Chapter 2
The Role of the Adeno-Associated Virus Capsid in Gene Transfer

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Abstract Adeno-associated virus (AAV) is one of the most promising viral gene transfer vectors that has been shown to effect long-term gene expression and disease correction with low toxicity in animal models, and is well tolerated in human clinical trials. The surface of the AAV capsid is an essential component that is involved in cell binding, internalization, and trafficking within the targeted cell. Prior to developing a gene therapy strategy that utilizes AAV, the serotype should be carefully considered since each capsid exhibits a unique tissue tropism and transduction efficiency. Several approaches have been undertaken in an effort to target AAV vectors to specific cell types, including utilizing natural serotypes that target a desired cellular receptor, producing pseudotyped vectors, and engineering chimeric and mosaic AAV capsids. These capsid modifications are being incorporated into vector production and purification methods that provide for the ability to scale-up the manufacturing process to support human clinical trials. Protocols for small-scale and large-scale production of AAV, as well as assays to characterize the final vector product, are presented here.

The structures of AAV2, AAV4, and AAV5 have been solved by X-ray crystallography or cryo-electron microscopy (cryo-EM), and provide a basis for rational vector design in developing customized capsids for specific targeting of AAV vectors. The capsid of AAV has been shown to be remarkably stable, which is a desirable characteristic for a gene therapy vector; however, recently it has been shown that the AAV serotypes exhibit differential susceptibility to proteases. The capsid fragmentation pattern when exposed to various proteases, as well as the susceptibility of the serotypes to a series of proteases, provides a unique fingerprint for each serotype that can be used for capsid identity validation. In addition to serotype identification, protease susceptibility can also be utilized to study dynamic structural changes that must occur for the AAV capsid to perform its various functions during the virus life cycle. The use of proteases for structural studies in solution complements the crystal structural studies of the virus. A generic protocol based on proteolysis for AAV serotype identification is provided here.

Keywords AAV; capsid; Proteolysis; capsid structure; Serotype; Vector production; Column chromatography; Gene therapy
1 Introduction

Adeno-associated virus (AAV) is a single-stranded DNA virus that is currently being utilized for gene therapy applications. AAV is a member of the family Parvoviridae, genus Dependovirus. Several features of AAVs that make them promising candidates as gene transfer vectors are as follows: (1) Good safety profile: the members of this genus are not associated with disease in humans. The virus is replication-deficient, and, except under special circumstances, it will not replicate or spread [1], and AAV has been well tolerated in human clinical trials. (2) Stable long-term expression of the transgene: AAV has successfully been used to express transgenes in several tissues, including brain, muscle, liver, lung, vascular endothelium, and hematopoietic cells [2–9]. (3) Ability to transduce dividing and nondividing cells [10]. (4) Episomal maintenance: wild-type AAV exhibits site-specific integration of its genome into chromosome 19; however, the majority of vector genomes appear to be maintained episomally [11–13]. Therefore, the risk of integration is minimal, compared with retroviral vectors that require integration into the host genome and have the potential to activate proto-oncogenes. (5) Low immunogenicity: unlike adenoviral vectors, a robust T cell response to the vector is not generated by AAV [14], although humoral immune responses are generated, which may result in viral neutralization [15]. The use of other AAV serotypes may circumvent this response and allow for repeated treatments [16]. Alternatively, immunosuppressive therapy may be used during treatment with AAV to avoid the immune response [17]. (6) Physicochemical stability: AAV virions are highly stable over a wide range of pH and temperature, a feature that is important for production and purification methods for clinical-grade AAV vectors, as well as for stability of the final vector product [18].

The genome of AAV is ~4,700 bases of linear, single-stranded DNA. There are two genes, rep and cap, which are flanked by the inverted terminal repeats (ITRs). The ITRs consist of 145 nucleotides, which form a characteristic T-shaped hairpin. These are the only sequences required in cis for viral DNA replication and packaging. The rep gene encodes four nonstructural proteins, Rep78, Rep68, Rep52, and Rep40, which play a role in viral genome replication and transcription, as well as packaging. Rep78 and Rep68 are translated from mRNAs transcribed from the p5 promoter, while Rep52 and Rep 40 are derived from mRNAs transcribed from the p19 promoter. Alternative splicing replaces a 92 amino acid C-terminal element in Rep78 and Rep52 with a 9 amino acid element in Rep68 and Rep40 [19]. The cap gene encodes the three structural proteins of the AAV capsid, VP1 (87kDa), VP2 (72kDa), and VP3 (63kDa), translated from mRNA transcribed from the p40 promoter. Differential splicing yields major and minor spliced products. VP1 is translated from the minor spliced mRNA, yielding less VP1 protein. VP2 and VP3 are both translated from the more abundant major spliced mRNA; however, VP2 is translated less efficiently because it initiates at an ACG codon, while VP3 is translated very efficiently because of a favorable Kozak context [20]. As a result, the AAV capsid proteins, which differ only in their N-terminal region, are present in the mature virion in a ratio of 1:1:10 (VP1:VP2:VP3). The single-stranded DNA genome of AAV and its products are depicted in Fig. 2.1.
2 The Role of the Adeno-Associated Virus Capsid in Gene Transfer

2.1 Capsid Assembly and Packaging

In 1980, the work of Myers and Carter provided evidence that the structural proteins for AAV assemble into empty capsids, and then the genome is packaged into these preformed capsids [21]. Pulse-chase experiments showed that empty particles rapidly accumulate (10–20 min), but that mature “full” virions accumulated more slowly (4–8 h). They also showed that the number of empty viral particles decreases at the same rate as the number of DNA-containing mature virions increases during the course of infection. Additionally, de la Maza and Carter showed that DI particles, particles with deletions in the AAV genome, are packaged into apparently normal capsids, indicating that full-length viral genomic DNA is not required for the assembly or structural integrity of the AAV capsid [22]. In the absence of capsid assembly, ssDNA does not accumulate, further suggesting that empty capsids form first. Interactions between the preformed AAV capsid and Rep52 provides a mechanism where the nonstructural protein’s helicase activity inserts the viral DNA [23]. Within the cell, capsid assembly occurs at centers within the nucleus where there is colocalization of Rep proteins, capsid proteins, and DNA [24]. Empty AAV capsids
can be produced by expressing the AAV cap gene in insect cells using a baculovirus expression system [25] or in mammalian cells utilizing a recombinant adenovirus expressing the AAV capsid proteins. These systems have advanced the structural studies of the AAV capsid as a result of the large amount of empty capsids or virus-like particles (VLPs) that can be produced. Studies of AAV assembly have demonstrated that VP3 alone is sufficient to form VLPs [26], but VP1 is required for infectivity [27]. Subsequently, it was shown that the unique N-terminus of VP1 has phospholipase A2 activity and contains a nuclear localization signal (NLS) [28]. The N-terminus of VP2 also has a NLS and may play a role in transporting VP3 into the nucleus; however, it has been shown that the N-terminus of VP2 is nonessential and that infectious virus can be produced that lack VP2 entirely [29]. Additionally, the N-terminus of VP2 has been replaced with green fluorescent protein and these capsids still assemble and maintain infectivity. This demonstrates that VP2 can tolerate peptide insertions and may be useful for incorporating peptides into the capsid for cell-specific targeting of AAV.

2.2 Capsid Structure

The adeno-associated virus capsid is ~25 nm in diameter and is composed of 60 subunits arranged in T = 1 icosahedral symmetry. Because AAV has a number of features that make it attractive as a gene transfer vector, many studies have focused on the basic biology of the virus, including studies that address the structural characteristics. Cryo-EM or crystal structures for AAV2, AAV4, and AAV5 have been determined [30–34], and the crystal structure for AAV8 is currently in progress [35]. Dependoviruses share the same subunit fold as the other members of the family Paroviridae, including the insect densoviruses, and the autonomously replicating paroviruses such as canine parovirus (CPV) and minute virus of mice (MVM), even though AAV shares low capsid primary sequence identity (7–22%) [36–38]. The monomeric subunit of AAV has a conserved β-barrel core that is common in viral capsid proteins. Figure 2.2 depicts the structure of a monomeric subunit of AAV2 as determined by Xie et al. [31].

