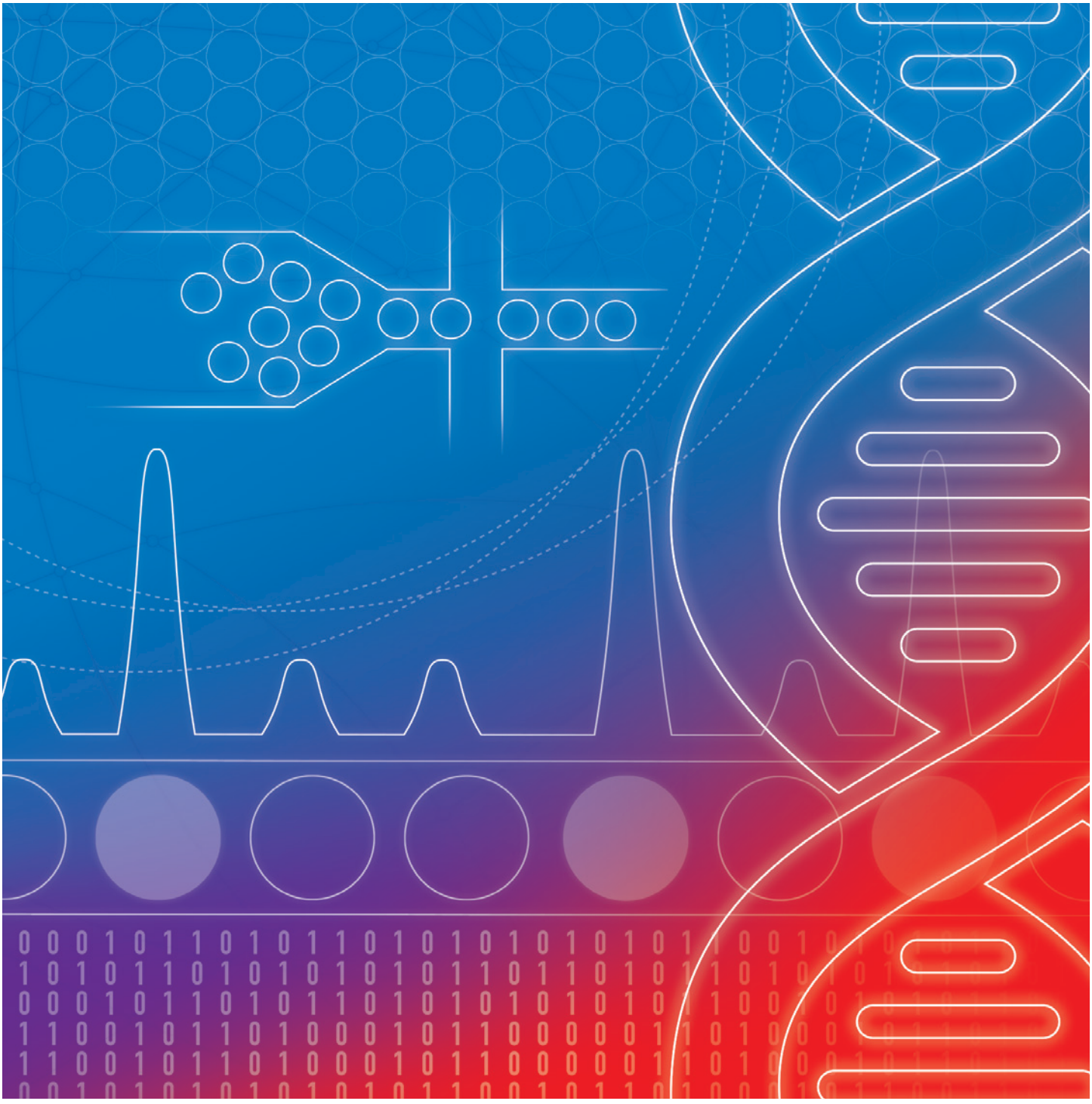
Droplet Digital PCR

Adeno-Associated Virus: Enabling Genomic Medicine

A Droplet Digital™ PCR eBook



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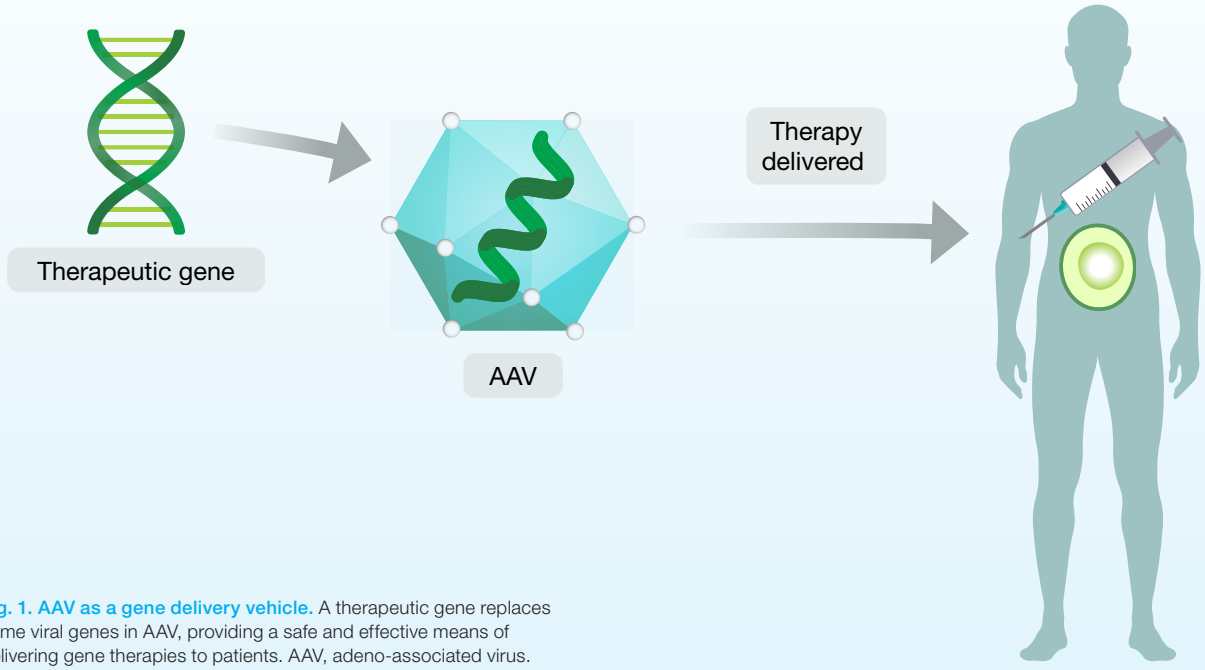

Fig. 1. AAV as a gene delivery vehicle. A therapeutic gene replaces some viral genes in AAV, providing a safe and effective means of delivering gene therapies to patients. AAV, adeno-associated virus.

From Contaminant to Therapeutic: The History of AAV

Today, adeno-associated virus (AAV) is the most popular vehicle for therapeutic in vivo gene delivery (Figure 1). Recent landmark regulatory approvals of AAV-based therapeutics like Zolgensma (onasemnogene abeparvovec-xioi) have only bolstered enthusiasm as well as the continued investment for the development of other AAV therapeutics to address diseases with high unmet medical need. However, the discovery and initial development of AAV was neither newsworthy nor intentional.

