Recombinant adeno-associated virus vectors: from virus to therapeutic vector

Gene therapies for hemophilia are currently being studied to determine their safety and efficacy. Approved gene therapies for hemophilia may have different labeling in different countries.

Pfizer

Contents

> Synopsis

Several characteristics of AAV make it amenable as a platform to produce recombinant vectors for gene therapy, including lack of pathogenicity and broad tissue specificity.[1] rAAV is the most commonly used vector for hemophilia gene therapies currently under investigation.[2] Approaches such as rational design and computer-guided design are currently being studied to potentially overcome limitations of the rAAV vector platform, including enhancing tissue tropism, optimizing transduction efficiency, and assisting with immune evasion.[3] Hemophilia gene therapy trials use different capsids.

The information included in this brochure is accurate as of August 2023. Check back regularly for further updates.

Vectors in gene therapy

Gene therapy is the introduction, removal or change in genetic material – specifically DNA or RNA – into the cells of a patient to treat a specific disease.[4]

> Hemophilia A and B gene therapies aim to deliver a functional copy of the *F8* or *F9* gene, respectively, to hepatocyte cells[5]

The functional gene must be packaged into a capsid, allowing for the delivery of the gene to the nucleus of the cell, where the cell can then work to express the therapeutic protein[5]

Therefore, the success of gene therapy is dependent on the development of effective vectors for gene delivery[5]

There are two main groups of gene therapy vectors under investigation:[1,6,7]

> **Non-viral vectors** – the application of non-viral vectors has been very limited to date

> **Viral-derived vectors**

Common viral-derived vector groups currently under investigation for gene therapy are: gammaretrovirus and lentivirus, which are both classified as belonging to the retrovirus group, adenovirus, and recombinant adeno-associated virus (rAAV).[1,3]

Common viral-derived vector groups under investigation

Image	Vector	Genetic material	Packaging capacity	Tropism	Vector genome forms	Select limitations	Select advantages
	**Gamma–retrovirus**[1,8,9]	RNA[1,9]	8 kb[1]	Dividing cells only[1]	Integrated[9]	Only transduces dividing cells, and integration into the host genome occurs at random sites[1]	Persistent gene transfer in dividing cells[1]
	**Lentivirus**[1,9]	RNA[1,9]	8–10 kb[1,9]	Broad (dividing and non-dividing cells)[1]	Integrated[1,9]	Random integration may cause potential complications[1]	Persistent gene transfer in most tissues[1]
	**Adenovirus**[1,9]	Double-stranded DNA[1,9]	Replication defective: 8 kb[1] Helper-dependent: 30–36 kb[1,9]	Broad (dividing and non-dividing cells)[1]	Mainly episomal[1,9]	Capsid mediates a potent inflammatory response[1,9]	Extremely efficient transduction of most tissues[1]
	**Recombinant adeno-associated virus**[1,9]	Single-stranded DNA[1,9]	<5 kb[1,9]	Broad (dividing and non-dividing cells)[1]	Predominantly episomal[1,9] Random integration events observed with a low frequency (0.1–1% of transduction events)[10]	Small packaging capacity[1]	Non-inflammatory; non-pathogenic[1,9]

Several characteristics of AAV make it amenable as a platform to produce recombinant vectors for the *in vivo* delivery of gene therapies:[1]

- Multiple serotypes permit broad tissue specificity[11*]
- Ability to establish potentially long-term transgene expression[2]
- Lack of pathogenicity[1,9,11]
- Predominately non-integrating[1,11]
- Defective replication[2]

*For hemophilia gene therapy the target is the liver.

Table developed from Thomas CE, et al. 2003,[1] Maetzig T, et al. 2011[8] and Colella P, et al. 2018.[10]

rAAV vectors are versatile vectors that can be engineered for specific functionality in gene therapy applications,[12] and are currently the most commonly used vector type for hemophilia gene therapy[2,13,14]

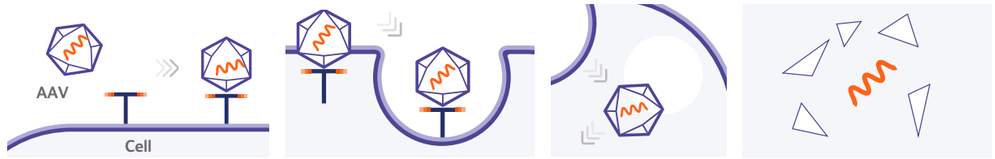
AAV biology

What is naturally occurring (wild-type) AAV?