The motif is an eight-stranded antiparallel β-barrel motif (jelly-roll β-barrel), with the β strands labeled B-I [36]. The β-strand labeled A is present in some paroviruses, including AAV. Not surprisingly, genetic capsid mutants of AAV2 in the conserved core β-barrel, such as mut19 (βA mutation), mut20 (βB mutation), mut25 (βD mutation), and mut46 (βI mutation), are unable to assemble into capsids [40]. AAV has long loop insertions between the strands of the core β-barrel that are labeled according to the β strands that they flank. These long interstrand loops contain β ribbons and elements of secondary structure that form much of the outer surface features of the AAV capsid. The GH loop is the longest interstrand loop, and three VPs interact extensively at each three-fold axis of symmetry, forming a prominent spike. Five DE loops each form an antiparallel β ribbon at the five-fold axis of symmetry that results in a cylindrical structure surrounding a canyon-like
depression. At the two-fold axis of symmetry there is a small depression, often referred to as the two-fold dimple [41, 42]. Analysis of newly discovered AAV genotypes identified a total of 12 hypervariable regions on the AAV capsid [43]. Overlaying these regions onto the X-ray crystallographic model of AAV2 showed that these regions are exposed on the capsid surface. Most of the variability is located between the G and H β strands, which are implicated in the formation of the valley and the peaks of the protruding three-fold axis of symmetry. These surface features of the virus are responsible for the interactions of the capsid with cellular receptors, as well as antibodies.

2.3 Cellular Receptors

Motifs on the capsid surface are critical for attachment to the host cell, which is the first step required for infection. Different serotypes of AAV utilize unique cellular receptors. The primary receptor for AAV2 is heparin sulfate proteoglycan (HSPG). After binding the cell surface, AAV2 can utilize secondary receptors, such as αVβ5 integrin, human fibroblast growth factor receptor-1 (FGFR-1), or hepatocyte growth factor (c-met), which mediate entry [44–47]. Recently, it was demonstrated that AAV2 utilizes α5β1 as an alternative co-receptor in HEK293 cells which lack αVβ5 integrin. The integrin recognition sequence, NGR, is at amino acid 511–513 within VP3, which, in assembled capsids, is located at the three-fold axis of symmetry,
adjacent to R585 and R588 of AAV2, which have previously been implicated in heparin binding [48]. The NGR motif is conserved among AAV serotypes 1–11, with the exception of AAV4, AAV5, and AAV11. Similar to AAV2, AAV3H has also been reported to bind heparin, heparan sulfate, as well as FGFR-1 [49]. The receptor for AAV5 has been shown to be PDGFR [50], and AAV5 binds to $\alpha$-2-3-$N$-linked sialic acids [51], while AAV4 binds to $\alpha$2-3-$O$-linked sialic acids [52]. AAV1 and AAV6 utilize $\alpha$-2-3-$N$-linked sialic acids, as well as $\alpha$-2-6-$N$-linked sialic acids; however, unlike AAV4, they are unable to utilize $O$-linked sialic acids [53]. Domains on the surface of the AAV capsid interact with the cellular receptors found on specific cells, resulting in the range of tissue tropism seen for the various AAV serotypes.

2.4 Serotypes

There are 11 known serotypes of AAV with different cellular targets and antigenic properties. Recently, about 100 genomic variants of these primary AAV serotypes have been discovered [54–56]. These new variants may provide expanded tropism and unique cellular targets for AAV-mediated gene delivery. Utilizing the natural differences in tropism of the AAV serotypes provides one strategy to efficiently deliver AAV vectors to specific target tissues, and selecting the appropriate capsid serotype for the target tissue is an important consideration. The rAAV genome can be packaged into capsids of its own serotype, “isotype,” or alternatively the rAAV genome can be “cross-packaged” into capsids derived from another serotype, a process called pseudotyping [57–59]. For pseudotyped vectors, the capsid gene of each serotype can be cloned with AAV2 rep, and the transgene is flanked by ITRs of AAV2. The resulting vector has a capsid of some serotype other than AAV2 and the packaged transgene flanked by the ITRs of AAV2. This provides a method to compare the transduction efficiency of various serotypes and the ability to choose the most efficient one for the specific application [58]. Table 2.1 lists several target tissues and the comparative transduction efficiency of selected AAV serotypes studied in animal models. Efficient transduction could involve cell entry associated with receptor binding and internalization, or post-entry events such as cellular processing pathways, intracellular transport, nuclear entry, or processing of virions and vector genomes. Thomas et al. showed that AAV6 and AAV8 vectors uncoat faster and, as a result, transgene expression is seen sooner than AAV2 [90, 91].

Repeated vector delivery might be needed in order to increase transgene expression or to deliver two genes in a coordinated fashion. For these applications, the same transgene can be packaged into capsids from different AAV serotypes for readministration and to circumvent an antibody neutralization response [92]. Differences in the amino acid composition of the viral capsid present unique epitopes to the host’s immune system. Studies with rAAV2 and rAAV8 vectors
Table 2.1 Transduction efficiencies of AAV serotypes in different tissues

| Tissue type          | Serotype                                                                 | Reference |
|----------------------|--------------------------------------------------------------------------|-----------|
| Muscle               | AAV1 > AAV2                                                              | [60]      |
|                      | AAV1-AAV6-AAV7 > AAV5 > AAV3 > AAV2 > AAV4                              | [58]      |
|                      | AAV6 > AAV5 or AAV2                                                     | [61]      |
|                      | AAV5 > AAV2                                                             | [62]      |
|                      | AAV7                                                                     | [63]      |
|                      | AAV8 highest systemic; AAV1 and AAV6 highest local injection             | [64]      |
|                      | Transducing remote sites when injected locally:                          |           |
|                      | AAV7 = AAV8 > AAV1, AAV5 and AAV6. AAV8 > AAV2; AAV1 > AAV2 and AAV8   |           |
| Liver (hepatocytes)  | AAV8 > AAV7 > AAV5 > AAV2; AAV1 > AAV5 > AAV3 > AAV2 > AAV4              | [58]      |
|                      | AAV5 > AAV2                                                             | [65]      |
|                      | AAV1 > AAV8 > AAV6 > AAV2                                              | [66]      |
|                      | AAV8                                                                     | [63]      |
|                      | AAV9 = AAV8                                                             | [67]      |
|                      | AAV8-AAV9 > AAV2                                                        | [54]      |
| Pancreas             | AAV8 > AAV2                                                             | [91]      |
|                      | AAV1 > AAV2                                                             | [68]      |
| Kidney               | AAV1 = AAV2 = AAV5                                                      | [69]      |
|                      | AAV2 transduces tubular epithelium but not glomerular, blood vessel or interstitial cells |           |
| Lung                 | AAV5 and AAV6                                                           | [70]      |
|                      | AAV9 > AAV5                                                             | [71]      |
| Retina               | AAV5 > AAV4 > AAV1 = AAV2 = AAV3                                       | [58]      |
| Photoreceptor cells  | AAV5 > AAV2                                                             | [72]      |
| Hematopoietic stem cells | AAV1 > AAV2-AAV5                                                        | [73]      |
| Dendritic cells      | AAV6                                                                     | [74]      |

