Proactive Progress

Current progress being made in gene therapy has shown that customised viral engineering is key to safe and effective adeno-associated virus gene delivery

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Although gene therapy is a promising tool within disease treatment, progress has been slow in developing effective clinical approaches. The issue lies in the difficulty to develop safe and efficient gene-delivery systems (1). An ideal vector system should deliver a certain amount of genetic material into the target cells. The transfer should be stable and cell-type-specific, allowing a high and controllable expression of the gene product without causing an immune response or other toxicity (2).

The revolutionary potential of adeno-associated viruses (AAV) is undisputed and underlined by the growing number of clinical trials using them as a central gene delivery tool. Recent groundbreaking success stories from clinical AAV applications have been reported in the media. The publication of one therapy successfully treating haemophilia B via liver-specific AAV vectors demonstrated their long-term potency, aside from a low number of temporary side effects (3). The first AAV FDA approval for an AAV-based therapy in history happened just before Christmas 2017, giving patients who suffer from bi-allelic RPE65 mutation-associated retinal dystrophy new hope that they may retain their eyesight (4). Impressive in their own right, these publications also help to uncover the secret to success when it comes to engaging in the development of any new AAV-based therapy: viral engineering – customising existing virus models to fit the specific goals of the therapeutic approach. More precisely, both the genetic strategy itself – in terms of plasmid manufacturing – and the viral capsid carrying it to the designated cell type or tissue need to be optimised to fit every aspect of their intended application, including the determination of the correct titre of the gene shuttle (see Figure 1).

A Solid Start

Among other applications, plasmid DNA is often used as starting material in the Good Manufacturing Practice-compliant (GMP) production of recombinant viruses, antibodies, and RNA, where these are the API used in clinical trials. In many cases, producing the plasmid DNA under GMP conditions is not necessary. An alternative is the high-quality grade plasmid DNA, which is both highly purified and well-characterised, thus meeting the requirements of most regulating agencies (5). High-quality grade plasmid DNA is produced based on a research cell bank and the patented ccc-grade DNA technology. A number of quality controls, both to the cell bank and to the plasmid DNA product, ensure that the final result is a product designed especially for the intended application that complies with the appropriate regulatory standards (5).

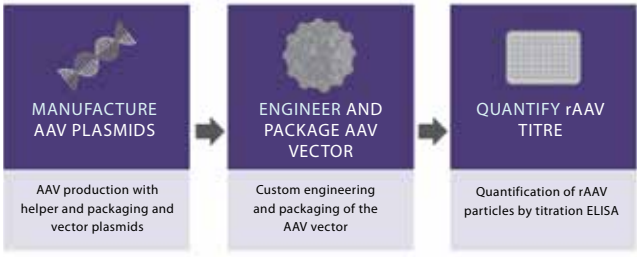

Figure 1: Necessary steps for the generation of a functional AAV vector, often applied for gene therapy

If plasmid DNA is used as starting material for AAV vector manufacturing, a 3-plasmid system or a 2-plasmid system is typically applied. While the 3-plasmid system comprises a co-transfection with three plasmids, the 2-plasmid system simply depends on a co-transfection with two plasmids (see Figure 2, page 26). The 3-plasmid system consists of a transfer (also known as a vector or genome) plasmid, which includes the gene of interest (GOI), flanked by the inverted terminal repeats sequences for successful viral packaging, a second plasmid composed of rep genes for virus lifecycle and sequences for virus capsids, so called cap genes, and a third plasmid that incorporates the adenoviral helper genes (6-7). The rep and cap genes, as well as the adenoviral helper genes, are located on one plasmid in case of the 2-plasmid system. These plasmids are called 'helper and packaging plasmids (pDG/pDP)' (8). To ensure tissue specificity, several serotypes of these plasmids are available. Furthermore, as an alternative, a patented 1-plasmid system that comprises all genes on one plasmid, including the GOI, exists (9).

A recent study showed that, in case of a 2-plasmid system, the transfer plasmid, as well as the helper and packaging plasmid, can be replaced by a corresponding *minicircle* (MC) construct (10).