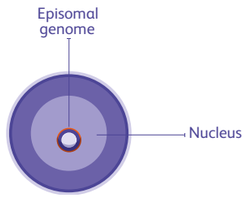
> Wild-type (WT) AAV is a single-stranded DNA parvovirus[3,12] belonging to the genus *Dependovirus,* the members of which require a helper virus to facilitate productive infection and replication[12,15]

> WT AAV comprises a protein shell (capsid) that surrounds and protects single-stranded DNA of approximately 4.8 kb[12]

> At the cellular level, AAV must undergo some major steps prior to achieving gene expression and the capsid is critical for the first:[16]



1. Binding or attachment to cellular surface proteins
2. Endocytosis
3. Trafficking to the nucleus and endosomal escape
4. Uncoating of the virus to release the genome



5. Conversion of the genome from single-stranded DNA (ssDNA) to double-stranded DNA for transcription in the nucleus occurs. The transgene is mostly maintained episomally as a concatemer of DNA (a DNA molecule made up of multiple copies of the same genome linked together in tandem) and is predominantly non-integrating

Figures developed from Naso, MF et al. 2017,[12] Colella P, et al. 2018,[10] Wu Z, et al. 2006[15] and Choi VW, et al. 2006.[16]

WT AAV genome

> The genome of WT AAV comprises two large open reading frames (ORFs) flanked by 145-base-pair inverted terminal repeats (ITRs)[3,15]

WT AAV protein shell (capsid)

> The capsid is responsible for cellular receptor binding and is the first element that the target cell receptor encounters.[16] It is composed of the virion structural proteins (VPs), VP1, VP2 and VP3.[3,12,16] Transcription of all VPs is controlled by the p40 promoter.[17] These proteins interact to form 60 protein subunits that assemble into icosahedral virion shells[3,12,15]

– Each subunit is composed at a 1:1:10 ratio of VP1:VP2:VP3[12]

– Each of the 60 subunits of the virion shell has variable regions, which have roles in receptor attachment, tissue tropism and transduction, and antigenicity[3]

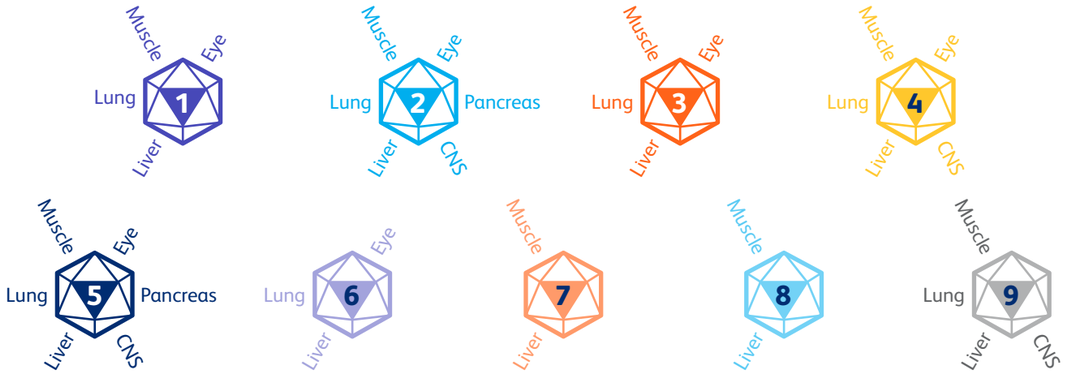
– rAAV vectors bearing differences in the virion structural proteins have different transduction abilities (i.e. resulting in varying cell tropism and kinetics of transgene expression)[3,8]

> Different AAV serotypes have different binding abilities and, in turn, varying tissue tropism[3,16]

– To date, at least 12 natural AAV serotypes have been isolated with unique tissue tropism[3]

– Most of these serotypes are currently used in clinical studies for various diseases[3]

