by improved analytical methods, such as HPLC, mass spectrometry for proteins. Nonetheless, with hundreds of patients enrolled in AAV trials up to date, no adverse events have been reported in relation to any of these impurities. In the near future, particular attention will be paid in those applications in which high doses of vectors will be administered systematically (>1 × 10^{14} vg/kg), for example, in Duchenne muscular dystrophy, because the total amount of impurities will be high and the route of administration is more prone to trigger immuno-toxicological responses. In the absence of comparative studies between manufacturing platforms, the field will have to rely on preclinical toxicology reports and phase I/II trial results to evaluate the safety and efficacy of the different production systems.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1. Weitzman, M.D., Kyöstö, S.R., Kotin, R.M., and Owens, R.A. (1994). Adeno-associated virus (AAV) Rep proteins mediate complex formation between AAV DNA and its integration site in human DNA. Proc. Natl. Acad. Sci. USA 91, 5808–5812.
2. Wistuba, A., Kern, A., Weger, S., Grimm, D., and Kleinschmidt, J.A. (1997). Subcellular compartmentalization of adeno-associated virus type 2 assembly. J. Virol. 71, 1341–1352.
3. Hermonat, P.L., and Muzyczka, N. (1984). Use of adeno-associated virus as a mammalian DNA cloning vector: transduction of neomycin resistance into mammalian tissue culture cells. Proc. Natl. Acad. Sci. USA 81, 6466–6470.
4. Gao, G., Zhong, L., and Danos, O. (2011). Exploiting natural diversity of AAV for the design of vectors with novel properties. Methods Mol. Biol. 807, 93–118.
5. Rabinowitz, J.E., Rollling, F., Li, C., Conrath, H., Xiao, W., Xiao, X., and Samulski, R.J. (2002). Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. J. Virol. 76, 791–801.

6. Wang, Q., Wu, Z., Zhang, J., Firman, J., Wei, H., Zhuang, Z., Liu, L., Miao, L., Hu, Y., Li, D., et al. (2017). A robust system for production of superabundant VP1 recombinant AAV vectors. Mol. Ther. Methods Clin. Dev. 7, 146–156.

7. Dong, B., Moore, A.R., Dai, J., Roberts, S., Chu, K., Kapranov, P., Moss, B., and Xiao, W. (2013). A concept of eliminating nonhomologous recombination for scalable and safe AAV vector generation for human gene therapy. Nucleic Acids Res. 41, 6609–6617.

8. Galli, A., Della Latta, V., Bologna, C., Pucciarelli, D., Cipriani, F., Backovic, A., and Cervelli, T. (2017). Strategies to optimize capsid protein expression and single-stranded DNA formation of adeno-associated virus in Saccharomyces cerevisiae. J. Appl. Microbiol. 123, 414–428.

9. Backovic, A., Cervelli, T., Salvetti, A., Zentlin, L., Giacca, M., and Galli, A. (2012). Capsid protein expression and adeno-associated virus like particles assembly in Saccharomyces cerevisiae. Microb. Cell Fact. 11, 124.

10. Thakur, S.S. (2012). Production of recombinant adeno-associated viral vectors in yeast. Ms thesis (Gainesville, FL: University of Florida). http://ufdc.ufl.edu/UF00044702/00001.

11. Snyder, R.O. April 2011. Production of recombinant AAV virions. U.S. patent 2011/0000001.

12. Mathews, L.C., Gray, J.T., Gallagher, M.R., and Snyder, R.O. (2002). Recombinant adeno-associated virus type 2 genome is involved in amplification and high-yield production of the integrated rep-cap genome. J. Gene Med. 4, 791–801.

13. Thomas, D.L., Wang, L., Niamke, J., Li, J., Kang, W., Scotti, M.M., Ye, G.J., Yeres, G., and Knop, D.R. (2009). Scalable recombinant adeno-associated virus production using recombinant herpes simplex virus type 1 confection of suspension-adapted mammalian cells. Hum. Gene Ther. 20, 861–870.

