

NOVEL COMPOSITIONS AND METHODS FOR PRODUCING RECOMBINANT AAV

CROSS REFERENCE TO RELATED APPLICATIONS

[001] The present application claims priority from U.S. Provisional Application 62/943,715, filed December 4, 2019, the content of which is incorporated herein by reference in its entirety.

SEQUENCE LISTING

[002] The instant application contains a Sequence Listing that has been submitted electronically in ASCII format. The Sequence Listing is hereby incorporated by reference in its entirety. The ASCII copy, created on November 30, 2020, is named 025297_WO015_SL.txt and is 111,015 bytes in size.

BACKGROUND OF THE INVENTION

[003] Adeno-associated virus (AAV) is a small non-enveloped virus belonging to the family *Parvoviridae* and the genus *Dependoparvovirus*. AAV is composed of a single-stranded DNA genome packaged into capsids assembled from three capsid proteins – viral protein (VP) 1, VP2, and VP3 – at an approximate molar ratio of 1:1:10 (Berns and Parrish (2007) *Parvoviridae* in Fields Virology (Knipe and Howley, eds., 5th ed. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins); Wang et al., *Nat Rev Drug Discov.* (2019) 18(5):358-78). The capsid proteins are encoded by a single capsid (*cap*) gene and are generated by alternative splicing and differential codon usage (*id.*). VP1 is the largest capsid protein (81.6 kD). VP2 (66.6 kD) is an N-terminal truncated form of VP1. VP3 (59.9 kD) is an N-terminal truncated form of VP2. *See, e.g.,* Cecchini et al., *Hum Gene Ther.* (2011) 22:1021-30.

[004] Recombinant AAV (rAAV) has been intensively explored as a vector for gene therapy and DNA vaccines in humans. For rAAV production in mammalian cells, a helper virus (e.g., adenovirus, vaccinia, or herpesvirus) is needed. Over the years, several modifications have been made to facilitate the production of rAAV, including (1) identifying and cloning the minimal set of helper proteins (adenovirus E1, E2A, E4, and VA), (2) providing the AAV Rep (Rep78,

Rep68, Rep52, and Rep40) and capsid proteins *in trans*, and (3) developing a transgene system that allows packaging of DNA sequences flanked by the AAV Inverted Terminal Repeats (ITRs) (Samulski et al., *J Virol.* (1989) 63(9):3822-8). When these three components are introduced into mammalian cells, rAAV containing a transgene of interest can be readily purified. Recombinant AAV so produced has been used in clinical trials (Clement and Grieger, *Mol Ther Methods Clin Dev.* (2016) 3:16002).

[005] The need for scaled-up rAAV production for larger clinical trials and commercialization has led to the development of insect cell-based production systems that utilize baculoviral vectors to express the Rep and capsid proteins and to carry the coding sequence for a transgene-containing AAV vector genome, which is packaged into rAAV capsids. Insect cell-based manufacturing of recombinant AAV (rAAV) offers several advantages over mammalian cell-based rAAV manufacturing, including scalability of non-adherent cells and cost savings due to the use of serum-free growth conditions. Such systems also do not require adenovirus helper functions (*see, e.g.*, Urabe et al., *Hum Gene Ther.* (2002) 13:1935-43; Urabe et al., *J Vir.* (2006) 80(4):1874-85; Chen et al., *Mol Ther.* (2008) 16(5):924-30; Smith et al., *Mol Ther.* (2009) 17(11):1888-96; and Mietzsch et al., *Hum Gene Ther Methods* (2017) 28(1):15-22). While the baculovirus-insect cell system has been successfully utilized for rAAV production at multiple scales, it has been observed that rAAV generated in insect cells has reduced VP1 content (*e.g.*, with a VP1:VP2:VP3 ratio of approximately 1:1:30 to 1:1:60) and consequently reduced potency, as compared to rAAV produced in mammalian cells (*see, e.g.*, Urabe et al., 2002, *supra*; Kohlbrenner et al., *Mol Ther.* (2005) 12(6):1217-25; Urabe et al., 2006, *supra*; Aslanidi et al., *Proc Natl Acad Sci USA* (2009) 106(13):5059-64; Kondratov et al., *Mol Ther.* (2017) 25(12):2661-75; and Mietzsch et al., *supra*). Several efforts have been made to improve the capsid ratios by modifying the start codon context of VP1 (*see, e.g.*, Kondratov et al., *supra*; Mietzsch et al., *supra*).

[006] Thus, there remains a need for an improved baculovirus-insect cell system for producing rAAV at industrial scales.

SUMMARY OF THE INVENTION

[007] The present disclosure provides baculovirus-insect cell systems for producing potent recombinant AAV at high yield. In one aspect, the present disclosure provides an insect cell

(e.g., an Sf9 or Sf21 cell) comprising a first baculoviral vector comprising an expression cassette for Rep78 and a second baculoviral vector comprising an expression cassette for Rep52, wherein the first vector and the second vector further comprise (i) an expression cassette for VP1 and an expression cassette for VP2/VP3, respectively; or (ii) an expression cassette for VP2/VP3 and an expression cassette for VP1, respectively. In some embodiments, one or both of the first and second vectors are stably integrated into the genome of the insect cell.

[008] In some embodiments, the Rep78 expression cassette and the Rep52 expression cassette comprise identical insect promoters. In some embodiments, the Rep78 expression cassette comprises a non-canonical start codon for the Rep78 coding sequence, wherein the codon is optionally ACG, TTG, GTG, or CTG.

[009] In some embodiments, the VP1 expression cassette and the VP2/VP3 expression cassette comprise identical insect promoters. In some embodiments, the VP1, VP2, and VP3 proteins comprise amino acid sequences from the same AAV serotype, or from more than one AAV serotype. In certain embodiments, the VP1, VP2, and/or VP3 proteins comprise amino acid sequences from AAV1, AAV2, AAV3 (e.g., AAV3B), AAV6, and/or AAV9. In certain embodiments, the Rep78 and Rep52 proteins are derived from a different AAV serotype from the VP1, VP2, and/or VP3 proteins.

[0010] In some embodiments, the Rep78, Rep52, VP1, and VP2/VP3 expression cassettes each comprise an insect promoter selected from a polyhedron promoter, an IE-1 promoter, and a p10 promoter.