AAVs were first discovered in the mid-1960s by electron microscopy–based screening of adenovirus preparations (Keeler and Flotte 2019). AAVs were initially described as a contaminant when they were accidentally discovered, and subsequent work was done purely for the sake of scientific curiosity (Wang et al. 2019, Pupo et al. 2022). Over the next 15–20 years, several crucial aspects of AAV biology were elucidated and the significance of this accidental discovery began to come into focus for the scientific community. Several landmark mechanistic studies eventually led to the successful cloning of wild-type AAV2 into plasmids (Wang et al. 2019). AAV2 was subsequently adapted for recombinant eukaryotic gene transfer by placing the required genes of the virus into plasmids. The vector set consisted of a two-plasmid system. The first plasmid contained the AAV2 inverted terminal repeats (ITRs) that flanked a genetic payload, and the second plasmid contained the genes necessary for viral replication and structural proteins (Keeler and Flotte 2019). These plasmids were cotransfected into HeLa cells that were infected with adenovirus to produce a recombinant AAV (rAAV) vector (Keeler and Flotte 2019).

Understanding of this novel gene delivery system progressed significantly to support clinical evaluation in a phase I trial for the treatment of cystic fibrosis (Keeler and Flotte 2019). As the scope and number of potential clinical applications of rAAV began to emerge, published data indicated that different AAV serotypes and capsid variants demonstrated higher gene transfer in select tissue types (Pupo et al. 2022). Investigators began cross-packaging the ITR-flanked rAAV2 genomes with a variety of other rAAV capsids by incorporating the desired *cap* gene to enhance gene transfer in the target cell type.

The past 50 years of rAAV research and development culminated in the 2012 approval of Glybera (alipogene tiparvovec), which is an rAAV1-based gene therapy for the treatment of lipoprotein lipase deficiency, by the European Medicines Agency. Luxterna (voretigene neparvovec-rzyl), a treatment for inherited retinal dystrophy, was the first rAAV-based therapy approved by the U.S. Food and Drug Administration (U.S. FDA) just 5 years later (Wang et al. 2019). Since then, application of gene therapies utilizing rAAV vectors has exploded and active clinical programs are being fast-tracked for clinical approval for congestive heart failure, hemophilia A and B, retinal disease, X-linked myotubular myopathy, and other indications (Pupo et al. 2022).

Clinical Applications of AAV

The rapid expansion of the scope and clinical utility of rAAV is due to several inherent clinical advantages of the vector itself. Specifically, these vectors are, for the most part, nonintegrative and have the capacity to transduce a variety of terminally differentiated cells to drive long-term therapeutic gene expression (Naso et al. 2017, Verdera et al. 2020). Further, rAAV are relatively nonimmunogenic (Naso et al. 2017). These advantages have translated to the clinical evaluation of rAAV with four overall therapeutic strategies:

- **Gene replacement** — first, rAAV can be used to deliver genes with the intent of compensating for a loss-of-function mutation. Such is the case where rAAV could be used to correct mutations that render proteins nonfunctional, including blood clotting factors VIII and IX in hemophilia A and B and SMN protein in spinal muscular atrophy (Nathwani 2019, Jablonka S et al. 2022)
- **Gene silencing** — inversely, rAAV can be used to silence genes in cases where gain-of-function–mediated toxicity is the underlying cause of disease progression. In the case of Huntington's disease, where the expansion of more than 39 CAG triplets in exon 1 of the huntingtin (*HTT*) gene produces a toxic gain-of-function HTT protein mutant, rAAV can deliver a microRNA precursor designed to silence this toxic mutant at the mRNA level (Miniarikova et al. 2018)
- **Gene addition** — outside of the context of monogenic disease, rAAV can be utilized to add genes that modulate progression in more complex human diseases. In the case of neurodegenerative syndromes, supplementation with rAAV-derived neurotrophic factors can slow progression and potentially improve the overall quality of life for the patient (Nam et al. 2021). Gene addition is also being investigated for cancer, heart failure, and infectious disease (Wang et al. 2019, Ishikawa et al. 2018, Santiago-Ortiz and Schaffer 2016)
- **Gene editing** — finally, rAAV can deliver gene editing machinery. This provides the opportunity to directly edit or repair pathogenic mutations. This approach is actively being investigated in lysosomal storage disorders (Wang et al. 2019)