MC DNA: The Effective Alternative

Manufacturing pure AAV vector preparations remains a difficult task. While empty capsids can be removed from vector preparations, best available purification strategies have thus far failed to remove antibiotic resistance genes or other plasmid backbone sequences (10). MC constructs could replace AAV transfer and helper and packaging plasmids for production of both single-stranded (ss) and self-complementary (sc) AAV

Figure 2: Transduction process of an AAV producer cell line with the 2-plasmid system

vectors. Due to the fact that bacterial backbone sequences are removed during MC production, encapsulation of prokaryotic plasmid backbone sequences is avoided (10). Particularly, this is of importance for scAAV vector preparations, which contain a high amount of plasmid backbone sequences. Replacing the plasmids by MC constructs not only allows one to reduce these contaminations below the quantification limit, but also improve transduction efficiencies of scAAV preparations up to 30-fold (10). Thus, MC technology significantly improves the quality of AAV vector preparations. As an added value, it is an easy-to-implement modification of standard AAV packaging protocols.

Purity of AAV Vectors

To build a competitive gene therapy strategy, a virus' affinities towards different types of tissue need to be considered and optimised to rule out unwanted side effects and decrease the loss of particles in a systemic application. Tissue specificity can be addressed two-fold: firstly, by application of tissue-specific promoters that elicit an expression only in therapeutically relevant cells, and, secondly, through the viral capsid itself that can be modified. Multiple innovative engineering techniques have emerged that can help address different aspects of the capsid's dynamics *in vivo*.

Next to the vector's tissue specificity, experimenters can consider additional parameters that may be decisive for a clinical application, such as a reduced immunogenicity or enhancement of particle productivity. While these techniques differ in their initial approach of how to identify and choose what areas of the viral package will be modified, most techniques utilise whole libraries of modified AAV capsids. These are first selected for their performance in relevant cell types or tissue models and subsequently applied in specific animal models to identify a high-performing capsid for the intended application. To use these libraries to their full potential, the demand for high-level production of large preclinical AAV batches is ever increasing – especially the selection of capsids with low immunogenicity that necessitate the highest purities, even during the early stages of production. Development standards that are key to avoid artefacts or off-side effects, at the latest once an R&D project moves into systemic applications in animal models, should be strictly standardised when going into high-level productions (11). This begins with the right choice and maintenance of highly standardised production cells. Up-to-date and with few exceptions, adherent cell clones generate higher and more dependable yields in relation to suspension cell clones. While this seems to make scalability more difficult, several 3D culturing systems, such as microcarrier or fixed-bed bioreactors, exist that serve well as a means to exploit 2D adherent cell systems more efficiently (12). They also serve as a scalable base for later full-GMP productions.

As important as the main packaging process, AAV protocols should include subsequent purification and concentration steps. Fast protein liquid chromatography (FPLC) affinity- or ion-exchange-columns have become state-of-the-art techniques that need to be adapted and optimised for every specific capsid variant to yield optimal results as gold standard for any ambitious development project (see Figure 3, page 28). This demand for higher purities in turn increases the demand for higher production yields to counter the loss of product that accompanies every concentration step. To date, preclinical production yields regularly fall between 10^{12} to 10^{13} particles. The newest refinements in plasmid technologies and packaging cell clones have helped increase the output to over

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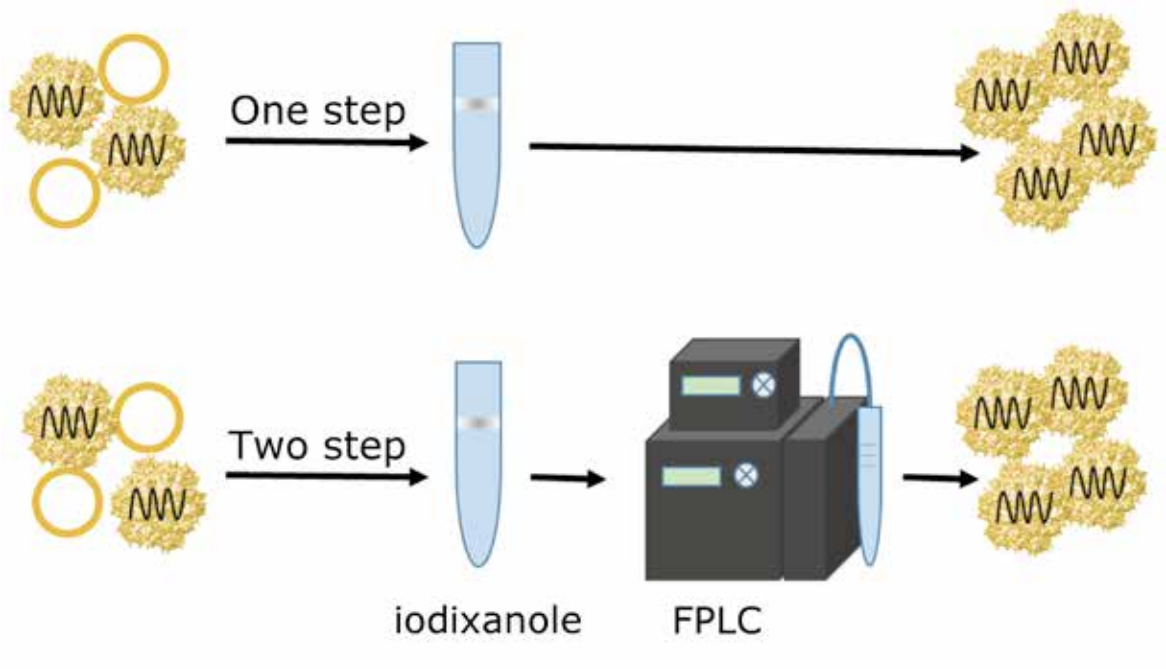