14. Adamsson-Small, L., Potter, M., Byrne, B.J., and Clément, N. (2016). A scalable method for the production of high-tier and high-quality adeno-associated virus type 9 vectors using the HSV platform. Mol. Ther. Methods Clin. Dev. 3, 16031.

15. Chalay, J.D., Ye, G.J., Thomas, D.L., Knop, D.R., Benson, J.M., Hutt, J.A., Wang, G., Humphries, M., and Flotte, T.R. (2011). Preclinical evaluation of a recombinant adeno-associated virus vector expressing human alpha-1 antitrypsin made using a recombinant herpes simplex virus production method. Hum. Gene Ther. 22, 155–165.

16. Clark, K.R., Voulgaropoulou, F., Frealey, D.M., and Johnson, P.R. (1995). Cell lines for the production of recombinant adeno-associated virus. Hum. Gene Ther. 6, 1329–1341.

17. Gao, G.P., Qu, G., Faust, L.Z., Engdahl, R.K., Xiao, W., Hughes, J.V., Zoltick, P.W., and Wilson, J.M. (1998). High-tier adeno-associated viral vectors from a Rep/Cap cell line and hybrid shuttle virus. Hum. Gene Ther. 9, 2353–2362.

18. Chadeau, G., Favre, D., TESSIER, J., Provost, N., Nony, P., Kleinschmidt, J., Moullier, P., and Salvetti, A. (2000). Efficient recombinant adeno-associated virus production by a stable rep-cap HeLa cell line correlates with adenovirus-induced amplification of the integrated rep.cap genome. J. Gene Med. 2, 266–268.

19. Matthews, L.C., Gray, J.T., Gallagher, M.R., and Snyder, R.O. (2002). Recombinant adeno-associated viral vector production using stable packaging and producer cell lines. Methods Enzymol. 346, 393–413.

20. Gao, G., Gu, G., Burnham, M.S., Huang, J., Chirmule, N., Joshi, B., Yu, Q.C., Marsh, J.A., Concio, C.M., and Wilson, J.M. (2000). Purification of recombinant adeno-associated virus vectors by column chromatography and its performance in vivo. Hum. Gene Ther. 11, 2079–2091.

21. Farson, D., Harding, T.C., Tao, L., Liu, J., Powell, S., Vimal, V., Yendluri, S., Koprinvnik, K., Ho, K., Twitty, C., et al. (2004). Development and characterization of a cell line for large-scale, serum-free production of recombinant adeno-associated viral vectors. J. Gene Med. 6, 1369–1381.

22. Tressier, J., Chadeau, G., Nony, P., Avet-Loiseau, H., Moullier, P., and Salvetti, A. (2001). Characterization of adenovirus-induced inverted terminal repeat-independent amplification of integrated adeno-associated virus rep-cap sequences. J. Virol. 75, 375–383.

23. Gao, G.P., Lu, F., Sanmiguel, J.C., Tran, P.T., Abbas, Z., Lynd, K.S., Marsh, J.A., Concio, C.M., and Wilson, J.M. (2002). Rep/Cap gene amplification and high-yield production of AAV in an A549 cell line expressing Rep/Cap. Mol. Ther. 5, 644–649.

24. Nony, P., Tressier, J., Chadeau, G., Ward, P., Giraud, A., Dugast, M., Linden, R.M., Moullier, P., and Salvetti, A. (2001). Novel cis-acting replication element in the adeno-associated virus type 2 genome is involved in amplification of integrated rep-cap sequences. J. Virol. 75, 9991–9994.

25. Francois, A., Guilbaud, M., Awedikian, R., Chadeau, G., Moullier, P., and Salvetti, A. (2005). The cellular TATA binding protein is required for rep-dependent replication of a minimal adeno-associated virus type 2 psi element. J. Virol. 79, 11082–11094.

26. Clark, K.R., Voulgaropoulou, F., Johnson, P.R. (1996). A stable cell line carrying adeno-associated virus-ducible rep and cap genes allows for infectivity titration of adeno-associated virus vectors. Gene Ther. 3, 1124–1132.