(continued)
Table 2.1 (continued)

| Tissue type            | Serotype                                                                 | Reference |
|------------------------|--------------------------------------------------------------------------|-----------|
| Cochlear inner ear cells | AAV1 and AAV2 > AAV5                                                    | [75]      |
| Solid tumors and melanoma                              | AAV2 > AAV1 and AAV3                                                    | [76]      |
| Glioblastoma           | AAV8 = AAV7 > AAV6 > AAV2 > AAV5                                        | [77]      |
| Glioma cells            | AAV2 > AAV4 and AAV5                                                    | [78]      |
| Brain                  | AAV5 > AAV1 > AAV2                                                      | [58]      |
|                        | AAV7 > AAV8 > AAV5 > AAV2 = AAV6                                        | [77]      |
|                        | AAV1 and AAV5 > AAV2; AAV1 and AAV5 transduced pars reticulate; AAV1 transduced entire midbrain; AAV1 and AAV5 transduced pyramidal cell layers; AAV2 transduced the dentate gyrus | [79]      |
|                        | AAV5 > AAV4 > AAV2                                                      | [80]      |
|                        | AAV1 > AAV2                                                            | [81, 82]  |
|                        | AAV8 > AAV1 or AAV2                                                    | [83]      |
|                        | AAV5 > AAV2                                                            | [84]      |
|                        | AAV2 transduces neurons                                                 | [85]      |
|                        | AAV5 and AAV1 transduce neurons and glial cells                         | [85]      |
|                        | AAV4 transduces ependymal cells                                         | [85]      |
|                        | AAV1 transduces glial cells and ependymal cells                         | [82]      |
| Cardiac tissue          | AAV8 > AAV2                                                            | [64]      |
|                        | AAV1 > AAV2 > AAV5 > AAV4 > AAV3                                       | [86]      |
|                        | AAV9 > AAV8                                                            | [67]      |
|                        | AAV9 > AAV8 > AAV1                                                     | [87]      |
|                        | AAV1 > AAV2                                                            | [88]      |
|                        | AAV6 > AAV2                                                            | [89]      |
expressing human factor IX indicate that the AAV2 capsid (amino acids 373–381) and the AAV8 capsid (amino acids 50–58) can elicit a cytotoxic T-lymphocyte (CTL) response [93]. In mice, the AAV8 capsid amino acids 126–140 elicited a Th1 response, whereas a Th2 response was elicited by AAV2 amino acids 475–489. The magnitude of the immune response depends on several factors, including vector dose, as well as the route and site of administration. Antibodies formed after transduction with one AAV serotype are likely to show only weak interaction with other serotypes, or may not cross-react. In animals that are transduced with AAV6 and then transduced with AAV2, delivery of the transgene was not hampered; however, animals transduced with AAV2 and then retransduced with AAV2 developed a neutralizing antibody response [92]. The region of the AAV2 capsid that has been shown to be responsible for generating a CD8⁺ T cell response is the RXXR motif in VP1, VP2, and VP3, which is involved in heparin binding for AAV2. This region was identified as being involved in uptake into dendritic cells, as well as the activation of capsid-specific T cells [94].

Alternative serotypes may also provide for treating cells that have heretofore been refractory to AAV infection, such as stem cells. It has been demonstrated that AAV2 is not efficient at transducing hematopoietic stem cells because of suboptimal levels of expression of the cell surface receptor for the viral vector (HSPG), impaired cellular trafficking of the vector, inefficient vector uncoating, and the lack of viral second-strand DNA synthesis [7]. However, AAV1 is able to transduce hematopoietic stem/progenitor cells when evaluated in short-term colony assays, as well as in long-term bone marrow transplantation assays in vivo [73]. There is also a low transduction efficiency for AAV2 in the airway epithelium of the lung due to a low abundance of HSPG on the apical surface; however, sialic acid is an abundant sugar on the apical surface of airway epithelium and pseudotyped AAV5 and AAV6 vectors have been shown to be efficient at gene transfer to murine airway epithelia in vivo [95, 96]. Encapsidated AAV6 vectors achieve transduction rates that should be sufficient for treating lung diseases, such as cystic fibrosis (CF). Recently, it was determined that the transduction efficiency of rAAV2 for the in vivo treatment of pancreatic and colon carcinoma is insufficient; however, other AAV serotypes, which have not yet been tested, may result in better transduction of this target tissue [97].

AAV2 and AAV1 bind to different cellular receptors, resulting in better transduction of muscle tissue for AAV1, and since the vectors in these studies contained the same cis elements, the AAV capsid is responsible for the differences in transduction efficiency between serotypes [98, 99]. Hauck and Xiao produced a series of capsid mutants to investigate the major regions of the AAV1 capsid responsible for the increased transduction efficiency of AAV1 in muscle tissue [100]; the major tissue tropism determinants were located in the surface region of VP1 (amino acid 350–423). Similar functional studies of the AAV capsid will provide a better understanding of the surface features and the specific requirements for engineering AAV capsids to efficiently target specific cells.
3 Methods

3.1 Retargeting

A receptor, like HSPG, which is present on a variety of cell types poses difficulties for targeting AAV2 vectors to a specific tissue. One approach to limiting gene expression in the target tissue is to utilize a viral vector that harbors a tissue-specific promoter [4, 72, 101]. Genetic modifications of the AAV capsid also provide an alternative approach for retargeting AAV2. Regions of the capsid have been identified that will accept insertion of heterologous peptides for retargeting. Girod et al., first demonstrated that the insertion of a 14 amino acid integrin binding peptide at amino acid 587 allowed for retargeting of AAV2 [102]. To obtain a genetic map of the AAV2 capsid, Wu et al. constructed alanine scanning mutants at 59 different positions in the AAV capsid gene by site-directed mutagenesis [103]. Studies of these mutants showed that the capsid can tolerate some modifications and still maintain infectivity. A 34 amino acid IgG binding domain was inserted into rAAV2 capsids at amino acid 587, and this, coupled with antibodies against B1 integrin, CXCR4, or the c-kit receptor, mediated the targeting of these rAAV vectors to specific cell surface receptors [104]. Endothelial cell binding peptides were identified using phage display and inserted into the AAV2 capsid at amino acid 587 to transduce venous endothelial cells and significantly reduce hepatocyte transduction [105]. Recently, it was demonstrated that AAV2 could be retargeted to the heart utilizing a capsid with mutations at amino acids 484 and 585 to eliminate heparin binding, which is required for AAV2 to infect the liver [106]. Perabo et al. also demonstrated that insertion at amino acid 587 with peptides that confer a net negative charge will ablate binding to HSPG, and this correlated with liver and spleen detargeting for AAV2. Insertion of peptides between 585 and 588 can either cause spatial separation or sterically block heparin binding of AAV2 (using bulky amino acids), and the insertion of positively charged peptides reconstitutes the ability to bind HSPG [107]. This provides a strategy to improve AAV targeting in other tissues. For retargeting AAV to tumor cells, an RGD-4C motif can be inserted at amino acids 520 and 584, or 588 to confer a novel tropism and eliminate heparin binding. The RGD motif binds the cellular integrin receptor, which is expressed on tumor cells, including ovarian cancer cells, but these cells express only low levels of HSPG and are nonpermissive for AAV2 transduction [108, 109].