[0011] The insect cell herein may further comprise a coding sequence for a recombinant AAV genome, wherein the recombinant AAV genome comprises an expression cassette for a transgene of interest that is under the transcriptional control of a mammalian promoter, and an AAV inverted terminal repeat (ITR) on both termini. In some embodiments, the coding sequence for the recombinant AAV genome is located on the first or second vector, or is located on a third vector. The transgene of interest may encode, for example, a therapeutic protein, including, without limitation: a protein whose function is lacking or deficient in a genetic disease (e.g., a lysosomal storage disease, or a hemophilia), such as an enzyme (for use in an enzyme replacement therapy) and a blood clotting factor (for use as replacement factor); and a protein for regulating gene expression (e.g., a zinc finger protein (ZFP) transcription factor). The transgene of interest may also encode a gene editing protein, such as a zinc finger nuclease (ZFN), a ZFP

deaminase, a ZFP recombinase, a TALEN, a CRISPR Cas protein, and a CRISPR Cpf protein. The transgene may also encode an interfering RNA molecule such as a small hairpin RNA.

[0012] In some embodiments, the VP1 protein and/or the VP2 protein comprise two or more mutations at residues 157, 162, 164, 179, 188, 194, 196, 197, 200, and 201 (numbering according to SEQ ID NO:1) relative to wildtype VP1 and VP2 proteins, respectively. As used herein, “numbering according to SEQ ID NO:1” means amino acid residue positions in SEQ ID NO:1, or the corresponding amino acid residue positions in a different VP1 sequence (e.g., VP1 sequence from a serotype other than AAV6). In some embodiments, the VP1 protein and/or the VP2 protein comprise two or more mutations selected from the group consisting of S157A, T162S, Q164A, S179T, L188I, T194A, A196S, A197G, P200S, and T201L (numbering according to SEQ ID NO:1). In further embodiments, the VP1 protein and/or the VP2 protein comprise all of these ten mutations.

[0013] In some embodiments, the VP1 protein further comprises one or more mutations at residues 67, 81, 84, 85, and 92 relative to wildtype VP1 protein (numbering according to SEQ ID NO:1). In certain embodiments, the VP1 protein comprises one or more mutations selected from the group consisting of A67E, Q81R, K84D, A85S, and R92K. In further embodiments, the VP1 protein comprises all of these five mutations.

[0014] In some embodiments, the VP1 protein and the VP2 protein disclosed herein are identical to the VP1 protein and the VP2 protein, respectively, of AAV6 but for the mutations. In particular embodiments, the VP1 protein and/or the VP2 protein are derived from AAV6 and comprise mutations S157A, T162S, Q164A, S179T, L188I, T194A, A196S, A197G, P200S, and T201L relative to wildtype AAV6 VP1 and VP2 proteins, respectively.

[0015] In some embodiments, the VP1-expressing vector also comprises an expression cassette for assembly-activating protein (AAP), wherein the AAP comprises one or more mutations at residues 8, 10, 12, 17, 21, and 22 (numbering according to SEQ ID NO:10) relative to wildtype AAP protein. As used herein, “numbering according to SEQ ID NO:10” means amino acid residue positions in SEQ ID NO:10, or the corresponding amino acid residue positions in a different AAP sequence (e.g., AAP sequence from a serotype other than AAV6). In certain embodiments, the AAP comprises one or more (e.g., all) mutations selected from the group consisting of P8Q, H10L, L12Q, Q17P, L21Q, and L22V. In particular embodiments, the AAP is identical to wildtype AAP of AAV6 but for the AAP mutations.

[0016] In particular embodiments, the VP1 expression cassette comprises a coding sequence for SEQ ID NO:7, with or without the first amino acid (e.g., nucleotides 18-151 of SEQ ID NO:14). In further embodiments, the capsid expression cassettes comprise 10 or more (e.g., 20 or more, 30 or more, 40 or more, 50 or more, 75 or more, 100 or more, 125 or more, or 150 or more) contiguous nucleotides, or the entire nucleotide sequence, of SEQ ID NO:14.

[0017] In some embodiments, the VP1 protein comprises one or more mutations at residues 81, 84, 85, and 92 relative to wildtype VP1 protein (numbering according to SEQ ID NO:1). In some embodiments, the VP1 protein comprises one or more mutations selected from the group consisting of Q81R, K84D, A85S, and R92K. In further embodiments, the VP1 protein comprises all of these four mutations. In particular embodiments, the VP1 protein further comprises a mutation at residue 67, e.g., an A67E mutation. In some embodiments, the VP1 protein is identical to the VP1 protein of AAV6 but for the mutation(s). In certain embodiments, the VP1 protein comprises mutations A67E, Q81R, K84D, A85S, and R92K relative to wildtype AAV6 VP1.

[0018] In particular embodiments of the present insect cell AAV production systems, the VP1 comprises SEQ ID NO:1, 7, or 16 with or without the first amino acid residue; the VP2 comprises amino acid residues 138-736 or 139-736 of SEQ ID NO:1 or 7, or comprises SEQ ID NO:18 with or without the first amino acid residue; the VP3 comprises amino acid residues of 204-736 or 205-736 of SEQ ID NO:1 or amino acid residues 203-736 or 204-736 of SEQ ID NO:7, or comprises SEQ ID NO:19 with or without the first amino acid residue.

[0019] In some embodiments, the VP1 comprises SEQ ID NO:24 with or without the first amino acid residue; the VP2 comprises amino acid residues 138-736 or 139-736 of SEQ ID NO:24; and the VP3 comprises amino acid residues of 203-736 or 204-736 of SEQ ID NO:24.

[0020] In some embodiments, the VP1 comprises SEQ ID NO:25 with or without the first amino acid residue; the VP2 comprises amino acid residues 138-735 or 139-735 of SEQ ID NO:25; and the VP3 comprises 203-735 or 204-735 of SEQ ID NO:25.

[0021] In some embodiments, the VP1 comprises SEQ ID NO:26 with or without the first amino acid residue; the VP2 comprises amino acid residues 138-736 or 139-736 of SEQ ID NO:26; and the VP3 comprises amino acid residues of 203-736 or 204-736 of SEQ ID NO:26.

[0022] In some embodiments, the VP1 comprises SEQ ID NO:27 with or without the first amino acid residue; the VP2 comprises amino acid residues 138-736 or 139-736 of SEQ ID NO:27; and the VP3 comprises amino acid residues of 203-736 or 204-736 of SEQ ID NO:27.

[0023] In some embodiments, the Rep78 comprises SEQ ID NO:21 with or without the first amino acid residue; and/or the Rep52 comprises SEQ ID NO:23 with or without the first amino acid residue.