27. Liu, X.L., Clark, K.R., and Johnson, P.R. (1999). Production of recombinant adeno-associated virus vectors using a packaging cell line and a hybrid recombinant adeno-virus. Gene Ther. 6, 293–299.
42. Toulouse, E., Benraiss, A., Bonnin, D., Blouin, V., Brum lent, N., Cartier, N., Epstein, A.L., Moullier, P., and Sal vetti, A. (2004). Identification of a replication-defective herpes simplex virus for recombinant adenassociated virus type 2 (AAV2) parti cle assembly using stable producer cell lines. J. Gene Med. 6, 555–564.

43. Yang, Q., Chen, F., and Trempe, J.P. (1994). Characterization of cell lines that inducibly express the adenassociated virus Rep proteins. J. Virol. 68, 4847–4856.

44. Saudan, P., Vlach, J., and Beard, P. (2000). Inhibition of S-phase progression by ad enoassociated virus Rep78 protein is mediated by hypophosphorylated pRB. EMBO J. 19, 4351–4361.

45. Li, Z., Brister, J.R., Im, D.-S., and Muryczka, N. (2003). Characterization of the aden associated virus Rep protein complex formed on the viral origin of DNA replication. Virology 313, 364–376.

46. Berthet, C., Raj, K., Saudan, P., and Beard, P. (2005). How adenassociated virus Rep78 protein arrests cells completely in S phase. Proc. Natl. Acad. Sci. USA 102, 13634–13639.

47. Schmidt, M., Afone, S., and Kotin, R.M. (2000). Adenoassociated virus type 2 Rep78 induces apoptosis through caspase activation independently of p53. J. Virol. 74, 9441–9450.

48. Chang, L.S., Shi, Y., and Shenk, T. (1989). Adeno-associated virus P5 promoter con tains an adenovirus E1A-inducible element and a binding site for the major late control system and its use in stable, high-titer 293 cell-based adenassociated virus packaging cell lines. J. Virol. 76, 13015–13027.

49. Yuan, Z., Qiao, C., Hu, P., Li, J., and Xiao, X. (2011). A versatile adenoassociated virus vector production. Mol. Biotechnol. 27, 7–14.

50. Martin, J., Frederick, A., Luo, Y., Jackson, R., Joubert, M., Sol, B., Poulin, F., Pastor, E., Armentano, D., Wadsworth, S., and Vincent, K. (2013). Generation and charac terization of a replication-defective AAV vector for treatment of achromatopsia. Hum. Gene Ther. Clin. Dev. 27, 165–176.

51. Ye, G.J., Scotti, M.M., Liu, J., Wang, L., Knop, D.R., and Veres, G. (2011). Characterization and characterization of residual HSV DNA in recombinant adenoassociated virus produced by an HSV complementation system. Gene Ther. 18, 135–144.

52. Nambiar, B., Cornell Sookdeo, C., Berthelette, P., Jackson, R., Piraino, S., Burnham, B., Nass, S., Souza, D., O’Riordan, C.R., Vincent, K.A., et al. (2017). Characteristics of minimally oversized adenoassociated virus vectors encoding human factor VIII generated using producer cell lines and triple transfection. Hum. Gene Ther. Methods 28, 148–162.

53. Ye, G.J., Budzynski, E., Sonnentag, P., Miller, P.E., Sharma, A.K., Ver Hoeve, J.N., Howard, K., Knop, D.R., Neuringer, M., McGill, T., et al. (2015). Safety and biodistribution evaluation in Cynomolgus macaques of rAAV2tYF-CB-hRS1, a recombinant adenoassociated virus vector expressing retinosin. Hum. Gene Ther. Clin. Dev. 26, 165–176.