3.2 Capsid Mosaics

Introduction of a peptide into the AAV2 capsid is a viable strategy for retargeting AAV2 to additional cell types; however, modifications in the AAV2 capsid often result in a reduction in vector yields, especially if the insertion is large. The unique N-terminal region of VP2 allows for peptide insertions without a loss of titer, when
VP1 and VP3 are supplied in trans. AAV2 mosaics that have ligand insertions in a subset of VP1, VP2, and VP3 molecules result in increased vector yields and transducing titers, compared with viruses that carry the insertion in all 60 capsid protein subunits [110]. Insertion of an HA epitope (YPVDVPDYA) at amino acid 522 or 533 of VP1 results in noninfectious particles [103]; however, the inclusion of wild-type capsid proteins can restore viral infectivity. Ried et al. [104] inserted an immunoglobulin-binding fragment of protein A (Z34C) that resulted in at least a tenfold reduction in particle titers, most likely due to its large size (34 amino acids). Compared with wild-type virus, infectious titers were 4 orders of magnitude lower. Gigout et al. generated mosaic viruses that contained between 25 and 75% of Z34C capsid proteins, with the rest of the capsid being composed of wild-type subunits to produce AAV2 mosaics that were infectious. Compared with wild-type virus, the Z34C mosaics showed up to a tenfold increase in titer, and those containing 25% Z34C capsid protein (an average of 15 subunits per capsid) were 4–5 orders of magnitude more infectious than all mutant viruses. By mutating R585 and R588 to alanine, they were able to eliminate HSPG binding and then the insertion of Z34C resulted in retargeting of AAV2 [110]. This work demonstrated for the first time that a combined approach of generating AAV2 mosaics to alter tropism and mutating two residues to reduce HSPG binding could be used to retarget AAV2 to specific cell types. For AAV1, incorporating an RGD4C motif in VP1 at amino acid 590 enables targeting to integrin receptors which are present on vascular endothelial cells [111]. It is also possible to incorporate a small biotin acceptor peptide (BAP) in this position for the purpose of metabolically biotinylating the AAV1 capsid for purification using a commercially available avidin affinity column. Mosaics with both of these modifications have been generated, which enable retargeting of the AAV1 capsid, and simplify purification.

3.3 Capsid Chimeras

Another strategy to broaden tissue tropism is to generate AAV with mixed or chimeric capsids [112]. This is accomplished by supplying the capsid gene from two distinct AAV serotypes during production. By varying the ratio of the two capsid genes, the resulting mixed virus may exhibit altered tropism. Rabinowitz et al. showed that mixed capsids of AAV2/5 at a ratio of 3:1 resulted in a loss of heparin binding. The ability of these virions to transduce HeLa cells, which have high levels of heparin sulfate, decreased, while the ability to transduce the heparin-sulfate-deficient cell line CHO pgsD increased from 2% to > 30% as the composition of the virion changed from AAV2-like to AAV5-like. Chimeras also may show combined tropism of both serotypes, broadening the tropism for these virions. Kohlbrenner et al. utilized chimeric capsids to improve infectivity when expressing AAV5 and AAV8 in insect cells [113]. It was determined that AAV5 and AAV8 vectors generated using the baculovirus system had low infectivity, which was due to insufficient phospholipase A2 activity. Substituting the entire VP1 protein from AAV2 in chimeric
AAV5 or AAV8 capsids resulted in increased phospholipase activity levels and enhanced transduction. Generating chimeras also provides a method to functionally define structural relatedness for newly discovered serotypes. The ability to generate stable chimeric capsids suggests that the subunits from these different serotypes are structurally compatible. Conversely, AAV2 when mixed with AAV4 inefficiently packages genomes. The inability to generate stable virions may reflect a failure of essential structural subunit interactions at one or more axes of symmetry. Alternatively, the nonstructural Rep protein interactions that occur on the surface of the capsid for packaging may not be compatible in the AAV2/AAV4 chimeras, as suggested by the structures for AAV2 and AAV4 [30, 31].

### 3.4 Vector Utility

The AAV capsid provides a potent gene delivery vehicle and has shown great promise in animal models, as well as in human clinical trials conducted to date. AAV vectors have been developed for a multitude of diseases, including disorders of the central nervous system for which other vectors and methods of treatment have been inadequate. Examples include Parkinson’s disease and Alzheimer’s disease. Gene therapy approaches utilizing AAV have also addressed classic genetic disorders, such as lysosomal storage disorders, hemophilia, and cystic fibrosis. Many diseases that are amenable to AAV gene therapy approaches are listed in Table 2.2. AAV vectors to treat these diseases have shown significant and persistent gene transfer without toxicity [3–5, 117–124, 138–141, 159–160] in human clinical trials. Vectors are administered to effect intracellular expression of proteins such as dystrophin or expression of secreted therapeutic proteins that result in cross correction of cells. AAV may also be utilized as a vaccine to deliver specific antigens; for example, AAV5 targets dendritic cells, allowing for antigen presentation, and AAV vectors have been produced that express components of papillomavirus, the causative agent of cervical cancer, as well as the receptor for the spike protein of SARS coronavirus [181, 182]. AAV-based gene transfer has the therapeutic potential to arrest or reverse the course of these inherited and acquired diseases.

### 3.5 Vector Production

An important consideration for the development of a suitable gene delivery vector is the ability to produce the quantities of vector required to treat human patients. Historically, production methods have been a limitation in the development of AAV vectors. Improvements in production and purification methods have resulted in better yields, increased purity, and higher titers through the development of scalable systems [113, 183–187]. Recombinant AAV vectors can be manufactured using a two-plasmid system, as shown in Fig. 2.3. One plasmid harbors the therapeutic
| Disorder                  | Target tissue                        | Species            | Serotype                      | Reference          |
|---------------------------|--------------------------------------|--------------------|-------------------------------|--------------------|
| Cystic fibrosis           | Nasal and lung epithelium            | Mice               | AAV2/5, AAV2/9                | [71]               |
|                           | Lung                                 | Mice               | AAV1, AAV2, AAV5              | [114]              |
|                           | Airway epithelium                    | Cell culture       | AAV5                          | [115]              |
|                           | Lung                                 | Mice               | AAV2, AAV5                    | [116]              |
|                           | Lung                                 | Mice               | AAV2, AAV6                    | [96]               |
|                           | Lungs – airway epithelium and maxillary sinus | Human | AAV2                          | [117–120, 121–124] |
| Hemophilia B              | Liver                                | Mice               | AAV8, AAV9                    | [67]               |
|                           | Liver                                | Mice               | AAV1, AAV2, AAV6              | [125]              |
|                           | Liver                                | Mice               | AAV5                          | [65]               |
|                           | Liver                                | Mice, Monkey       | AAV2, AAV5, AAV2/8            | [126]              |
|                           | Liver                                | Mice               | AAV2                          | [127, 128]         |
|                           | Liver                                | Dog                | AAV2/8                        | [129]              |
|                           | Liver                                | Dog                | AAV2                          | [130, 131]         |
|                           | Liver                                | Human              | AAV2                          | [4]                |
|                           | Muscle                               | Mice               | AAV1-AAV2 hybrid              | [60]               |
|                           | Muscle                               | Mice               | AAV1                          | [132, 133]         |
|                           | Muscle                               | Mice               | AAV1, AAV2, AAV3, AAV4, AAV5  | [98]               |
|                           | Muscle                               | Mice; Dog          | AAV1                          | [134]              |
|                           | Muscle                               | Dog                | AAV2                          | [135–137]          |
|                           | Muscle                               | Human              | AAV2                          | [138–141]          |
| Anemia                    | Muscle                               | Mice               | AAV2                          | [142, 143]         |
| Parkinson’s disease       | Brain                                | AAV2               |                               | [144]              |
|                           | Brain                                | Monkey             | AAV2                          | [145–149]          |
|                           | Brain                                | Human              | AAV2                          | [5]                |
| Lysosomal storage disease | Muscle                               | Mice               | AAV2                          | [150]              |
|                           | Muscle                               | Mice               | AAV1 and AAV2                 | [151]              |
|                           | Muscle                               | Mice               | AAV2/6                        | [152]              |