[0024] These embodiments encompass helper proteins (Rep78, Rep52, VP1, VP2, and VP3) that do not contain the first amino acid residue encoded by the coding sequence (i.e., the amino acid encoded by the start codon) because the producing cell cleaves off that first amino acid residue. These embodiments also encompass helper proteins whose first amino acid residues are encoded by a non-canonical start codon such as those described herein or otherwise known in the art.

[0025] In another aspect, the present disclosure also provides a recombinant AAV virion produced in the insect cell herein, its use in treating a human patient in need of the transgene, its use in the manufacture of a medicament for such treatment, and a pharmaceutical composition comprising the recombinant AAV virion and pharmaceutically acceptable carrier.

[0026] In another aspect, the present disclosure provides a method of producing a recombinant AAV virion, comprising providing an insect cell herein, culturing the insect cell under conditions that allow expression of the recombinant AAV genome and packaging of the recombinant AAV genome within an AAV capsid comprising the VP1, VP2, and VP3 proteins, and isolating the recombinant AAV from the culture. Also provided are a combination (e.g., a composition) of the expression cassettes for making the AAV virion in insect cells.

[0027] Other features, objectives, and advantages of the invention are apparent in the detailed description that follows. It should be understood, however, that the detailed description, while indicating embodiments and aspects of the invention, is given by way of illustration only, not limitation. Various changes and modification within the scope of the invention will become apparent to those skilled in the art from the detailed description.

BRIEF DESCRIPTION OF THE FIGURES

[0028] **FIG. 1A** shows the alignment of the VP1/VP2 amino acid sequences from AAV serotypes 3B, 5, 6, 8, and 9 between amino acid residues 155 and 207 (numbering based on

consensus sequence). N-terminal Edman degradation sequencing was performed on the proteolytic cleavage products cored from an SDS-PAGE gel to identify the proteolytic cleavage site in AAV6 VP1/VP2. The cleavage site maps to residues G₁₉₀E₁₉₁. Multiple sequence alignment was generated using the Geneious software package and default settings for ClustalW alignment. The VP3 start site is indicated by the triangle at residue 205. **FIG. 1A** discloses SEQ ID NOs:28-33, respectively, in order of appearance.

[0029] **FIG. 1B** shows the alignment of the VP1/VP2 amino acid sequences from AAV serotypes 1, 2, 3B, 4, and 6-11 between amino acid residues 155 and 205 (numbering based on consensus sequence). Multiple sequence alignment was generated using the Geneious software package and default settings for ClustalW alignment. **FIG. 1B** discloses SEQ ID NOs:34-38, 73, and 39-43, respectively, in order of appearance.

[0030] **FIG. 1C** is a table comparing corresponding amino acid positions from AAV serotypes 1, 4, 6, 7, 8, and 11 between amino acid residues 158-203 as aligned in **FIG. 1B**. The table indicates amino acid mutations that may prevent proteolytic cleavage in these serotypes. Amino acid residue numbers are based on the consensus sequence shown in **FIG. 1B**.

[0031] **FIG. 2** shows the substitution of a 45-amino acid stretch of an AAV9 VP1/VP2 (SEQ ID NO:4) for the corresponding region in the AAV6 VP1/VP2 sequence (SEQ ID NO:3), which abolishes proteolytic cleavage of the resultant chimeric VP1/VP2 proteins (“AAV6/9 VP1/VP2”). The figure shows the alignment of the transplanted AAV9 *Cap* nucleotide sequence and the AAV6 *Cap* nucleotide sequence that is replaced in AAV9 Transplant; the arrow “A” indicates the AAP start codon. AAV9 Transplant: baculoviral helper vector whose AAV6 *Cap* gene contains a sequence from the AAV9 *Cap* gene. **FIG. 2** discloses SEQ ID NOs:44-45, respectively, in order of appearance.

[0032] **FIGs. 3A** and **3B** illustrate variants of AAV6/9 VP1/VP2 prevented cleavage. **FIG. 3A**: a sequence alignment of Variants 1, 2, 3, and 4 of AAV6/9 VP1/VP2 (numbering according to SEQ ID NO:1). **FIG. 3B**: partial alignment of the *cap/AAP* nucleotide sequences and the AAP amino acid sequences (in rectangle) of AAV6, AAV9 Transplant, Variant 1, Variant 2, Variant 3, and Variant 4. The AAP amino acid sequence of AAV9 Transplant has six mutations (P8Q, H10L, L12Q, Q17P, L21Q, and L22V) relative to that of AAP6 in the indicated region, while the AAP amino acid sequences of Variants 1-3 each have only two mutations (L21Q and L22V). Variant 4 has the native AAP6 sequence in the indicated region. **FIG. 3A** discloses

SEQ ID NOs:46-51 and **FIG. 3B** discloses SEQ ID NOs:52, 67, 53, 68, 54, 69, 55, 70, 56, 71, 57, and 72, all respectively, in order of appearance.

[0033] **FIG. 4A** shows the alignment of the assembly-activating protein (AAP) amino acid sequences from AAV serotypes 1, 2, 3B, 4, and 6-11 in the indicated region. Amino acid residue numbers are based on the consensus sequence. Multiple sequence alignment was generated using the Geneious software package and default settings for ClustalW alignment. **FIG. 4A** discloses SEQ ID NOs:58-62, 8, 63-64, 9, and 65-66, respectively, in order of appearance.

[0034] **FIG. 4B** shows the amino acid differences in the indicated AAP region (consensus numbering) between (i) AAV1, AAV2, AAV4, AAV6, AAV7, AAV8, AAV10, or AAV11 and (ii) AAV9 or AAV3B. Amino acid residue numbers are based on the consensus sequence shown in **FIG. 4A**.

[0035] **FIG. 5** is a schematic diagram showing two baculoviral helper systems for producing AAV in insect cells. The start codons for the Rep78, Rep52, VP1, and VP2/VP3 coding sequences, along with their transcription direction, are shown. Both systems involve two baculoviral vectors. A coding sequence for a recombinant AAV genome comprising a gene of interest (GOI) flanked by a pair of AAV inverted terminal repeats (ITR) can be placed on a separate baculoviral vector, or placed on the same vector as the Rep52 coding sequence.