54. Ye, G.J., Budzynski, E., Sonnentag, P., Nork, T.M., Miller, P.E., McPherson, L., Ver Hoeve, J.N., Smith, L.M., Arndt, T., Mandapati, S., et al. (2016). Safety and biodistribution evaluation in CNGB3-deficient mice of rAAV2tYF-PR1.7-hCNGB3, a recombinant AAV vector for treatment of achromatopsia. Hum. Gene Ther. Clin. Dev. 27, 26–37.

55. Ye, G.J., Budzynski, E., Sonnentag, P., Nork, T.M., Miller, P.E., Sharma, A.K., Ver Hoeve, J.N., Smith, L.M., Arndt, T., Mandapati, S., et al. (2016). Safety and biodistribution evaluation in Cynomolgus macaques of rAAV2tYF-PR1.7-hCNGB3, a recombinant AAV vector for treatment of achromatopsia. Hum. Gene Ther. Clin. Dev. 26, 177–184.

56. Ye, G.J., Conlon, T., Erger, K., Sonnentag, P., Sharma, A.K., Howard, K., Knop, D.R., and Chulay, J.D. (2015). Safety and biodistribution evaluation of rAAV2tYF-CB-hRS1, a recombinant adenoassociated virus vector expressing retinosin, in RS1-deficient mice. Hum. Gene Ther. Clin. Dev. 26, 177–184.

57. Wright, J.F., Tappnell, B.C., Humphries, M., Carey, B., Calcedo, R., Rouhani, F., Campbell-Thompson, M., Yachnis, A.T., Sandhaus, R.A., McElvaney, N.G., et al. (2011). Phase 2 clinical trial of a recombinant adenoassociated viral vector expressing z1-antitrypsin: interim results. Hum. Gene Ther. 22, 1239–1247.

58. Smith, P.H., Wright, F.J., Qu, G., Patarroyo-White, S., Parker, A., and Sommer, J.M. (2003). Packaging of host cell and plasmid DNA into recombinant adenoassociated virus particles produced by triple transfection. Mol. Ther. 7, 5348.
88. Thorne, B.A., Quigley, P., Nichols, G., Moore, C., Pastor, E., Price, D., Ament, J.W., Muruve, D.A. (2004). The innate immune response to adenovirus vectors. Hum. Gene Ther. 24 (Suppl 1), S218–S219.

89. Denard, J., Jenny, C., Blouin, V., Moullier, P., and Svinartchouk, F. (2014). Different protein composition and functional properties of adeno-associated virus-6 vector manufactured from the culture medium and cell lysates. Mol. Ther. Methods Clin. Dev. 1, 14031.

90. Grimm, D., Kern, A., Pawlita, M., Ferrari, F., Samulski, R., and Kleinschmidt, J. (1999). Titration of AAV-2 particles via a novel capsid ELISA: packaging of genomes can limit production of recombinant AAV-2. Gene Ther. 6, 1322–1330.

91. Kost, T.A., and Comreday, J.P. (2002). Recombinant baculoviruses as mammalian cell gene-delivery vectors. Trends Biotechnol. 20, 173–180.

92. Laakkonen, J.P., Kaikkonen, M.U., Ronkainen, P.H.A., Ihailainen, T.O., Niskanen, E.A., Hakkinen, M., Salminen, M., Kulomaa, M.S., Ylä-Herttuala, S., Airenne, K.J., and Vihinen-Ranta, M. (2008). Isolation of highly infectious and pure adeno-associated virus type 2 (AAV-2) capsid. Virolology 357, 373–382.

93. Johnson, J.S., and Samulski, R.J. (2009). Enhancement of adeno-associated virus infection by mobilizing capsids into and out of the nucleus. J. Virol. 83, 2632–2644.

94. Bevington, J.M., Needham, P.G., Verrill, K.C., Collaco, R.F., Basrur, V., and Trempe, J.P. (2007). Adeno-associated virus interactions with B23/Nucleophosmin: identification of sub-nuclear virion regions. Virolgy 357, 102–113.

95. Aucoin, M.G., Perrier, M., and Kamen, A.A. (2006). Production of adeno-associated virus vectors with recombinant adeno-associated virus type 6. J. Virol. 80, 6620–6631.