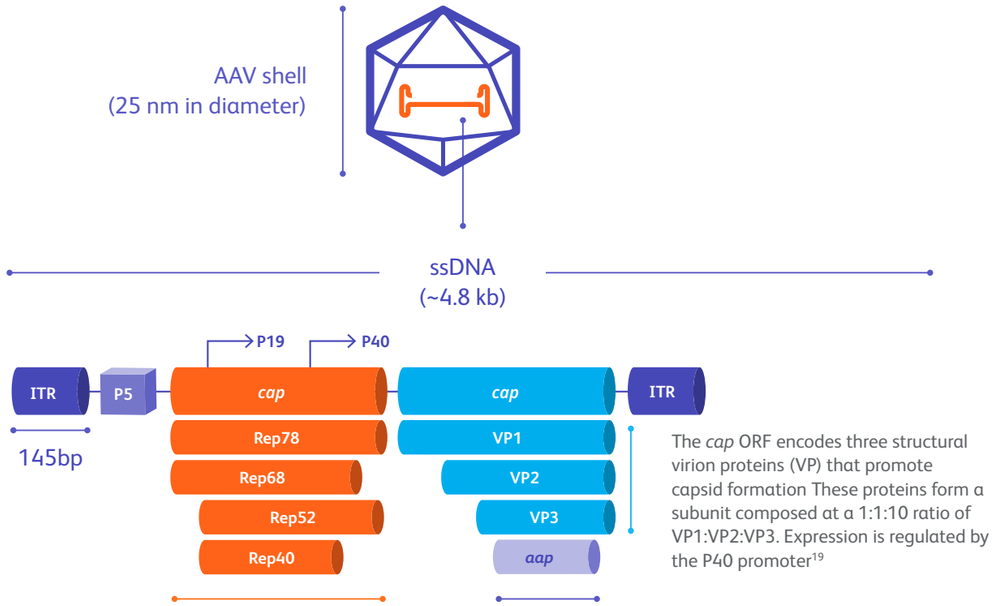
Examples of AAV serotype vector tropism



CNS: Central nervous system.
Image developed from Wu Z, et al. 2006[15] and Grimm D, Kay MA. 2003.[18]

- **Vectors are based on viral platforms, but are <u>not</u> viruses[1]**
- **In the rAAV vector, the WT AAV *rep* and *cap* genes have been replaced with the transgene of interest[2]**

Key components of wild-type AAV



The *cap* ORF encodes three structural virion proteins (VP) that promote capsid formation These proteins form a subunit composed at a 1:1:10 ratio of VP1:VP2:VP3. Expression is regulated by the P40 promoter[19]

The *rep* ORF encodes a family of non-structural replication proteins (Rep) required for viral transcription control and replication, as well as packaging of viral genomes into newly produced, pre-assembled capsids.[19] Expression is controlled by the P5 and P19 promoters[19]

The assembly-activating protein (AAP) is encoded by an alternate reading frame of the *cap* gene. It is required for stabilizing and transporting newly produced VP from the cytoplasm into the cell nucleus[3,19]

AAV: Adeno-associated virus; ITR: Inverted terminal repeat; ORF: Open reading frame; ssDNA: Single-stranded DNA.
Figure developed from: Ayuso E, et al. 2010,[17] Li C, Samulski RJ. 2020,[3] Büning H, Srivastava A. 2019,[19] Naso MF, et al. 2017[12] and Pfeifer A, Verma IM. 2001.[21]

rAAV composition

An overview of rAAV

- The rAAV vector, which lacks viral DNA, is a protein-based nanoparticle that has been engineered to transverse the cell membrane where it can traffic and deliver its DNA cargo into the target cell nucleus[12]
  - Both the *rep* and *cap* genes have been removed and replaced with the gene of interest in the rAAV vector,[12] resulting in its inability to replicate within the host cells[2]
  - Substitution of the *rep* and *cap* ORFs with an expression cassette containing a promoter (e.g. a liver-specific promoter), a therapeutic gene (e.g. *F8* or *F9*, which encode FVIII or FIX) and a transcription terminator polyadenylation signal (PolyA) forms the basis of all rAAV vectors[3,10]
  - In the absence of the *rep* gene, the ITR-flanked transgenes can form circular episomal DNA in the nucleus (i.e. rAAV vectors may not have the ability to integrate site-specifically into a chromosome)[12,20]
- Notably, only the 145-bp inverted terminal repeats (ITRs) from WT AAV, which induce transgene expression and play essential roles in vector production and cell transduction, are necessary for the rAAV vector[3]
  - ≈96% of the genome can be removed to permit engineering of the rAAV vector for gene therapy[3]