DETAILED DESCRIPTION OF THE INVENTION

[0036] The present disclosure provides improved baculovirus-insect cell systems and related compositions for producing potent rAAV capsids at high yield. The present rAAV production systems utilize dual baculoviral helper vectors. The first vector carries an expression cassette for Rep78 and an expression cassette for VP1 or VP2/VP3, while the second vector carries an expression cassette for Rep52 and an expression cassette for the remaining capsid protein(s) (VP2/VP3 if the first vector expresses VP1, or VP1 if the first vector expresses VP2/VP3). In some embodiments, the Rep78 cassette uses a promoter identical (or having similar strength) to the promoter in the Rep52 cassette, and optionally has a suboptimal start codon, such that the Rep78 and Rep52 proteins can be produced with an optimal stoichiometry. In other embodiments, the Rep78 cassette uses a promoter weaker than the promoter in the Rep52 cassette, but has a canonical start codon, such that the Rep78 and Rep52 proteins can be produced with an optimal stoichiometry.

[0037] In some embodiments, the VP1 cassette uses a promoter identical (or having similar strength) to the promoter in the VP2/VP3 cassette, and optionally has a suboptimal start codon, such that the three capsid proteins can be produced with an optimal stoichiometry. In other embodiments, the VP1 cassette uses a promoter weaker than the promoter in the VP2/VP3 cassette, but has a canonical start codon, such that the capsid proteins can be produced with an optimal stoichiometry.

[0038] To reduce the number of baculoviral vectors needed to transduce the rAAV-producing cells, the coding sequence for the rAAV genome may also be included in one of the two vectors, for example, in the second vector (i.e., the vector that carries the Rep52 expression cassette).

[0039] The present inventors have discovered that expressing Rep78 and Rep52 from separate cassettes and expressing VP1 and VP2/VP3 from separate cassettes greatly improve the potency and/or yield of the rAAV produced in the insect cell systems. As demonstrated in the Working Example below, the present systems produce rAAV with a multi- fold increase in potency and/or yield as compared to production systems in which the capsid proteins are produced from the prevailing one-vector system, in which the capsid proteins are produced from a single *Cap* gene and the Rep proteins are produced from a single *Rep* gene.

[0040] The present production systems can be used to produce rAAV of any serotype, such as AAV1, AAV2, AAV3, AAV3A, AAV3B, AAV4, AAV5, AAV6, AAV7, AAV8, AAV8.2, AAV9, AAVrh10, or an engineered serotype. The coding sequences of the capsid proteins may be derived from any desired serotype.

[0041] The present systems also can be used to produce rAAV of a pseudotype such as AAV2/8, AAV2/5, or AAV2/6. By “pseudotype,” “pseudotyped,” or “cross-packaged” rAAV is meant a recombinant AAV whose capsid is replaced with the capsid of another AAV serotype, to, for example, alter transduction efficacy or tropism profiles of the virus (*see, e.g., Balaji et al., J Surg Res. (2013) 184(1):691-8*). For example, an AAV2/8 pseudotyped AAV contains capsids of AAV8 and the ITRs derived from AAV2.

[0042] The present systems also may be used to produce a chimeric or hybrid AAV. By “chimeric” or “hybrid” rAAV is meant a recombinant AAV whose capsid is assembled from capsid proteins derived from different serotypes and/or whose capsid proteins are chimeric proteins with sequences derived from different serotypes (e.g., serotypes 1 and 2; *see, e.g., Hauck et al., Mol Ther. (2003) 7(3):419-25*).

I. Capsid Protein Expression Cassettes

[0043] In mammalian cells, the *Cap* gene is transcribed into two mRNAs under the control of the mammalian p40 promoter. One mRNA is translated to VP1, and the other into VP2 and VP3. The translation start codon for VP2 is ACG, a suboptimal start codon that often causes ribosome skip, whereas the start codon for VP3 is the canonical ATG. Through an interplay of alternative splicing and the weak VP2 start codon, the *Cap* gene produces VP1, VP2, and VP3 in an apparent protein ratio of 1:1:10 (*see, e.g., Berns and Parrish, supra*).

[0044] Failures of prior insect cell systems to produce rAAV with high yield and potency have been attributed in part to the inability of these systems to achieve an optimal stoichiometry of the capsid proteins. In the present rAAV production systems, two separate expression cassettes are used for expressing VP1 and VP2/VP3. The use of separate expression cassettes for VP1 and VP2/VP3 surprisingly leads to high yield production of potent rAAV.

[0045] In some embodiments, the expression cassette for VP1 and the expression cassette for VP2/VP3 use insect promoters of the same or similar strengths. For example, the two cassettes may use identical promoters. Examples of insect promoters that can be used are a p10 promoter, a p35 promoter, a polyhedron (polyh) promoter, an E1 promoter, a Δ E1 promoter, a 4 $\times$ Hsp27 EcRE+minimal Hsp70 promoter, and a basic promoter.

[0046] The expression cassettes may contain additional regulatory elements, such as Kozak sequences; transcription initiation and termination sites (either modified or unmodified); mRNA splice sites (either modified or unmodified, within or adjacent to the polypeptide coding sequence; and viral, eukaryotic, or prokaryotic RNA elements that control splicing, nuclear export, localization, stabilization, or translation of mRNAs (e.g., Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) (Zufferey et al., *J Virol.* (1999) 73(4):2886-92), MMLV/MPMV, eukaryotic constitutive transport element (CTE) (Li et al., *Nature* (2006) 443(7108):234-7), RNA zipcodes (Jambhekar and DeRisi, *RNA* (2007) 13(5):625-42), and omega or other 5'-UTR RNA elements that increase translational efficiency).

[0047] In some embodiments, the coding sequences for the capsid proteins may be modified to further enhance AAV yield and potency. For example, the coding sequences may be codon-modified to increase mRNA stability, translation efficiency, and/or DNA vector stability in insect cells. The start codon regions of the coding sequences may also be modified, to further

fine tune expression levels of the capsid proteins; for example, the VP1 start codon may be changed from wildtype ATG to a suboptimal codon, such as the start codon of VP2 (ACG), such that VP1 expression levels are lower. The VP1 start codon may also be changed to other suboptimal start codons such as TTG, CTG, and GTG.

[0048] To avoid production of VP2 and VP3 or any other by-product peptide from the VP1 expression cassettes, the VP1 coding sequence may be mutated to remove the native start codons for the embedded VP2 and VP3 ORFs, any out-of-frame ATG sites, any undesired splice acceptor sites, any cryptic promoter sequences, and/or any destabilizing elements (*see, e.g.*, Smith et al., *supra*).