96. Aucoin, M.G., Perrier, M., and Kamen, A.A. (2007). Improving AAV vector yield in mammalian cells. J. Virol. 81, 985–990.

97. Connor, E., Gao, G.P., and Wilson, J.M. (2001). Improvement of a single-step gravity-sedimentation purification process for recombinant adeno-associated virus vectors using high hydrostatic pressure to selectively inactivate helper adenovirus. Biotechnol. Bioeng. 64, 207–217.

98. Hermens, W.T.J.M.C., & Smith, I.P. March 2013. Removal of contaminating viruses from AAV preparations. World Intellectual Property Organization Patent 2013/036118A1.

99. Tijal, S.T., zu Altenschildseck, G.M., and Doerrer, W. (1983). Autographa californica nuclear polyhedrosis virus (AcNPV) DNA does not persist in mass cultures of mammalian cells. Virology 125, 107–117.

100. Rice, S.A., and Knipe, D.M. (1990). Genetic evidence for two distinct transactivation functions of the herpes simplex virus alpha protein ICP27. J. Virol. 64, 1704–1715.

101. Knop, D.R., and Harrell, H. (2007). Bioreactor production of recombinant herpes simplex virus vectors. Biotechnol. Prog. 23, 715–721.

102. Ye, G.I., Scotti, M.M., Thomas, D.L., Wang, L., Knop, D.R., and Chulay, J.D. (2014). Herpes simplex virus clearance during purification of a recombinant adeno-associated virus serotype 1 vector. Hum. Gene Ther. Clin. Dev. 25, 212–217.

103. Jenny, C., Toublanc, E., Danos, O., and Merten, O.W. (2005). Evaluation of a serum-free medium for the production of rAAV-2 using HEk293 derived producer cells. Cytotherapy 7, 49–112.

104. Leonard, J.N., Fersht, P., Delgado, A., and Schaffer, D.V. (2007). Enhanced preparation of adeno-associated viral vectors by using high hydrostatic pressure to selectively inactivate helper adenovirus. Biotechnol. Bioeng. 97, 1170–1179.

105. Grimm, D., Kern, A., Pawlita, M., Ferrari, F., Samulski, R., and Kleinschmidt, J. (1999). Titration of AAV-2 particles via a novel capsid ELISA: packaging of genomes can limit production of recombinant AAV-2. Gene Ther. 6, 1322–1330.

106. Jenny, C., Toublanc, E., Danos, O., and Merten, O.W. (2005). Evaluation of a serum-free medium for the production of rAAV-2 using HEk293 derived producer cells. Cytotherapy 7, 49–112.

107. Grimm, D., Kern, A., Pawlita, M., Ferrari, F., Samulski, R., and Kleinschmidt, J. (1999). Titration of AAV-2 particles via a novel capsid ELISA: packaging of genomes can limit production of recombinant AAV-2. Gene Ther. 6, 1322–1330.

108. Van Loo, N.D., Fortunati, E., Ehleret, E., Rabelin, M., Grosfeld, V., and Scholte, B.J. (2008). Baculovirus-mediated immediate-early gene expression. J. Virol. 82, 6620–6631.

109. Burnham, B., Nass, S., Kong, E., Mattingly, M., Woodcock, D., Song, A., Wadsworth, S., Cheng, S.H., Scaria, A., and O’Riordan, C.R. (2015). Analytical ultra centrifugation as an approach to characterize recombinant adeno-associated viral vectors. Hum. Gene Ther. Methods 26, 228–242.

110. Aucoin, M.G., Perrier, M., and Kamen, A.A. (2006). Production of adeno-associated viral vectors in insect cells using triple infection: optimization of baculovirus concentration ratios. Biotechnol. Bioeng. 95, 1081–1092.

111. Aucoin, M.G., Perrier, M., and Kamen, A.A. (2007). Improving AAV vector yield in insect cells by modulating the temperature after infection. Biotechnol. Bioeng. 97, 1501–1509.