Figure 3: AAV purification and concentration. Either a one-step method using an iodixanole gradient or a two-step method with a subsequently FPLC purification step can be applied

10^{14} particles for some serotypes. With further adjustments, the 10^{15} mark as a new preclinical standard is within a reach that can be scaled further when entering GMP production.

Importantly, the standard for empty-to-full capsids has also increased considerably in the past 10-14 months, setting the minimal benchmark at a 20/80 relation. The quantification of purified AAV vector preparations concludes the *in vitro* phase of the AAV production protocol. This step is of particular importance for clinical applications that demand a dependable and reproducible quantification of accurate recombinant AAV (rAAV) titres to ensure a safe and reliable gene transfer.

AAV Titration ELISA

Quantification methods for rAAV vector preparations include quantitative PCR (qPCR), digital droplet PCR (ddPCR) for measuring DNA, dot blot, and enzyme-linked immunosorbent assay (ELISA) for intact viral capsid protein (see Figure 4, page 30). Other methods using protein chromatography, flow cytometry, or virus particle counting instruments have also been described, but are generally only applicable for highly skilled users and/or require specialised and expensive equipment. Electron microscopy can be used to determine the ratio of empty and full viral capsids, but is not useful for an absolute quantification of viral particles.

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Each of these techniques has its pros and cons: qPCR is widely used, but suffers from several issues such as sample preparation, primer design, or PCR efficiency that can lead to high inter-laboratory variation of results for identical samples (13-14). Digital droplet PCR methods overcome some of the limitations of qPCR (15). There is no need for a dilution series of standard DNA with known concentrations to measure an unknown sample, and limited PCR-efficiency is not an issue as it is for qPCR. However, variations between labs can still occur due to different sample processing protocols. Dot blot is a relatively simple and quantitative method, but works only with reliable reference material, while suffering from the limited linearity and dynamic range of Western blotting in general.

Given the practical drawbacks of the aforementioned techniques, a conventional sandwich ELISA currently appears to be the best format for the quantification of rAAV preparations: a microtitre plate coated with a monoclonal antibody specific for a conformational epitope on intact, assembled AAV capsids reliably captures AAV particles from a given rAAV preparation. Using the established biotin/streptavidin peroxidase colour reaction of the secondary antibody, the precise titre of infective vectors can be determined photometrically, delivering robust

	ELISA	qPCR	ddPCR	Dot blot
Measured parameter	Viral particles	Viral gene	Viral gene	Viral particles (non-denaturing)
Accuracy	High	Medium	Medium-high	Medium
Interlab variability	Low	High	Medium	Medium
Time	Hours	Hours	Hours	Hours
Investment for equipment	Low	Medium	High	Low
Cost per sample	Low	Low	Low	Low

Figure 4: Comparison of current methods for the quantification of rAAV titres

and reproducible data. As a fast, sensitive, reliable, cost-effective, and well-established format, the ELISA is ideal for standardised therapy protocols.

ELISAs for the quantitative determination of AAV particles in cell culture supernatants and purified virus preparations are on the market for all AAV serotypes that are under investigation for gene delivery (1-2,5-6,8-9). The assay performance critically depends on the quality of the primary capture antibody that should be designed to detect conformational epitopes not present on unassembled capsid proteins. Quality attributes for AAV ELISAs include validation in published studies and alignment with international reference standards (available for AAV2 and AAV8).

On The Rise

With the recent launch of the first AAV-based gene therapy and several late stage clinical studies, a growing AAV community of academic and industrial labs are using AAV vectors for the development of gene therapies with an increasing demand for useful and reliable analytical AAV tools for R&D and manufacturing. The need for standardisation will favour those methods that facilitate the complete workflow from manufacturing of the plasmid to engineering and production of the vector up to the quantification of infective vectors, thereby enabling safe and effective AAV gene therapies.

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