[0049] In some embodiments, the present production systems produce rAAV6. The complete amino acid sequence of an AAV6 VP1 protein is shown below, where the start site of VP2 (T) and the mutated start site of VP3 (changed from the native M) are boldfaced and underlined:

```

1      XAADGYLPDW LEDNLSEGIR EWWDLKPGAP KPKANQQKQD DGRGLVLPGY KYLGPFNGLD
61     KGEPVNAADA AALEHDKAYD QQLKAGDNPY LRYNHADAEF QERLQEDTSF GGNLGRAVFQ
121    AKKRVLEPFG LVEEGAKTAP GKRRPVEQSP QEPDSSSGIG KTGQQPAKKR LNFGQTGDSE
181    SVPDPQPLGE PPATPAAVGP TTVVASGGGAP MADNNEGADG VGNASGNWHC DSTWLGDRVI
241    TTSTRTWALP TYNNHLYKQI SSASTGASND NHYFGYSTPW GYFDFNRFHC HFSPRDWQRL
301    INNNWGFRPK RLNFKLFNIQ VKEVTTNDGV TTIANNLTST VQVFSDSEYQ LPYVLGSAHQ
361    GCLPPFPADV FMIPQYGYLT LNNGSQAVGR SSFYCLEYFP SQMLRTGNNF TFSYTFEDVP
421    FHSSYAHSQS LDRLMNPLID QYLYYLNRTQ NQSGSAQNKD LLFSRGSPAG MSVQPKNWLP
481    GPCYRQQRVS KTKTDNNNSN FTWTGASKYN LNGRESIINP GTAMASHKDD KDKFFPMGSV
541    MIFGKESAGA SNTALDNVMI TDEEEIKATN PVATERFGTV AVNLQSSSTD PATGDVHVMG
601    ALPGMWQDR DVYLQGPiWA KIPHTDGHFH PSPLMGGFGL KHPPPQILIK NTPVPANPPA
661    EFSATKFASF ITQYSTGQVS VEIEWELQKE NSKRWNPEVQ YTSNYAKSAN VDFTVDNNGL
721    YTEPRPIGTR YLTRPL (SEQ ID NO:1)

```

In the sequence above, X in position 1 may be M (wildtype; source: GenBank AAB95450.1), T, L, or V. In other embodiments, the VP1 start residue may be another amino acid encoded by a non-canonical start codon such as a suboptimal start codon (*see, e.g.*, Kearse et al., *Genes Dev.* (2017) 31:1717-31). In some embodiments, the mutated VP3 start site in the VP1 protein is an amino acid other than the V shown above. In some embodiments, the AAV6 VP2 protein produced herein comprises a sequence spanning amino acids 138 to 736 of SEQ ID NO:1 where the VP3 start site is not mutated (i.e., is the native methionine) and the AAV6 VP3 protein comprises a sequence spanning amino acids 204-736 of SEQ ID NO:1 where the N-terminal amino acid is the native M.

[0050] Additional, non-limiting examples of coding sequence modifications are described below. *See also* WO 2020/168145, the disclosure of which is incorporated hereby by reference in its entirety.

A. Modifications to Reduce Proteolytic Degradation of VP1 and VP2

[0051] In some embodiments, the VP1 and VP2 coding sequences may be mutated in two or more codons such that the VP1 and VP2 proteins encoded thereby are resistant to proteolytic degradation during the production process. Because the VP1 coding sequence also contains the open reading frame for AAP, the codon changes may also cause mutations in the AAP. These mutations in VP1 and VP2 further improve the rAAV's infectivity, i.e., potency.

[0052] In some embodiments, the present improved baculovirus-insect cell systems can be used to produce any AAV serotype that is susceptible to proteolysis in insect cells. Such AAV serotypes may include, for example, AAV1, AAV6, AAV8, or variants thereof, or any pseudotyped or chimeric rAAV whose VP1 and VP2 proteins are susceptible to proteolysis in insect cells.

[0053] In an improved AAV production system of the present disclosure, two or more point mutations are introduced to AAV VP1 and VP2 proteins derived from, e.g., AAV1, AAV4, AAV6, AAV7, AAV8, or AAV11, to remove the sites susceptible to proteolysis in insect cells. The introduced point mutations may be residues identical to those at the corresponding positions in AAV2, AAV3, AAV5, AAV9, or AAV10.

[0054] By "corresponding" amino acid residue or region is meant an amino acid residue or region that aligns with (though not necessarily identical to) the reference residue or region, when the subject sequence and the reference sequence containing the residues or regions are aligned to achieve maximum homology (allowing gaps that are recognized in the art). For example, amino acid residue 189 (L) of AAV8 VP1 corresponds to amino acid residue 188 of AAV6 VP1.

[0055] In these systems, the overlapping region shared by the VP1 and VP2 proteins may be mutated to remove proteolytic sites. For example, the rAAV6 VP1 and/or VP2 may comprise mutations relative to the wildtype at two or more residues in a region corresponding to residues 138-203 (e.g., residues 151-201, residues 157-201, or residues 185-194 (i.e., PQPLGEPPAT (SEQ ID NO:2), where cleavage has been shown between G and E)) of SEQ ID NO:1, where the mutated residues are selected from a group consisting of residues 157, 162, 164, 179, 188, 194, 196, 197, 200, and 201. In some embodiments, the AAV6 VP1 and/or VP2 comprise two or

more mutations selected from the group consisting of S157A, T162S, Q164A, S179T, L188I, T194A, A196S, A197G, P200S, and T201L (numbering according to SEQ ID NO:1). For example, an AAV6 VP1 and/or VP2 may have the mutations (i) S179T, L188I, T194A, A196S, A197G, P200S, and T201L (“Variant 1”); (ii) S179T, L188I, T194A, A196S, and A197G (“Variant 2”); (iii) T194A, A196S, A197G, P200S, and T201L (“Variant 3”); (iv) S157A, T162S, and Q164A (“Variant 4”); or (v) P200S and T201L. For convenience, only the residue numbers in VP1 are referred to herein. The numbers of the corresponding residues in VP2 can be readily discerned from SEQ ID NO:1 above. For example, residue S157 in VP1 is residue S20 in VP2.

[0056] In particular embodiments, the AAV6 VP1 and/or VP2 comprise the mutation L188I and one or more other mutations in the group. For example, the AAV6 VP1 and/or VP2 may have the mutations L188I, P200S, and T201L.

[0057] In some embodiments, the AAV6 VP1 and/or VP2 comprise all ten mutations of S157A, T162S, Q164A, S179T, L188I, T194A, A196S, A197G, P200S, and T201L, such that their sequence in the region corresponding to residues 157-201 in SEQ ID NO:1 is as follows.

AGIGKSGAQP AKKRLNFGQT GDTEVPDPQ PIGEPPAAPS GVGSL (SEQ ID NO:4)

This sequence is identical to the sequence in the corresponding region in AAV9 VP1 and VP2.