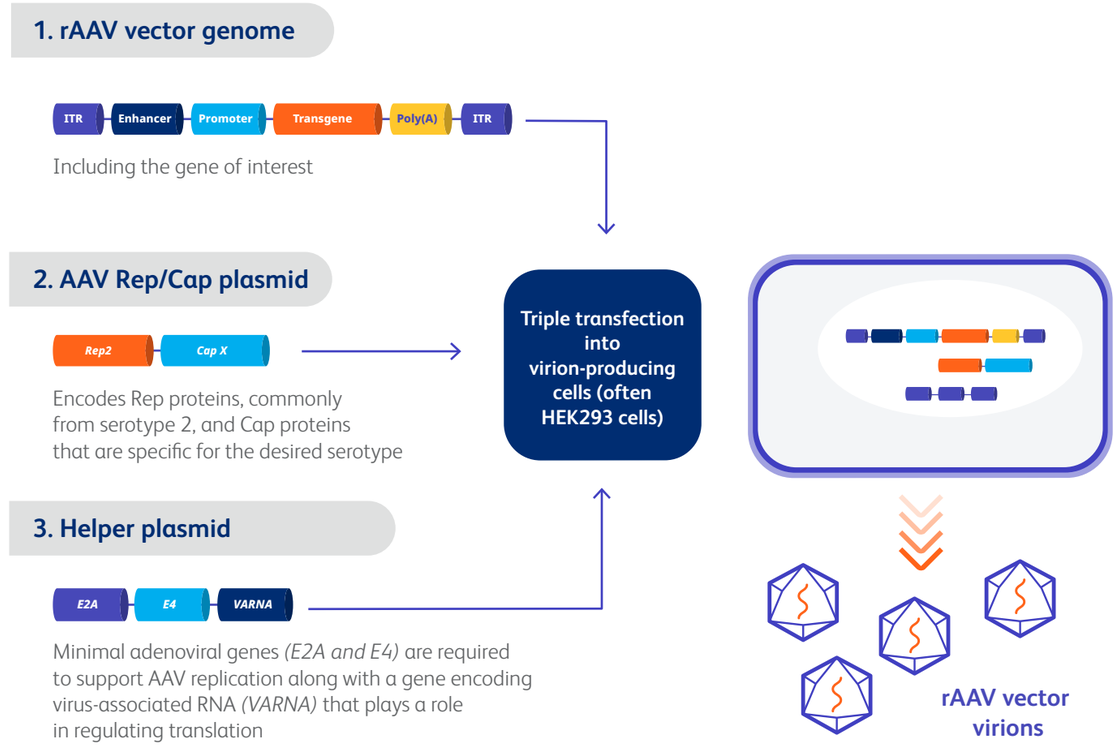
AAV: Adeno-associated virus; ITR: Inverted terminal repeat; P: Promoter; Poly(A): Polyadenylation A tail; rAAV: Recombinant AAV; ssDNA: Single-stranded DNA.
Adapted from Pfeifer A, Verma IM. 2001[21] and Naso MF, et al. 2017.[12]

Our knowledge and understanding of virus dynamics and function can facilitate the rational engineering of virus particles to develop recombinant gene therapy vectors[22]

Production of rAAV vectors for gene therapy

- rAAV vectors are commonly produced using transient transfection protocols, where a recombinant plasmid containing gene(s) of interest subcloned within the ITRs of WT AAV is co-transfected with a recombinant helper plasmid containing the WT AAV and adenoviral genes necessary for the rescue, replication, and packaging of rAAV in an appropriate packaging cell line[17,23]
- This triple transfection protocol is the most common approach, although other protocols are also used:[24]
  - Double infection with herpesvirus into mammalian cells[24]
  - Double infection with baculovirus into insect cells[24]

Triple transfection protocol to produce an rAAV vector



AAV: Adeno-associated virus; ITR: Inverted terminal repeat; Poly(A): Polyadenylation A tail; rAAV: Recombinant AAV.
Figure adapted from Ayuso E, et al. 2010[17] and Aponte-Ubills JJ, et al. 2018.[24]