112. Vandenberghe, L.H., Xiao, R., Lock, M., Lin, J., Korn, M., and Wilson, J.M. (2010). Efficient serotype-dependent release of functional vector into the culture medium during adeno-associated virus manufacturing. Hum. Gene Ther. 21, 1251–1257.

113. Dias Florencio, G., Pecquet, G., Beley, C., Buclet, P.O., Garcia, L., and Benchaour, R. (2015). Simple downstream process based on detergent treatment improves yield and in vivo transduction efficacy of adeno-associated virus vectors. Mol. Ther. Methods Clin. Dev. 2, 15024.

114. Ayuso, E., Minguez, F., Montane, J., Leon, X., Anguela, X.M., Hauvet, G., Edmonson, S.A., Africa, L., Zhou, S., High, K.A., et al. (2010). High AAV vector purity results in serotype- and tissue-independent enhancement of transduction efficiency. Gene Ther. 17, 503–510.

115. Zolotukhin, S., Byrne, B.J., Mason, E., Zolotukhin, I., Potter, M., Chensu, K., Summerford, C., Samulski, R.J., and Muzyczka, N. (1999). Recombinant adeno-associated virus purification using novel methods improves infectious titers and yield. Gene Ther. 6, 973–985.

116. Auricchio, A., Hildinger, M., O’Connor, E., Gao, G.P., and Wilson, J.M. (2001). Isolation of highly infectious and pure adeno-associated virus type 2 vectors with a single-step gravity-flow column. Hum. Gene Ther. 12, 71–76.
117. Anderson, R., Macdonald, I., Corbett, T., Whiteway, A., and Prentice, H.G. (2000). A method for the preparation of highly purified adeno-associated virus using affinity column chromatography, protease digestion and solvent extraction. J. Virol. Methods 85, 23–34.

118. Wang, Q., Lock, M., Prongay, A.J., Alvira, M.R., Petkov, B., and Wilson, J.M. (2015). Identification of an adeno-associated virus binding epitope for AVB sepharose affinity resin. Mol. Ther. Methods Clin. Dev. 2, 15040.

119. Cecchini, S., Virag, T., and Kotin, R.M. (2011). Reproducible high yields of recombinant adeno-associated virus produced using invertebrate cells in 0.02- to 200-liter cultures. Hum. Gene Ther. 22, 1021–1030.

120. Chahal, P.S., Aucoin, M.G., and Kamen, A. (2007). Primary recovery and chromatographic purification of adeno-associated virus type 2 produced by baculovirus/insect cell system. J. Virol. Methods 139, 61–70.

121. Smith, R.H., Yang, L., and Kotin, R.M. (2008). Chromatography-based purification of recombinant adeno-associated virus. Methods Mol. Biol. 434, 37–54.

122. Venkatkrishnan, B., Yarbrough, J., Domisc, J., Bennett, A., Rothner, B., Kozyreva, O.G., Samulsli, R.J., Muszyczka, N., McKenna, R., and Agbandje-McKenna, M. (2013). Structure and dynamics of adeno-associated virus serotype 1 VP1-unique N-terminal domain and its role in capsid trafficking. J. Virol. 87, 4974–4984.

123. Urabe, M., Nakakura, T., Xin, K-Q., Obara, Y., Mizukami, H., Kume, A., Kotin, R.M., and Ozawa, K. (2006). Scalable generation of high-titer recombinant adeno-associated virus type 5 in insect cells. J. Virol. 80, 1874–1885.

124. Okada, T., Nonaka-Sarukawa, M., Uchihori, R., Kinoshita, K., Hayashi-Kinoh, H., Nitahara-Kasahara, Y., Takeda, S., and Ozawa, K. (2009). Scalable purification of adeno-associated virus serotype 1 (AAV1) and AAV8 vectors, using dual ion-exchange adsorptive membranes. Hum. Gene Ther. 20, 1013–1021.