(continued)
| Disorder               | Target tissue                                      | Species     | Serotype | Reference |
|------------------------|----------------------------------------------------|-------------|----------|-----------|
| Brain                  | Mice                                               | AAV2        |          | [153]     |
| Brain                  | Mice                                               | AAV2, AAV5  |          | [154]     |
| Brain                  | Human                                              | AAV2        |          | [3]       |
| Brain                  | Mice                                               | AAV8        |          | [155]     |
| Brain                  | Mice                                               | AAV2/8      |          | [156]     |
| Canavan disease        | Brain                                              | Mice        | AAV2     | [157]     |
| Canavan disease        | Brain                                              | Rodents, monkey, human | AAV2     | [158]     |
| Canavan disease        | Brain                                              | Human       | AAV2     | [159, 160]|
| Type I diabetes        | Pancreas – acinar cells, beta cells                | Mice        | AAV6, AAV8 | [161]     |
|                        | Islets                                             | Mice        | dsAAV2, dsAAV6, dsAAV8 | [162]     |
|                        | Islet cells                                         | Mice        | AAV1, AAV2 | [68]      |
|                        |                                                    |             |          | [68]      |
| Alzheimer’s disease    | Brain – Abeta vaccine                              | Mice        | AAV2     | [163–165] |
|                        | Brain                                              | Rat         | AAV2/5   | [84]      |
|                        | Brain                                              | Rat         | AAV1, AAV2, AAV4, AAV5 | [85]      |
| Cardiovascular         | Cardiac tissue                                     | Rodent      | AAV2     | [166]     |
|                        | Cardiac tissue                                     | Mice        | AAV2/1, AAV2/8, AAV2/9 | [87]      |
|                        | Cardiac muscle                                     | Mice        | AAV2     | [151, 167]|
|                        | Cardiac tissue                                     | Rat         | AAV6, AAV2 | [89]      |
| Cancer                 | Glioma                                             | Tumor cell lines | AAV2, AAV4, AAV5 | [78]      |
|                        | Ovarian carcinoma                                  | Ovarian carcinoma cell line | RGD modified AAV2 | [168]     |
|                        |                                                    | Mice        | AAV2     | [169]     |
|                        | Breast carcinoma                                   | Mice        | AAV2     | [170]     |
|                        | Glioblastoma                                       | Mice        | AAV2     | [171]     |
|                        |                                                    | Rat         | AAV2     | [172]     |
| Vaccine development                                                                 | Species | Vector | Ref       |
|------------------------------------------------------------------------------------|---------|--------|-----------|
| Prostate cancer                                                                     | Mice    | AAV2   | [173]     |
| Liver cancer                                                                        | Mice    | AAV2   | [174, 175]|
| Lung adenocarcinoma                                                                 | Mice    | Hybrid AAV2/5 | [176]     |
| Bone-marrow-derived dendritic cells                                                | Mouse   | AAV6   | [74]      |
| Papillomavirus antigen HPV16 L1 protein delivery                                   | Mice – intranasal delivery | AAV5   | [182]     |
| Dendritic cells or muscle, HIV gp160 protein delivery                               | Mice    | AAV1, AAV5, AAV7 and AAV8 | [181]     |
| Dendritic cells, siRNA delivery for dengue virus vaccine                            | Human cells | AAV2   | [177]     |
| Obesity                                                                            |         |        |           |
| Vector expressing leptin                                                           | Mouse   | AAV2   | [178]     |
| Vector expressing leptin receptor                                                   | Rat     | AAV2   | [179]     |
| Vector expressing adiponectin                                                      | Rat     | AAV1, AAV2, AAV3, AAV4, AAV5 | [180]     |
gene flanked by the ITRs and the other one contains the AAV rep and cap genes, as well as the helper functions for AAV2 replication. The adenovirus helper functions are provided by expression of E2A, E4ORF6, and VA RNAs in the helper plasmid, while the adenovirus E1A and E1B gene products are expressed in the HEK293 cells utilized for production [188]. The two plasmids are co-transfected into 293 cells, and this results in production of an AAV virion that contains the therapeutic gene flanked by the ITRs [189]. The ITRs are the only viral sequences remaining after purification of rAAV vectors.

Protocol 1 (see Appendix) describes the small-scale production and purification of rAAV vectors as described by Zolotukhin et al. [190]. Generally, HEK293 cells are expanded, transfected, and harvested at 72h post-transfection. The cells are then lysed, and loaded onto either a cesium chloride or an iodixanol gradient to separate infectious virions from empty capsids and other cellular proteins. Virus is then purified using either heparin affinity chromatography or ion-exchange chromatography. The chromatography method used is dependent on the natural receptors for AAV, as well as the charge characteristics of the viral particle, as shown in Fig. 2.4. Following column chromatography, the virus is concentrated and characterized. These protocols yield vector stocks that are relatively pure; however, owing to the density gradient requirement, these methods are not easily scalable.

Protocols 2 and 3 describe the large-scale production and purification of rAAV vectors as described by Snyder et al. [18]. Protocol 3 provides a method suitable for large-scale production of rAAV2 vectors that is scalable for the production of vectors under cGMP conditions for clinical trials [191]. Cell factories of HEK293 cells are transfected, incubated for 72h, and then harvested. The cell pellet is resuspended in lysis buffer containing 0.5% deoxycholate to reduce viral particle aggregation and 50 U Benzonase/ml to degrade cellular, plasmid, and nonpackaged nucleic acid. A microfluidizer is utilized to lyse the cells in order to form a fine suspension that can be loaded directly onto a Streamline Heparin column. The column is washed and the virus is eluted from the column with phosphate-buffered saline (PBS) containing 0.2 M NaCl (for a total ionic strength of 350 mM). The peak fraction from the Streamline Heparin column is brought to 1 M NaCl and loaded on a Phenyl
2 The Role of the Adeno-Associated Virus Capsid in Gene Transfer

Fig. 2.4 Flowchart of the steps for rAAV production as described in Protocol 1. HEK293 cells are expanded, transfected, and harvested at 60 h posttransfection. The cells are lysed, and loaded onto either a cesium chloride gradient or an iodixanol gradient to separate infectious virions from empty capsids. Virus is purified using either heparin affinity chromatography or ion-exchange chromatography. Virus preparations are formulated and concentrated, and characterized.

Sepharose column. This column is a hydrophobic interaction column (HIC) and the virus will remain in the flow through. The flow through from the Phenyl Sepharose column is diluted using sterile water to bring the salt concentration to 0.150 M NaCl. This is loaded onto a SP Sepharose column, washed in column buffer, and eluted in PBS with 0.135 M NaCl (for a total ionic strength of 0.285 M).

3.6 Vector Characterization

Several assays to characterize the final product with respect to titer, purity, identity, potency, particle to infectious ratio, and stability are listed in Table 2.3. In addition, vector product safety testing should be conducted prior to animal or human studies. Assays used for product characterization and safety testing are included in Table 2.3.
Table 2.3 Assays used for vector characterization and safety testing of rAAV vectors produced for clinical trials under cGMP conditions

| Assay type   | Method and reference | Purpose                                                                 |
|-------------|----------------------|-------------------------------------------------------------------------|
| **Titer**   |                      |                                                                         |
| Infectious  | Infectious Center Assay (ICA) [192, 193] | Determine titer of infectious particles produced |
|             | Serial Dilution Replication Assay (dRA) [194] |                                                                         |
| Vector genome | Dot-blot hybridization | Determine genome-containing vector concentration |
|             | PCR [195]            |                                                                         |
| Capsid      | ELISA [24]           | Determine total capsid protein concentration – enables a determination of empty particles in the prep |
|             | Bradford Western     |                                                                         |
|             | Electron microscopy [196] |                                                                         |
|             | Optical density (OD) [197] |                                                                         |
| **Purity**  |                      |                                                                         |
| Protein     | SDS-PAGE             | Determine the presence or absence of contaminating proteins             |
| Cellular DNA | DNA hybridization or PCR | Determine the presence or absence of cellular DNA                        |
| **Identity**|                      |                                                                         |
| Transgene cassette | DNA sequencing or restriction enzyme digestion | Verification of the transgene |
| Capsid      | SDS-PAGE with silver and Coomassie stain | Expected AAV banding pattern |
|             | Limited proteolysis  | Serotype identification                                                  |
| **Potency** |                      |                                                                         |
| Transgene expression | Transduction assay in cells or animals | Ensure that the active transgene product is expressed |
| **Safety**  |                      |                                                                         |
| Adventitious agents | qPCR-based assays to detect infectious adventitious viral agents | Detect contaminating infectious viral agents of human or animal origin (serum, trypsin) |
| Mycoplasma  | Growth assays on cells in antibiotic-free conditions, followed by dye or PCR to detect mycoplasma | Determine the presence or absence of mycoplasma |
|             | Growth assay in appropriate agar media |                                                                         |
| Endotoxin   | Rabbit pyrogen assays | Determine the presence or absence of endotoxin                          |