Considerations when developing rAAV capsids

When developing rAAV capsids for gene transfer, there are several aspects to consider, including transduction efficiency, seropositivity and immune responses

Parameters that may impact transduction efficiency and the delivery of rAAV vector gene therapy

Tissue tropism and cell transduction

- AAV serotypes exhibit preferential tropism for different tissue types[18]
- Transduction efficacy of different tissues / cells varies between serotypes[18]
- For example, AAV4 exhibits tropism for various tissues (muscle, central nervous system [CNS], liver, lung and eye), with preferential tropism for the CNS and muscle[18]

Seropositivity

- WT AAV, in the absence of a helper virus, does not itself cause human infection; however, natural exposure is high in the general population (asymptomatic infection), and a large proportion of the human population have neutralizing antibodies against various AAV serotypes[25–27]
- Individuals often present with neutralizing antibodies against several different serotypes due to homology in amino acid sequence and the polyclonal response in humans[28]
- This pre-existing immune response may prevent transduction by binding to the capsid before transducing cells[27]

Immune responses

- Components of the rAAV capsid (and possibly the transgene) are detectable on the surface of transfected cells and may be seen as ‘foreign’ to the immune system
- Transduced cells present capsid antigen on their surface by major histocompatibility complex (MHC) class I and can be targeted by capsid-specific CD8+ T cells eliciting an immune response leading to their destruction[29]
- Response can be long-lasting and may preclude further treatment with the same vector[18,30,31]

Bioengineering the rAAV capsid can enhance tissue tropism, improve transduction efficiency, and allow evasion of the immune response[3]

Engineering the rAAV capsid

The following approaches for engineering gene therapy capsids may be applicable to other gene therapies and are not specific to hemophilia gene therapies

To optimize rAAV vector gene transfer and overcome the limitations of this platform, the capsid can be modified to redirect or expand tropism, enhance transduction and evade the host immune response.[19] Four main approaches are currently used to engineer and optimize the rAAV capsid:[32]

- **Rational design**
- **Directed evolution**
- **Computer-guided design**
- **Natural discovery**

Rational design

How does rational design work?

Rational design is the engineering of the rAAV capsid based on current scientific knowledge, including the sequence, structure and function of AAV, to further modulate, enhance and optimize the performance of rAAV vectors to achieve the desired function.[26]

Through this approach, rAAV vectors can be engineered to enhance tropism and evade neutralizing antibodies and the cytotoxic T-cell response.[3]

Rational design methods may include receptor engraftment, peptide insertion, peptide loop swap and point mutations.[3]

Example of rational design

Tyrosine residues on the capsid surface are susceptible to ubiquitylation tagging them for proteasome-mediated capsid degradation.[33] Ubiquitylation is one of the key cellular regulatory steps that guides protein degradation through regulation of proteasome activity.[34] Engineering of capsid proteins to delete the tyrosine residues enables evasion of a capsid-specific cytotoxic T-cell response potentially increasing transduction efficiency.[3]