125. Pacouret, S., Bouzelha, M., Shelke, R., Andres-Mateos, E., Xiao, R., Maurer, A., Mevel, M., Turunen, H., Barungi, T., Penaud-Budloo, M., et al. (2017). AAV-1D: a rapid and robust assay for batch-to-batch consistency evaluation of AAV preparations. Mol. Ther. 25, 1357–1366.

126. Bennett, A., Patel, S., Mietzsch, M., Jose, A., Lins-Austin, B., Yu, J.C., Bothner, B., McKenna, R., and Agbandje-McKenna, M. (2017). Thermal stability as a determinant of AAV serotype identity. Mol. Ther. Methods Clin. Dev. 6, 171–182.

127. Bennett, A.M., Su, L., Su, Q., Gao, G., Mueller, C., and Flotte, T.R. (2015). Stability and compatibility of recombinant adeno-associated virus under conditions commonly encountered in human gene therapy trials. Hum. Gene Ther. Methods 26, 71–76.

128. Wright, J.F., Le, T., Prado, J., Bahr-Davidson, J., Smith, P.H., Zhen, Z., Sommer, J.M., Pierce, G.F., and Qu, G. (2005). Identification of factors that contribute to recombinant AAV2 particle aggregation and methods to prevent its occurrence during vector purification and formulation. Mol. Ther. 12, 171–178.

129. Turnbull, A.E., Skulimowski, A., Smythe, J.A., and Alexander, I.E. (2000). Adeno-associated virus vectors show variable dependence on divalent cations for thermostability: implications for purification and handling. Hum. Gene Ther. 11, 629–635.

130. Guo, P., El-Gohary, Y., Prasadan, K., Shiota, C., Xiao, X., Wiersch, J., Paredes, J., Tulachan, S., and Gates, G.K. (2012). Rapid and simplified purification of recombinant adeno-associated virus. J. Virol. Methods 183, 139–146.

131. Xiao, R., Andres-Mateos, E., Barungi, T., Qiu, H., and Vandenberghe, L.H. (2017). Downstream AAV recovery dramatically increased by non-ionic surfactant P68. Mol. Ther. 25 (Suppl 1), S329.

132. Louis, N., Evedogh, C., and Graham, F.L. (1997). Cloning and sequencing of the cellular-viral junctions from the human adenovirus type 5 transformed 293 cell line. Virology 233, 423–429.

133. Duffridge, R.B., Tang, P., Hsia, H.C., Leong, P.M., Miller, J.H., and Calos, M.P. (1987). Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Mol. Cell. Biol. 7, 379–387.

134. Giard, D.J., Aaronson, S.A., Todaro, G.J., Arnstein, P., Kersey, J.H., Dosik, H., and Parks, W.P. (1973). In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J. Natl. Cancer Inst. 51, 1417–1423.

135. Stoker, M., and MacPherson, I. (1964). Syrian hamster fibroblast cell line Bhk21 and its derivatives. Nature 203, 1355–1357.

136. Wright, J.F. (2008). Manufacturing and characterizing AAV-based vectors for use in clinical studies. Gene Ther. 15, 840–848.

137. Snyder, R.O., Audit, M., and Francis, J.D. (2011). rAAV product characterization and stability studies. Methods Mol. Biol. 807, 405–428.

138. Robert, M.A., Chahal, P.S., Audy, A., Kamen, A., Gilbert, R., and Galillet, B. (2017). Manufacturing of recombinant adeno-associated viruses using mammalian expression platforms. Biotechnol. J. 12, 1860193.

139. Samulski, R.J., Chang, L.S., and Shenk, T. (1987). A recombinant plasmid from herpes simplex virus type 1 containing the late region of adeno-associated virus and its derivatives. Nature 330, 581–584.

140. Robert, M.A., Chahal, P.S., Audy, A., Kamen, A., Gilbert, R., and Galillet, B. (2017). Manufacturing of recombinant adeno-associated viruses using mammalian expression platforms. Biotechnol. J. 12, 1860193.