(continued)
Table 2.3 (continued)

| Assay type | Method and reference | Purpose |
|------------|----------------------|---------|
| Sterility  | Bacteriostasis/fungistasis | Determine the presence or absence of microbial contaminants |

**Stability**

Physicochemical

SDS-PAGE

Demonstrate that the product is not degrading over time

Infectious

ICA

Demonstrate that infectivity is maintained over time

Sterility

Bacteriostasis/fungistasis

Demonstrate the integrity of final product container over time

3.7 Proteolytic Structural Mapping

In the near future, customized AAV gene therapy vectors may consist of modified capsids that allow for specific targeting to treat patients with various diseases. The 3D structures of the AAV capsids will provide a basis for rational vector design; however, the 3D structures available for autonomous parvoviruses and dependoviruses provide only a “snapshot” of the capsid topology in a low energy conformation. Our knowledge about the AAV viral capsid structure in solution is limited; however, this structure must be dynamic to carry out the various functions required for viral attachment and entry, as well as trafficking within the cell. Cryo-EM studies have shown that the unique N-terminus of VP1 is internal to the capsid based on additional density at the two-fold axis of symmetry [198]. *In vitro*, upon heat treatment of AAV capsids, it has been shown that this region can be externalized. Mutagenesis experiments have shown that this externalization occurs through the pore at the five-fold axis of symmetry [199]. Previously, antibodies have been produced that detect various regions of the capsid proteins [200]. The B1 antibody epitope is on the C-terminal end of the capsid protein, and is primarily internal at the two-fold axis of symmetry in assembled capsids. This antibody is useful in detecting denatured AAV proteins and the epitope is highly conserved among the AAV serotypes. A1 antibody recognizes the unique N-terminal region of VP1, while the A69 epitope is in the N-terminal region of VP2 for AAV2. Polyclonal antibodies have been produced to AAV2 capsids, as well as to other serotypes. In addition, antibodies have been produced that recognize conformational epitopes that are present only on assembled capsids for AAV2, as well as AAV1. Capsid antibodies can be utilized for serotype identity testing of the final vector product; however, many of these antibodies cross-react with multiple serotypes. Historically,
AAV has been shown to be remarkably stable and is generally resistant to proteases. However, we have demonstrated that when exposed to proteases, specific regions of the capsid are susceptible to proteolysis [201]. Owing to differences in the primary sequence of the capsid proteins of different serotypes, as well as the resulting differences in capsid structure, proteolysis can be utilized for AAV serotype determination. Different serotypes provide unique fragmentation patterns when cleaved with protease, with some serotypes such as AAV5 being relatively resistant, while others such as AAV2 are more susceptible to trypsin and chymotrypsin. Figure 2.5 depicts protease mapping of the AAV capsid for capsid serotype determination. A generic assay is described in Protocol 4 where proteolysis is used together with specific antibodies to provide a powerful mapping technique and a method to identify and confirm the serotype of the AAV capsid.

![Protease mapping of the AAV capsid for capsid serotype determination](image)

**Fig. 2.5** Protease mapping of the AAV capsid for capsid serotype determination. A Samples are digested with a protease, in this case trypsin for one set of samples and chymotrypsin for the other set. A Western blot is performed using polyclonal antisera to AAV capsids, and based on the fragmentation pattern, a serotype determination can be made. Undigested sample (T0) and samples digested for 12 h (T12) for AAV2, AAV1, and AAV5 are shown. AAV5 is resistant to these proteases, while AAV1 and AAV2 exhibit differences in their fragmentation patterns. T0 samples represent the undigested capsid proteins VP1, VP2, and VP3. B Different AAV serotypes demonstrate different susceptibilities to proteases due to the differences in their primary amino acid sequences. This differential susceptibility provides a unique signature for each serotype and allows for capsid serotype identification.
3.8 Concluding Remarks on AAV Capsid

In summary, the AAV capsid is the major determinant in gene transfer efficiency and targeting. Several approaches are available to target AAV viral vectors to specific cell types, including utilizing natural serotypes that target a desired cellular receptor, producing pseudotyped vectors, as well as engineering chimeric and mosaic capsids. Genetic mutations of the AAV capsid have identified regions that will accommodate peptide insertions and modifications for specific targeting. The use of modified capsids may enhance efficiency and target specificity to make a more potent and safe vector. Several genetic diseases are amenable to treatment with AAV vectors, making AAV a valuable gene delivery vehicle, especially for disorders for which current therapies are inadequate or non-existent. Current production methods allow for the generation of quantities of virus needed for clinical trials, and purification methods have been developed for several serotypes. In addition, assays have been developed for final product characterization to ensure patient safety.

Appendix

Protocol 1: AAV Small-Scale Production (~3 × 10⁸ cells/prep)

Transfection Protocol

1. Seed [20] 15-cm plates with 293 cells so that they will be 75–80% confluent the following day (12–16 h prior to transfection). 293 cells are cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin.
2. Check plates prior to transfection to ensure optimal confluency, and change media to ensure cells are in a volume of 20 ml. Be careful not to disrupt the monolayer.
3. For transfection, prepare the DNA mixture at room temperature in a 50-ml conical tube as follows: Mix 900µg of helper plasmid with 300µg rAAV vector plasmid. This is 60µg DNA per plate in a 1:1 molar ratio. Bring the final volume of the DNA mixture to 22.5 ml with sterile dH₂O. Add 2.5 ml of 2.5 M CaCl₂ to the DNA for a CaCl₂ concentration of 0.250M.
4. Prepare ten 15-ml conical tubes each with 2.5 ml 2× HBS to transfect the 20 plates (25 ml 2× HBS total). Transfect two plates at a time by adding 2.5 ml of DNA mixture to a tube of 2.5 ml 2× HBS, mix, and incubate for 1 min at room temperature to allow precipitate to form. Add 5 ml of media to the tube and mix. (Total volume in the tube is 10 ml.) Pipette 5 ml onto each of the two plates. Repeat for the remainder of the plates.
5. Incubate at 37°C for 72 h.
6. At 72h, harvest cells. Use media to dislodge the cells. Collect media and cells and centrifuge at 1,000 × g, for 10 min at 4°C to pellet the cells. Discard the media. Resuspend pellet in PBS to wash the pellet. Centrifuge at 1,000 × g, for 10 min at 4°C. Discard PBS and freeze pellet. Frozen pellets can be stored at −20°C for processing at a later time.

Transfection Reagents and Materials

- 15-cm tissue culture plates.
- 293 cells.
- Culture media: DMEM + 5% FBS + 1% pen/strep.
- Trypsin for splitting cells.
- PBS without magnesium and calcium.
- Vector plasmid, 300 µg.
- Helper plasmid, 900 µg.
- Sterile dH₂O.