[0058] Thus, to generate a modified VP1 coding sequence, one can replace the coding sequence for residues 157-201 of VP1 (i.e., residues 20-64 of VP2; SEQ ID NO:3) with the coding sequence for the corresponding amino acid sequence (SEQ ID NO:4) of AAV9, using well known molecular clone techniques; the resultant modified baculoviral AAV6 helper vector is referred to herein as “AAV9 Transplant” and the resultant VP1 proteins are referred to herein as “AAV6/9 VP1.” The same transplant may be made to mutate the same region in VP2, resulting in an AAV6/9 VP2. In some embodiments, the portion of the AAV6 VP1 gene sequence that is replaced comprises the underlined sequence shown below (*see also* FIG. 2):

AAGAGCCAGACTCCTCCTCGGGCATTGGCAAGACAGGCCAGCAGCCCGCTAAAAAGAGACTCAATTTTGG
TCAGACTGGCGACTCAGAGTCAGTCCCCGACCCACAACCTCTCGGAGAACCTCCAGCAACCCCGCTGCT
GTGGGACCTACTACAATGGCTTCAGGCGGTGGCGCACCAATGG (SEQ ID NO:13)

[0059] In certain embodiments, the underlined portion in SEQ ID NO: 13 is replaced by a corresponding *Cap* gene sequence from AAV9 to generate an AAV9 Transplant disclosed

herein. In particular embodiments, the AAV9 *Cap* sequence transplanted to the AAV6 *Cap* gene in an AAV9 Transplant comprises the underlined sequence shown below (*see also* FIG. 2):

AAGAGCCAGACTCCTCCGCGGGTATTGGCAAATCGGGTGCACAGCCCGCTAAAAAGAGACTCAATTTCCG
TCAGACTGGCGACACAGAGTCAGTCCCAGACCCTCAACCAATCGGAGAACCTCCCGCAGCCCCCTCAGGT
GTGGGATCTCTTACAATGGCTTCAGGCGGTGGCGCACCAATGG (SEQ ID NO:14)

[0060] In other embodiments, a modified *cap6* gene in the baculoviral AAV6 helper construct can be generated by replacing the coding sequence for residues 157-201 of VP1 in SEQ ID NO:1 (i.e., residues 20-64 of VP2; SEQ ID NO:3) with the coding sequence for the corresponding amino acid sequence from a serotype whose VP1 and/or VP2 are resistant to proteolytic cleavage in insect cells, e.g., AAV2, AAV3, AAV5, or AAV10.

[0061] In other embodiments, the VP1/VP2 proteins of AAV serotypes, such as AAV1, AAV4, AAV7, AAV8, or AAV11, can be mutated such that they contain one or more mutations shown in FIG. 1C. The resultant proteins are expected to be more resistant to proteolysis in insect cells. Unlimiting examples of engineered VP1/VP2 proteins from various AAV serotypes are shown below:

(1) the engineered AAV1 VP1/VP2 proteins may contain one or more of the following mutations: S158T/A, I160T/V, T163A/S/K, Q165K/A, K169R, S180A/T, L189E/I, T195A, A197S/T, A198S/G, V199L, P201T/S, and T202N/L;

(2) the engineered AAV4 VP1/VP2 proteins may contain one or more of the following mutations: T158S/A, I160T/V, K163A/S, K165Q/A, K169R, K171R, V173N, E175G, D176Q/E, E177T, T178G, G179D/E, A180S/T, G181D/E, D182S, G183V, E189L/I, S191Q/E, T192P, S193P, G194A, M196P, S197T, D200G, D201T/S, S202N/L, and E203T;

(3) the engineered AAV6 VP1/VP2 proteins may contain one or more of the following mutations: S158T/A, I160T/V, T163A/S/K, Q165K/A, K169R, S180A/T, L189E/I, T195A, A197S/T, A198S/G, V199L, P201T/S, and T202N/L;

(4) the engineered AAV7 VP1/VP2 proteins may contain one or more of the following mutations: T158S/A, I160T/V, K163A/S, Q165K/A, R169K, S180A/T, L189E/I, S197T, S198G, V199L, and G202N/L;

(5) the engineered AAV8 VP1/VP2 proteins may contain one or more of the following mutations: T158S/A, I160T/V, K163A/S, Q165K/A, R169K, S180A/T, L189E/I, S197T, G198S, V199L, P201T/S, and N202L; and

(6) the engineered AAV11 VP1/VP2 proteins may contain one or more of the following mutations: S158T/A, I160T/V, K163A/S, K165Q/A, R169K, E175G, E176Q, D177T, T178G, G179D/E, A180S/T, G181D/E, D182S, G183V, E189L/I, S191Q/E, D192P, T193P, S194A, M196P, S197T, S200G, D201T/S, I202N/L, and E203T (consensus numbering; *see* FIG. 1C).

[0062] In some embodiments, the VP1/VP2 proteins from an AAV serotype susceptible to proteolysis in insect cells are mutated to contain one or more mutations at the following amino acid residues (consensus numbering in FIG. 1C): 158, 163, 165, 180, 189, 195, 197, 198, 201, and 202. (These residues correspond respectively to amino acid residues 157, 162, 164, 179, 188, 194, 196, 197, 200, and 201 according to the numbering of SEQ ID NO:1 (AAV6) or a corresponding sequence from another serotype aligned to show maximal homology to SEQ ID NO:1.) In further embodiments, the VP1/VP2 proteins may additionally contain one or more mutations at the following amino acid residues (consensus numbering in FIG. 1C): 160, 169, 171-179, 181-183, 191-194, 196, 199, and 203.

[0063] In some embodiments, the VP1/VP2 proteins from an AAV serotype susceptible to proteolysis in insect cells are mutated to contain one or more mutations: S158T/A, I160T/V, K/T163S/A/T, Q165K/A, K169R, K171R, V173N, E175G, D176Q/E, D/E177T, T178G, G179D/E, S180A/T, G181D/E, D182S, G183V, L189E/I, Q/S191E, T/D192P, S/T193P, G/S194A, T/G195A, M196P, A197S/T, A198G/S, V199L, S200G, D/P201S/T, T/S202N/L, and E203T (consensus numbering in FIG. 1C).