AAV Biology: Structure and Function of AAV Vectors

Wild-type AAV belong to the genus *Dependoparvovirus* within the family *Parvoviridae*. Collectively, more than 100 naturally occurring AAV variants have been isolated to date, but only a handful have been developed for therapeutic purposes (Santiago-Ortiz and Schaffer 2016). The wild-type virion consists of an icosahedral protein capsid that is ~26 nm in diameter. The protein coat encapsulates a 4.7 kb single-stranded DNA genome that can either be the sense or antisense strand (Wang et al. 2019). The viral genome consists of three genes: *rep* (replication), *cap* (capsid), and *aap* (assembly) (Bolt et al. 2021). The viral *cap* gene consists of three subunits: VP1, VP2, and VP3 (Wang et al. 2019). The *rep* gene encodes for a total of four proteins that are required for viral genome replication and packaging (Bolt et al. 2021). These proteins, dubbed Rep78, Rep68, Rep52, and Rep40, are all named for their molecular mass. The viral genome is flanked by two ITRs that serve as the viral origin of replication. Finally, the *aap* gene encodes the assembly and nuclear localization of the cap subunit proteins.

In the context of rAAV, the overall structure of the assembled virus is the same as the wild type. However, the entire genome that contains the AAV protein-coding sequences has been replaced with the therapeutic gene expression cassette (Wang et al. 2019). This cassette needs to be under 5 kb and typically consists of a promoter, the gene sequence, and a termination sequence (Figure 2) (Pupo et al. 2022). Promoters utilized in these cassettes are typically strong enough to generate high transgene expression. Examples include cytomegalovirus (CMV) or chicken β-actin fused with the CMV enhancer. Other genetic elements can also be included to enhance or regulate gene expression. Examples include the woodchuck hepatitis posttranscriptional regulator element (WPRE) or others (Wang et al. 2019). The ITRs, which flank the therapeutic gene cassette, are the only elements that remain

from the wild-type genome (Bolt et al. 2021). The structural and replication genes (*rep* and *cap*) that were removed from the wild-type viral genome are provided in another plasmid during production of the rAAV. Finally, AAV replication and packaging require elements from a helper virus, such as adenovirus or herpes simplex virus (Wang et al. 2019). For rAAV production, these elements are provided in a helper plasmid.

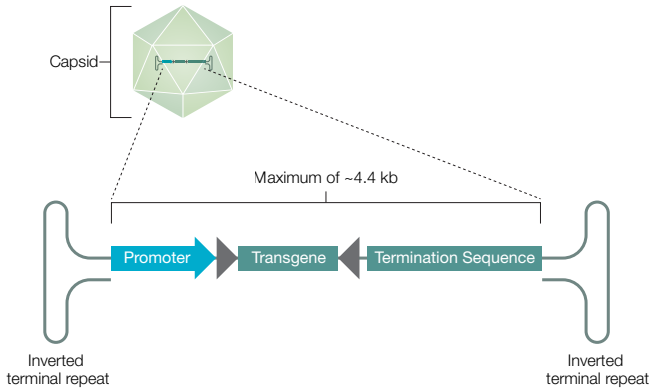

Fig. 2. General physical and genomic structure of rAAV. rAAV, recombinant adeno-associated virus.

Transduction of host cells by rAAV occurs via the AAV transduction pathway (Figure 3). The rAAV will first interact with carbohydrates on the host cell's surface. Most commonly these carbohydrates are sialic acid, galactose, and heparin sulfate (Naso et al. 2017). Different serotypes have natural tropism for different cells and tissues based on sugar-binding preferences and therefore can influence transduction preferences (Naso et al. 2017). Capsids can be further engineered to enhance selectivity based on this preferential carbohydrate binding (Pupo et al. 2022). The virion is subsequently internalized by the host and transported to the nucleus. The ITR-flanked coding sequence forms circular concatemers that exist as episomes in the nucleus of the host cell (Bolt et al. 2021).