2.5 M CaCl₂ – (stock 147.02 g/mol calcium chloride dihydrate) – for 1 l add 367.55 g CaCl₂·2H₂O. Prepare 10-ml aliquots and store at −20°C.

2× HBS (HEPES-buffered saline; prepare 1 l):

0.274 M NaCl (stock 58.44 g/mol) – 16.0 g.
0.010 M KCl (stock 74.56 g/mol) – 0.75 g.
0.002 M Na₂HPO₄ anhydrous (stock 141.96 g/mol) – 0.28 g.
0.011 M dextrose (stock 180.16 g/mol) – 2.0 g.
0.042 M HEPES (stock 238.3 g/mol) – 10.0 g.

Adjust the pH to 7.05 with 0.5 N NaOH, bring to a final volume of 1 l with dH₂O, and sterile filter. Store in 50-ml aliquots at −20°C. (Note: the optimal pH range for 2× HBS is 7.05–7.12. The pH of the 2× HBS is a key factor that influences precipitate formation.)

Cell Lysate and Cesium Chloride Gradient Protocol

7. Resuspend pellet in 30 ml lysis buffer. Lyse cells by three freeze/thaw cycles. Thaw pellet in 37°C water bath and freeze cells in EtOH/dry ice bath; repeat twice.

8. Transfer the lysate to a 40-ml Dounce homogenizer. Homogenize the lysate with 20 strokes to shear cellular DNA.

9. For each 10 ml of lysate, add 5 g CsCl and homogenize until the CsCl is dissolved completely.

10. Fill six 12.5 ml ultra clear ultracentrifuge tubes (Beckman) with 10 ml of lysate and underlay with 0.5 ml each of CsCl (1.5 g/ml). Balance the tubes using CsCl (1.37 g/ml) prior to ultracentrifugation.
11. Centrifuge for 24 h at 265,000 × g (40,000 rpm in SW 41 rotor), 21°C.
12. Collect AAV from the gradient by dripping 1-ml fractions, and verify the density of the fractions by refractometry. The density of infectious AAV virions is 1.40–1.42 g/cm³. Empty AAV particles and DI particles have a density of 1.32–1.35 g/cm³.
13. Dialyze AAV-containing fractions into the buffer that will be used for column chromatography (1× TD buffer for heparin column or 15 mM NaCl, 20 mM Tris (pH 8.5) for Q Sepharose purification).
14. Purify using either heparin affinity chromatography (for AAV2, AAV3, or AAV6) or ion-exchange Q Sepharose chromatography (for AAV1, AAV4, AAV5, AAV8, or AAV9). Use a 1-ml column for 20–40 plates, as described in steps 1–7 under “AAV Purification by Heparin Chromatography” or “AAV Purification by Q Sepharose Chromatography.” The purification method is as described by Zolotukhin et al. [190].

Cell Lysate and Cesium Chloride Gradient Reagents

Lysis buffer (prepare 1 l):
- 150 mM NaCl (stock 58.44 g/mol) – 8.766 g.
- 50 mM Tris (stock 121.14 g/mol) – 6.055 g.
- Adjust pH to 8.5 with HCl, bring to a final volume of 1 l with dH₂O, and filter sterilize. Store at room temperature.

1.37 g/ml CsCl in PBS – Dissolve 50 g CsCl in PBS and adjust the final volume to 100 ml. Weigh 1 ml to verify the density is 1.37 g/ml. Filter sterilize. Store at room temperature.

1.5 g/ml CsCl in PBS – Dissolve 67.5 g CsCl in PBS and adjust the final volume to 100 ml. Weigh 1 ml to verify the density is 1.5 g/ml. Filter sterilize. Store at room temperature.

5× TD buffer (prepare 1 l):
- 5× PBS −500 ml of 10× PBS (Invitrogen).
- 5 mM MgCl₂·6H₂O (stock 203.3 g/mol) – 1.0165 g.
- 12.5 mM KCl (stock 74.56 g/mol) – 0.932 g.

1× TD buffer – Prepare from 5× TD buffer stock: 200 ml 5× TD stock, 800 ml dH₂O.

Q Sepharose low salt column buffer:
- 15 mM NaCl (stock 58.44 g/mol) – 0.877 g.
- 20 mM Tris (stock 121.14 g/mol) – 2.423 g.
- Adjust pH to 8.5 with HCl and bring to a final volume of 1 l with dH₂O.
Alternative Cell Lysate and Iodixanol Gradient Protocol

7a. Resuspend pellet in 30 ml lysis buffer. Lyse cells by three freeze/thaw cycles. Thaw pellet in 37°C water bath and freeze cells in EtOH/dry ice bath; repeat twice.

8a. To the cell suspension, add 3 µl of 4.82 M MgCl₂ and vortex. Add 1 µl of Benzonase (250 U/µl) and vortex. Incubate at 37°C for 30 min. Centrifuge for 20 min at 1,000 × g.

9a. Pipette the supernatant into two quick seal tubes for a 70 Ti rotor. Underlay lysate with iodixanol gradient:
   - 7.5 ml of 15% iodixanol
   - 5.0 ml of 25% iodixanol
   - 7.5 ml of 40% iodixanol
   - 5.0 ml of 60% iodixanol

10a. Weigh tubes, cap, and seal with heat sealer.

11a. Centrifuge at 350,333 × g, 18°C, for 1 h (69,000 rpm in a 70 Ti rotor for 1 h).

12a. Collect the 40% iodixanol band and the interface between the 60% and the 40% bands. This is done by setting up a ring stand and placing the tube in a clamp. Swab the top and bottom of the tube with an alcohol swab. Vent the top of the tube with a needle and collect fractions from bottom of the tube.

13a. Dialyze virus-containing fractions into the buffer that will be used for column chromatography (1× TD buffer for heparin column or 15 mM NaCl, 20 mM Tris (pH 8.5) for Q Sepharose purification).

14a. Purify using either heparin affinity chromatography (for AAV2, AAV3, or AAV6) or ion-exchange Q Sepharose chromatography (for AAV1, AAV4, AAV5, AAV8, or AAV9). Use a 1-ml column for 20–40 plates, as described in steps 1–7 under “AAV Purification by Heparin Chromatography” or “AAV Purification by Q Sepharose Chromatography.” The purification method is as described by Zolotukhin et al. [190].

Cell Lysate and Iodixanol Gradient Reagents

Lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.5) (prepare 11):
   - 150 mM NaCl (stock 58.44 g/mol) – 8.766 g.
   - 50 mM Tris (stock 121.14 g/mol) – 6.055 g.
   - Adjust pH to 8.5 with HCl, bring the final volume to dΗ₂O with 11, and filter sterilize. Store at room temperature.

5 M NaCl (stock 58.44 g/mol) – 292.2 g and dΗ₂O to 11.
   For iodixanol gradient, mix the following:
### Heparin Column Chromatography Reagents

1. **1× TD buffer** – prepare from 5× TD buffer stock: 200 ml 5× TD stock, 800 ml dH₂O.
2. **1× TD/1 M NaCl buffer**: 200 ml 5× TD buffer, 58.44 g NaCl; bring to 1 l with dH₂O.
3. **Storage buffer:**
   - 100 mM NaCl (stock 58.44 g/mol) – 5.844 g.
   - 50 mM Tris (stock 121.14 g/mol) – 6.055 g.
   - Adjust pH to 8.0 and bring to 1 l with dH₂O.