[0064] In some embodiments, the engineered VP1/VP2 proteins may contain one or more of the following mutations: S158A, T163S, Q165A, S180T, L189I, T195A, A197S, A198G, P201S, and T202L (consensus number in FIG. 1C). (These residues correspond respectively to amino acid residues S157A, T162S, Q164A, S179T, L188I, T194A, A196S, A197G, P200S, and T201L according to the numbering of SEQ ID NO:1 (AAV6) or a corresponding sequence from another serotype aligned to show maximal homology to SEQ ID NO:1.)

[0065] The mutations described herein remove sites in the AAV capsid proteins susceptible to proteolytic cleavage in insect cells. Thus, a helper construct containing the modified *Cap* gene will give rise to rAAV products of higher purity and uniformity, as well as improved capsid protein.

[0066] It has been surprisingly found that the point mutations introduced herein to the AAV VP1/VP2 unique region (i.e., region common to VP1 and VP2 but absent in VP3) can also

significantly improve the rAAV's potency through a mechanism that does not rely on solely on the prevention of proteolytic cleavage.

B. Modifications to Further Improvement of rAAV Production Yield

[0067] Additionally or alternatively, the VP1 coding sequence in the baculoviral helper construct is mutated in one or more codons in the region coding for the VP1 PLA2 domain such that the engineered PLA2 domain acquires higher enzymatic activity. It has been shown that higher PLA2 enzymatic activity may lead to a higher yield of rAAV in the insect cell production system.

[0068] The present disclosure provides a baculovirus-insect system in which the VP1 coding sequence is altered in the VP1 unique region (i.e., region present in VP1 but not in VP2 or VP3) to improve the production yield of the rAAV in insect cells. The VP1 unique region (corresponding to residues 1-137 of SEQ ID NO:1) contains the PLA2 domain, and the mutations in the region may increase the enzymatic activity of the PLA2 domain in the resultant VP1 protein.

[0069] In some embodiments, the baculoviral helper construct of the present disclosure provides helper functions for the production of rAAV6 or rAAV9 and the VP1 expression cassette on a helper construct includes a modified AAV6 or AAV9 *Cap* gene (*Cap6* or *Cap9*, respectively) with a mutated PLA2 domain. The mutated PLA2 domain may comprise mutations relative to the wildtype at one or more positions in a region corresponding to residues 1-137 (e.g., 52-97 or 67-92) of SEQ ID NO:1, where the positions are selected from a group consisting of residues 67, 81, 84, 85, and 92 (numbering of SEQ ID NO:1). In some embodiments, the AAV6 VP1 protein comprises one or more mutations selected from the group consisting of A67E, Q81R, K84D, A85S, and R92K (numbering according to SEQ ID NO:1). In particular embodiments, the AAV6 VP1 protein comprises all of the five mutations, such that its sequence in the region corresponding to residues 52-97 (SEQ ID NO:5) in SEQ ID NO:1 is as follows.

YLGPFGNGLDK GEPVNEADAA ALEHDKAYDR QLDSGDNPYL KYNHAD (SEQ ID NO:6)

This sequence is identical to the sequence in the corresponding region in AAV2 VP1. Thus, to generate a modified *cap6* gene in the baculoviral AAV6 helper construct, one can replace the coding sequence for residues 52-97 or 67-92 of VP1 with the coding sequence for the corresponding amino acid sequence of AAV2, using well known molecular cloning techniques.

[0070] It has been surprisingly found that the point mutations introduced herein to the AAV VP1 unique region can significantly improve the VP1 PLA2 domain's enzymatic activity and also lead to significant improvement (e.g., two or more fold, three or more fold, four or more fold, or five or more fold) in the yield of the rAAV produced in insect cells.

[0071] In particular embodiments, the engineered *Cap* gene encodes an AAV6 VP1 protein containing both the aforementioned mutations in the PLA2 domain and the aforementioned mutations that remove the proteolytic sites. One exemplary modified AAV6 VP1 protein ("AAV6/2/9 VP1") has the following sequence (where V203 may be M203 or another amino acid instead):

```

1      MAADGYLPDW LEDNLSEGIR EWWDLKPGAP KPKANQQKQD DGRGLVLPGY KYLGPFNGLD
61     KGEPVNEADA AALEHDKAYD RQLDSGDNPY LKYNHADA EF QERLQEDTSF GGNLGRAVFQ
121    AKKRVLPEPFG LVEEGAKTAP GKRRPVEQSP QEPDSSAGIG KSGAQPAKKR LNFQQTGDTE
181    SVPDPQPIGE PPAAPSGVGS LTVASGGGAP MADNNEGADG VGNASGNWHC DSTWLGDRVI
241    TTSTRTWALP TYNNHLYKQI SSASTGASND NHYFGYSTPW GYFDNFRFHC HFSPRDWQRL
301    INNNWGFRPK RLNFKLFNIQ VKEVTNDGV TTIANNLTST VQVFS DSEYQ LPYVLGSAHQ
361    GCLPPFPADV FMIPQYGYLT LNNGSQAVGR SSFYCLEYFP SQMLRTGNNF TFSYTFEDVP
421    FHSSYAHSQS LDRLMNPLID QYLYYLNRTQ NQSGSAQNKD LLFSRGSPAG MSVQPKNWLP
481    GPCYRQQRVS KTKTDNNNSN FTWTGASKYN LNGRESIINP GTAMASHKDD KDKFFPMSGV
541    MIFGKESAGA SNTALDNVMI TDEEEIKATN PVATERFGTV AVNLQSSSTD PATGDVHVMG
601    ALPGMVWQDR DVYLQGIWA KIPHTDGHFH PSPLMGGFGL KHPPPQILIK NTPVPANPPA
661    EFSATKFASF ITQYSTGQVS VEIEWELQKE NSKRWNPEVQ YTSNYAKSAN VDFTVDNNGL
721    YTEPRPIGTR YLTRPL (SEQ ID NO:7)

```

Other exemplary modified AAV6 VP1 protein has the same sequence as SEQ ID NO:7 except that the start residue in position 1 is not present, or is T, L, V or another amino acid encoded by a non-canonical start codon such as a suboptimal start codon. A baculoviral helper construct encoding one of these modified AAV6 VP1 proteins can be used to produce rAAV6 or a pseudotyped or chimeric rAAV with improved potency and yield in insect cells.