Manufacturing and Quantification of Fully Potent AAV

Recombinant AAVs are produced using transient triple transfection of plasmids that contain the *rep* and *cap*, elements of a helper virus, and the transfer gene into adherent or suspension human embryonic kidney 293 (HEK293) cells. Although transient transfection presents a straightforward approach to AAV manufacturing, the process is somewhat inefficient and plasmid imbalances may contribute to the generation of virions that lack genomic content or contain incomplete genomes (Srivastava et al. 2021). These product-related impurities, known as empty capsids, represent a major quality issue in AAV manufacturing. Empty capsids or capsids with altered or truncated payloads can constitute from 20% to over 98% of the total rAAV particles in vector preps prior to purification (Wang et al. 2019). The presence of these empty or altered capsids affects the efficacy and safety of the AAV vector by blunting in vivo gene delivery as well as increasing the risk of immunogenicity (Srivastava et al. 2021).

Quantification of fully loaded and functional titers is crucial for AAV potency determination. Since the rAAV viral genome (VG) is thought to be the bioactive agent, most titration methods are centered on genomic quantification (Sanmiguel et al. 2019). Multiple methods have been successfully utilized, including dot hybridization, Southern blotting, ultraviolet (UV) spectrophotometry, PicoGreen based fluorimetry, and quantitative PCR (qPCR) (Dobnik et al. 2019). The most

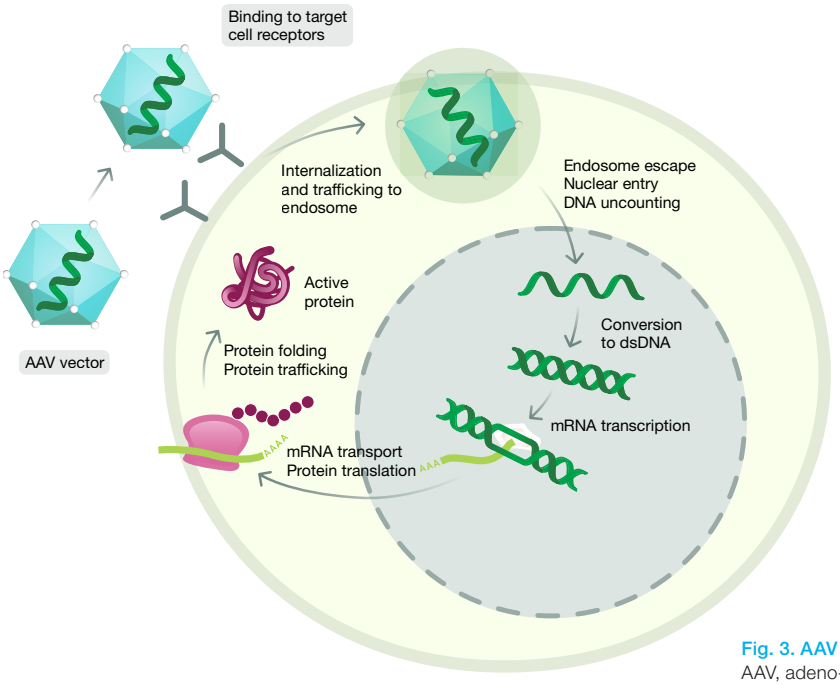

Fig. 3. AAV transduction pathway.
AAV, adeno-associated virus.

common method is qPCR because it is simple and robust under suitable and ideal conditions (Dobnik et al. 2019). However, the limitations of qPCR can introduce significant variability when determining VG titer. Primarily, qPCR requires a standard curve to be generated with a purified and well-characterized reference standard. Since only two well-characterized AAV reference standards are currently available (AAV2 and AAV8), plasmid DNA is typically utilized as a substitute (Dobnik et al. 2019; Furuta-Hanawa et al. 2019). The secondary structure of plasmid DNA has been noted to adversely affect qPCR, as DNA supercoiling suppresses annealing of the primers (Hou et al. 2010; Furuta-Hanawa et al. 2019). As a result, this attenuated primer binding will shift the regression line and cause a significant overestimation of VG titer (Hou et al. 2010). In addition, utilization of different target areas of the same rAAV genome with different primer sets can produce dramatically different VG titers, even in the presence of the same reference standard (Wang et al. 2013).