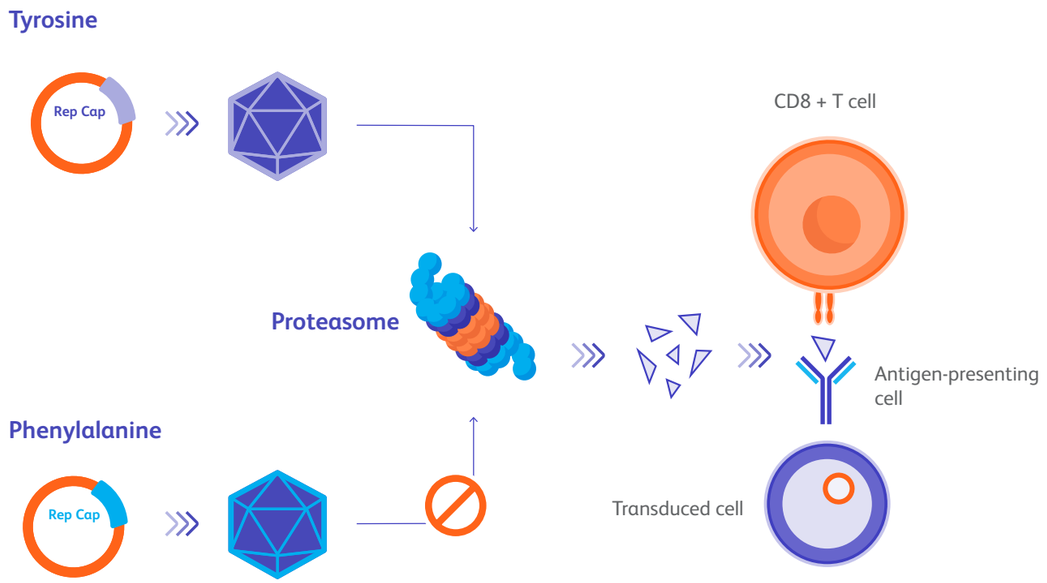


Figure developed from Wang D, et al. 2019[26] and Rabinowitz J et al. 2019.[35]

Creating rAAV vector hybrid serotypes through rational design

- Structural analysis of natural AAV serotypes suggests that their capsid topology differs, which may account for their unique tropism[16,19]
- Understanding the tropism of each serotype helps us to understand the modifications that can be applied to enhance the efficiency of gene transfer[16,19]
- By swapping domains from one serotype capsid with another, it is possible to create **hybrid vectors** with desirable qualities suited to the overall aim of the gene therapy[19]

rAAV hybrid vector serotypes can be created in the following ways:

Transcapsidation[16]

- Also known as pseudotyping, this approach involves packaging an AAV genome containing an ITR from one serotype into the capsid of another serotype. AAV2 ITR is well-understood and is commonly cross packaged into different serotype capsids to better target cell types that do not express the receptors that AAV2 utilizes

Absorption modification[16]

- This method is used to alter capsid tropism by using an AAV-specific antibody that is chemically linked to another antibody that binds specifically to a cellular receptor known to be expressed on the targeted cell type

Mosaic capsid[16]

- A mosaic capsid AAV is a single virion that is composed of a mixture of viral unmodified capsid proteins from different serotypes

Chimeric capsid[16]

- A chimeric capsid AAV involves the insertion of a foreign protein sequence, either from another WT AAV or an unrelated protein into the open reading frame of the capsid gene

Directed evolution

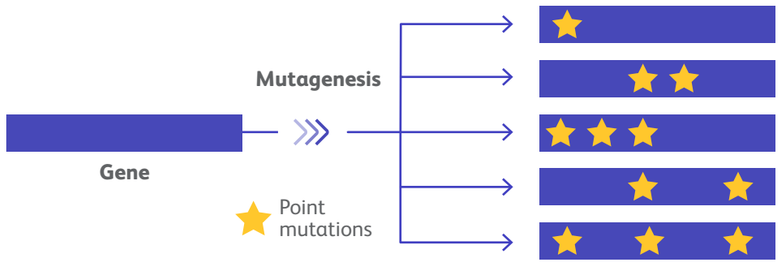
How does directed evolution work?

Emulates the process of natural evolution, in which repeated genetic diversification and selection enable the accumulation of key mutations or genetic modifications that progressively improve a molecule's function, even without knowledge of the underlying mechanistic basis for the problem.[26,36]

Directed evolution is a high-throughput molecular engineering approach that can be used to generate highly diverse mutant rAAV vectors, with the potential ability to evade the immune response or with enhanced transduction.[3]

Directed evolution approaches include error-prone polymerase chain reaction (PCR) and gene shuffling.[3,26,36]

Error-prone PCR



Error-prone PCR can be used to generate rAAV vector variants that may potentially evade neutralizing antibodies[36]

Gene shuffling

Gene shuffling has led to the creation of diverse rAAV libraries from which improved capsids can be selected.[26]

PCR: Polymerase chain reaction; rAAV: Recombinant adeno-associated virus. Figure developed from Wang D, et al. 2019.[26]

Computer-guided design

How does computer-guided design work?

Using computational design for machine-guided engineering of rAAV capsids not seen in nature can provide a more efficient approach to previously explored engineering techniques.