### AAV Purification by Q Sepharose Chromatography

1. Equilibrate a 1 ml HiTrap Q HP column (GE Healthcare) by washing with ten column volumes of low salt Q column buffer (0.020 M Tris, 0.015 M NaCl, pH 8.5). The flow rate for all chromatography steps is 1 ml/min.
2. Activate the column by washing with five column volumes of high salt Q column buffer (0.020 M Tris, 1.0 M NaCl, pH 8.5).
3. Re-equilibrate the column by washing in ten column volumes of low salt Q column buffer.
4. Apply the dialyzed virus from either the cesium or iodixanol gradient to the column. (Note: After recovering the virus from the cesium or iodixanol gradient, virus must be dialyzed into low salt buffer or a buffer exchange into low salt buffer must have been performed in order to bind to the Q column.)
5. Wash the column with ten volumes of low salt Q column buffer.
6. Elute with five column volumes of elution buffer (20 mM Tris, 0.5 M NaCl, pH 8.5) [190] and collect 5 1-ml fractions.
7. Dialyze the virus preparation into storage buffer (50 mM Tris, 100 mM NaCl, pH 8.0) or suitable formulation aliquot, and freeze at −20°C.

**Q Sepharose Chromatography Reagents**

**Q Sepharose low salt column buffer:**
- 15 mM NaCl (stock 58.44 g/mol) – 0.877 g.
- 20 mM Tris (stock 121.14 g/mol) – 2.423 g.
- Adjust pH to 8.5 and bring the final volume to 1 l with dH₂O.

**Q Sepharose high salt column buffer:**
- 1 M NaCl (stock 58.44 g/mol) – 58.44 g.
- 20 mM Tris (stock 121.14 g/mol) – 2.423 g.
- Adjust pH to 8.5 and bring the final volume to 1 l with dH₂O.

**Q Sepharose elution buffer:**
- 0.5 M NaCl (stock 58.44 g/mol) – 29.22 g.
- 20 mM Tris (stock 121.14 g/mol) – 2.423 g.
- Adjust pH to 8.5 and bring the final volume to 1 l with dH₂O.

**Storage buffer:**
- 100 mM NaCl (stock 58.44 g/mol) – 5.844 g.
- 50 mM Tris (stock 121.14 g/mol) – 6.055 g.
- Adjust pH to 8.0 with HCl and bring to 1 l with dH₂O.
Protocol 2: AAV Large-Scale Production (10 Cell Factories ∼1 × 10¹⁰ cells)

Transfection Protocol

1. Seed [10] ten-layer cell factories (Nunc) each with 5 × 10⁸ 293 cells so that each will be 75–80% confluent the next day. (Incubate 12–16 h prior to transfection.) 293 cells are cultured in DMEM supplemented with 5% fetal bovine serum, 1% penicillin, and 1% streptomycin.

2. Check each cell factory microscopically prior to transfection to ensure optimal confluency.

3. For transfection, prepare the DNA mixture in a 250-ml conical tube: Each cell factory requires 2,400 µg total DNA: 1,800 µg helper plasmid and 600 µg rAAV vector plasmid. (This is a 1:1 molar ratio.) Calculate the volume of total input DNA required. Bring the final volume of the DNA mixture to 46.8 ml with sterile dH₂O. Add 5.2 ml 2.5 M CaCl₂ to the DNA for a final CaCl₂ concentration of 0.25 M. The total volume of the DNA/CaCl₂/dH₂O is 52 ml.

4. Add the DNA to 52 ml of 2× HBS, mix well, and incubate for 1 min at room temperature to allow precipitate to form. Add the transfection mix to 1,000 ml of prewarmed DMEM-Complete Media. Discard media from cell factory. Pour media with transfection mix into the cell factory. Repeat for all cell factories.

5. Incubate at 37°C for 72 h.

6. At 72 h, harvest cells. Collect media and cells and centrifuge at 1,000 × g, for 10 min at 4°C to pellet the cells. Discard the media. Resuspend pellet in 300 ml PBS to wash the pellet. Centrifuge at 1,000 × g, for 10 min at 4°C. Discard PBS and freeze pellet. Frozen pellets can be stored at −20°C for processing at a later time.

Protocol 3: Large-Scale Purification Protocol for AAV2

1. Resuspend the cell pellet from one cell factory in 60 ml large-scale lysis buffer (20 mM Tris-Cl, 150 mM NaCl, 0.5% deoxycholate, and Benzonase (50 U/ml)). Deoxycholate is used in the lysis buffer to reduce aggregation.

2. For ten cell factories, lyse the cells in a single-pass by using a microfluidizer (Microfluidics M-110S). This will form a fine suspension that can be loaded onto the column.

3. Load the lysate onto a 150-ml Streamline Heparin column (Pharmacia) at a flow rate of 20 ml/min using an AKTA-FPLC (Pharmacia).

4. Wash the column with four column volumes of lysis buffer, followed by five column volumes of PBS.

5. Elute the virus with PBS containing 0.2 M NaCl (0.350 M total ionic strength) and monitor the absorbance at 280 λ. The peak fraction will be ∼90 ml.
6. Bring the NaCl concentration of the peak fraction to 1 M NaCl and load this onto a Phenyl Sepharose column (5 ml column, Pharmacia). This is a hydrophobic interaction column and the flow-through that contains the virus is collected.
7. The flow-through is diluted to 150 mM NaCl with sterile water.
8. The virus is loaded onto a 5 ml SP Sepharose column (Pharmacia) at a flow rate of 5 ml/min.
9. Wash the column with ten column volumes of PBS.
10. Elute the virus with PBS containing 0.135 M NaCl (285 mM total ionic strength) and monitor the absorbance at 280 \( \lambda \). Aliquot and store at −20°C.
11. Dialyze the virus prep into storage buffer (50 mM Tris-Cl, 100 mM NaCl, pH 8.0), aliquot, and freeze at −20°C.

**Protocol 4: Proteolytic Digestion for AAV Capsid Serotype Validation [201]**

1. Dialyze AAV vector into protease digestion reaction buffer, 50 mM Tris-Cl, 100 mM NaCl, pH 8.0, if needed.
2. Digest 0.8 µg (≈1.2 \times 10^{11}) capsids with either 5 µg (0.02% final concentration) of trypsin, 1 µg of proteinase K, or 80 µg of \( \alpha \)-chymotrypsin in a 25 µl reaction at 37°C for up to 24 h. For each serotype, an undigested sample should be included as a control. These proteases are commercially available from Sigma.
3. Add an equal volume of Laemmli sample buffer containing 1% sodium dodecyl sulfate (SDS) and 655 mM \( \beta \)-mercaptoethanol and boil the samples at 100°C for 5 min.
4. Separate the proteolytic fragments on a 10% SDS-PAGE gel for 90 min at 125 V (constant voltage) until the dye front reaches the bottom of the gel.
5. Transfer the proteins to nitrocellulose (Western blot) in transfer buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, and 20% methanol) for 2 h at 0.5 A (constant amperes).
6. Probe the membrane with rabbit polyclonal antisera to AAV capsids. Duplicate samples can be run to probe with other AAV antibodies to determine the origin of the fragments that are produced. For example, a signal with monoclonal B1 antibody demonstrates that the fragment is from the common C-terminal end of VP1, VP2, and VP3. Alternatively, by probing with monoclonal A1 antibody, a signal on the Western blot would demonstrate that the origin of the fragment is the unique N-terminus of VP1. The B1 epitope is conserved among most AAV serotypes and polyclonal antibodies have been developed for a few AAV serotypes. Anti-AAV capsid antibodies are available from Progen or American Research Products, Inc.

**Acknowledgments** We thank Dr. Philippe Moullier at the Laboratoire de Therapie Genique, INSERM U649, CHU Hotel Dieu, Nantes Cedex 1, France. R.S. owns equity in a gene therapy company that is commercializing AAV for gene therapy applications. This project was funded by
UF College of Medicine start-up funds, The Department of Molecular Genetics and Microbiology, Association Nantaise de Thérapie Génique (ANTG), and Association Française contre les Myopathies (AFM) Award 12263 to R.O.S, and by CLINIGENE, an EC-funded Network of Excellence.

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