Droplet Digital PCR (ddPCR™) is a viable alternative for rAAV titering with several inherent benefits. First, the method can deliver absolute quantification of VG titer in the absence of a standard curve. The lack of suitable vector reference standards and potential issues with using plasmid DNA makes this feature of ddPCR technology extremely attractive. When using different primer sets targeting various segments of the genes, ddPCR technology produces similar titers. This indicates that ddPCR technology is unaffected by different primer sets or secondary conformation of the VG (Furuta-Hanawa et al. 2019). In addition, ddPCR technology can determine the integrity of the vector genome using a 2-D approach, where two different probes targeting different positions of the rAAV genome are utilized (Figure 4). Indeed, the titer of intact rAAV measured by 2-D Droplet Digital PCR was highly correlated with functional virus (rAAV transduction activity) in an accelerated stability study (Furuta-Hanawa et al. 2019).

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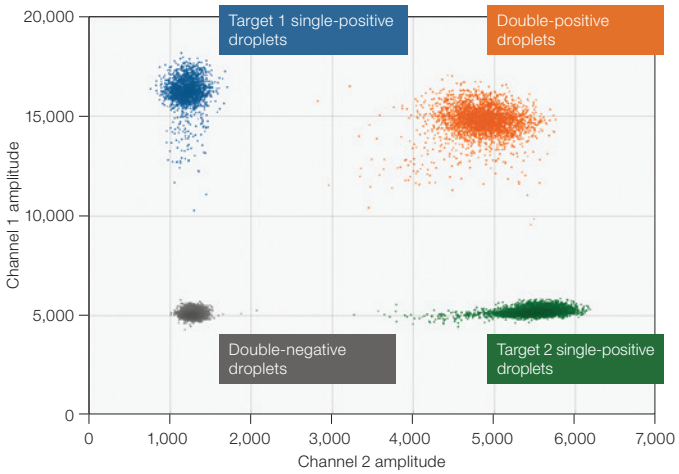

Fig. 4. Example 2-D amplitude plot. Two targets can be quantified and visualized simultaneously using 2-D Droplet Digital PCR.

Summary and Conclusions

The accidental discovery of AAV during the 1960s was the start of a new era in modern medicine. AAV is now the leading vector for in vivo gene therapy. Recent approvals of rAAV-based therapeutics have only bolstered confidence and have widened the scope of clinical rAAV utilization. Currently, the primary rAAV manufacturing method is transient transfection of HEK293 cells. Though this method represents an extremely rapid option for manufacturing, transient transfection can be inconsistent and as a result can lead to variability in rAAV quality and potency during the manufacturing process. One byproduct of this transfection heterogeneity is empty capsids. Empty capsids or capsids containing truncated genomes can blunt rAAV-mediated transduction and can also cause additional immunogenicity.

Quantification of complete VG concentration is crucial in all aspects of rAAV development. Currently the gold standard for VG quantification is qPCR, which is robust and straightforward in ideal conditions. However, the availability of a known and well-characterized reference standard is paramount. Unfortunately, plasmid DNA is typically used as a reference standard since these well-characterized reference standards are extremely limited. VG quantification using plasmid DNA can be problematic, producing variable results if primers bind promiscuously to reference standards. Because of the benefit of absolute quantification in the absence of standard curves, ddPCR technology is an attractive alternative for VG titering. Further, 2-D Droplet Digital PCR can determine vector integrity through multiplexing of different probe sets targeting different areas of the VG. With accurate assessment of VG titer using ddPCR technology, rAAV-based therapeutics can deliver on their promise in the treatment of genetic diseases.

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