New capsid variants with enhanced transduction can be generated using computational design, which uses knowledge of DNA sequences and phylogenic analysis between AAV serotypes to construct a potential ancestral AAV capsid library.[3,26] Phylogenetics refers a systematic approach to reconstructing the past evolutionary history of species, based on present day data.[37]

Such capsid libraries can be used to generate alternative rAAV capsid variants with enhanced transduction.[3,26]

Example of computer-guided design

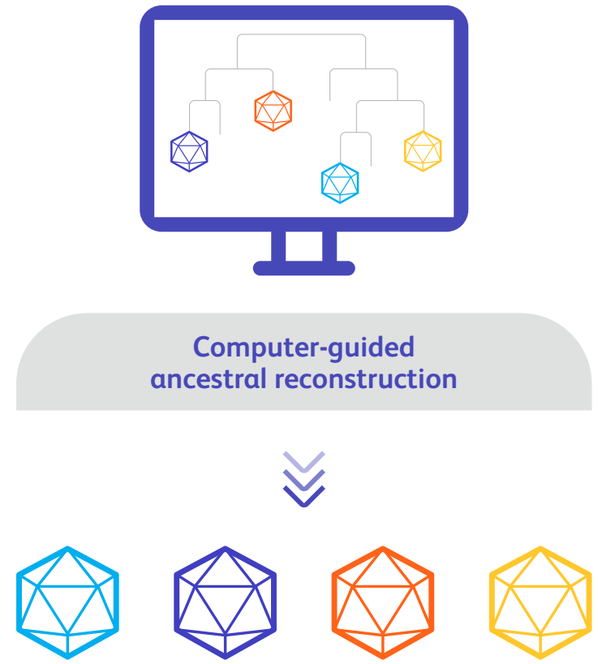


Figure developed from Wang D, et al. 2019.[26]

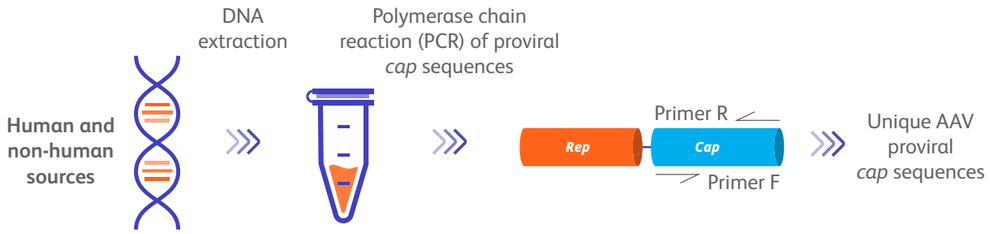
Anc80, the predicted ancestor of the widely studied AAV serotypes 1, 2, 8 and 9, was designed using computer-guided engineering.[3,26,38] This is an *in vivo* gene therapy vector for targeting the liver, muscle, and retina in adult mice and non-human primates.[3]

Natural discovery

How does natural discovery work?

AAV was originally discovered as a cell culture contaminant.[26] To date, the most clinically promising vectorized serotypes have been isolated from natural sources, including AAV2, which is the most used serotype.[26]

The aim of the natural discovery approach is to discover naturally occurring AAV through surveying proviral sequences present in a host tissue that may have been infected with WT AAVs.[26]



AAV: Adeno-associated virus.
Figure developed from Wang D, et al. 2019.[26]

Due to the high prevalence of AAV antibodies against various serotypes in the general population, isolation of novel capsids from non-human sources may offer the potential to overcome pre-existing immunity.[26]

Example of natural discovery

AAV9 was isolated from human liver tissue and was demonstrated to bypass the blood–brain barrier, providing an option for gene therapies that can transduce the CNS.[26] AAV9 is the most widely studied capsid for CNS gene therapy applications.[39]

Key take-home messages

- A necessary component of gene therapy depends on effective vehicles for gene transfer, termed 'vectors'[5]
- The main viral vector groups currently under investigation for gene therapy are gammaretrovirus and lentivirus, which are both classed as belonging to the retrovirus group, adenovirus, and rAAV[1]
- Several characteristics of AAV make it amenable as a platform to produce recombinant vectors for gene therapy including defective replication and its lack of pathogenicity – AAV is one of the most commonly used vectors for hemophilia gene therapy[1]
- When constructing a rAAV vector the following considerations are important:[3]
  - Target tissue
  - Transduction efficiency
  - Immune evasion
- Our knowledge and understanding of virus dynamics and function can facilitate the engineering of virus particles to develop rAAV vectors with specific functionality for gene therapy applications[22]

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