[0072] In some embodiments, the engineered *Cap* gene encodes an AAV9 VP1 protein containing an AAV2 PLA2 domain. The wildtype AAV9 VP1 sequence (UniProtKB - Q6JC40 (Q6JC40_9VIRU)) is shown below, where the five amino acid residues differing from the corresponding ones in the AAV9/2 VP1 in the PLA2 domain are indicated with boldface and boxes:

```

1      MAADGYLPDW LEDNLSEGIR EWWALKPGAP QPKANQQHQD NARGLVLPGY KYLGPFNGLD
61     KGEPVNADA AALEHDKAYD QQLKAGDNPY LKYNHADA EF QERLKEDTSF GGNLGRAVFQ
121    AKKRLLLEPLG LVEEAAKTAP GKRRPVEQSP QEPDSSAGIG KSGAQPAKKR LNFQQTGDTE
181    SVPDPQPIGE PPAAPSGVGS LTMASGGGAP VADNNEGADG VGSSSGNWHC DSQWLGDRI
241    TTSTRTWALP TYNNHLYKQI SNSTSGGSSN DNAYFGYSTP WGYFDNFRFH CHFSPRDWQR
301    LINNNWGFRP KRLNFKLFNI QVKEVTDNNG VKTIANNLTS TVQVFTDSY QLPYVLGSAH
361    EGCLPPFPAD VEMIPQYGYL TLNDGSQAVG RSSFYCLEYF PSQMLRTGNN FQFSYEFENV

```

```

421 PFHSSYAHSQ SLDRLMNPLI DQYLYYLSKT INSGGQNQQT LKFSVAGPSN MAVQGRNYIP
481 GPSYRQQRVS TTVTQNNNSE FAWPGASSWA LNGRNSLMNP GPAMASHKEG EDRFFPLSGS
541 LIFGKQGTGR DNVDADKVM I TNEEEIKTTN PVATESYQGV ATNHQSAQAQ AQTGWVQNQG
601 ILPGMVWQDR DVYLQGP IWA KIPHTDGNFH PSPLMGGFGM KHPPPQILIK NTFVPADPPT
661 AFNKDKLNSF ITQYSTGQVS VEIEWELQKE NSKRWNPEIQ YTSNYYKSN VEFVNTTEGV
721 YSEPRPIGTR YLTRNL (SEQ ID NO:11)

```

[0073] The wildtype AAV9 and AAV2 sequences in this region (residues 52-97 of SEQ ID NO:11) are shown below, where the five amino acid changes relative to wildtype AAV9 after the AAV2 sequence transplant are boldfaced and boxed:

```

AAV9: YLGPNGLDK GEVNAADAA ALEHDKAYDQ QLKAGDNPYL KYNHAD (SEQ ID NO:12)
AAV2: YLGPENGLDK GEVNEADAA ALEHDKAYDR QLDSGDNPYL KYNHAD (SEQ ID NO:6)

```

[0074] In some embodiments, M203 in SEQ ID NO:11 may be replaced by V203 or another amino acid encoded by a noncanonical start codon.

C. Modifications to AAP

[0075] In some embodiments, the VP1 coding sequence also encodes an engineered AAP that has improved abilities to stabilize capsid protein and to facilitate capsid assembly. It has been shown that when residues 1-30 of AAV6 AAP (“AAP6”) are changed to residues 1-30 AAV9 AAP (“AAP9”), the potency of the rAAV produced with the engineered helper construct is significantly increased. Residues 1-30 of AAP6 and AAP9 are shown below, where the six amino acid differences in wildtype AAP9 relative to wildtype AAP6 are indicated by boldface and boxes:

```

AAV6 AAP: MATQSQSPTH NLSENLQQPP LLWDLLQWLQ (SEQ ID NO:8)
AAV9 AAP: MATQSQSQTL NQSENLPQPP QVWDLLQWLQ (SEQ ID NO:9)

```

The complete AAV6 AAP wild-type sequence is shown below:

```

MATQSQSPTH NLSENLQQPP LLWDLLQWLQ AVAHQWQTIT KAPTEWVMPQ EIGIAIPHW
ATESSPPAPE HGPCPPITTT STSKSPVLQR GPATTTTSTA TAPPGILIS TDSTAISHHV
TGSDSSTTIG DSGPRDSTSS SSTSRSRRSR RMMASRPSLI TLPARFKSSR TRSTSCRTSS
ALRTRAASLR SRRTCS (SEQ ID NO:10)

```

[0076] In some embodiments, the engineered AAP protein comprises one or more mutations at residues 8, 10, 12, 17, 21, and 22 (numbering based on consensus sequence in FIG. 4A). In further embodiments, the engineered AAP protein may comprise one or more of the following amino acid residues: Q8, L10, Q12, P17, Q21, and V22. In some embodiments, the engineered AAP protein may comprise one or more of the following mutations relative to the wildtype AAP sequence: P8Q, H10L, L12Q, Q17P, L21Q, and L22V. Unlimiting examples of engineered, improved AAP proteins are shown below:

(1) engineered AAP1 (AAP of serotype 1) comprising one or more of the following mutations: P8Q, I9T, H10L, L12Q, L16H, Q17P, L21Q, L22V, and L26I;

(2) engineered AAP2 (AAP of serotype 2) comprising one or more of the following mutations: E2A, T5S, Y7S, L8Q, P10L, S11N, L12Q, D14E, S15N, H16L, Q17P, L21Q, E24D, I26L, and R27Q;

(3) engineered AAP4 (AAP of serotype 4) comprising one or more of the following mutations: E2A, Q3T, A4Q, T5S, D6Q, P7S, L12Q, R13S, D14E, Q15N, L16H, P17Q, E18Q, C22V, L23W, M24D, T25L, V26I/L, R27Q, and C28W;

(4) engineered AAP6 (AAP of serotype 6) comprising one or more of the following mutations: P8Q, H10L, L12Q, L16H, Q17P, L21Q, L22V, and L26I;

(5) engineered AAP7 (AAP of serotype 7) comprising one or more of the following mutations: P8Q, L12Q, L16H, Q17P, L21Q, L22V, and L26I;

(6) engineered AAP8 (AAP of serotype 8) comprising one or more of the following mutations: F7S, L12Q, L16H, Q17P, R19P, L21Q, and I26L;

(7) engineered AAP10 (AAP of serotype 10) comprising one or more of the following mutations: S3T, P8Q, Q12L, L16H, Q17P, A19P, L21Q, and V26I/L; and

(8) engineered AAP11 (AAP of serotype 11) comprising one or more of the following mutations: E2A, P3T, E4Q, T5S, D6Q, P7S, L12Q, R13S, D14E, Q15N, I16L/H, P17Q, A18Q, C22V, L23W, Q24D, T25L, L26I, K27Q, and C28W (*see also* FIG. 4B).

[0077] To generate engineered AAP protein, one can perform point mutations. Because the open reading frame encoding AAP is embedded in the *Cap* gene and is translated merely by a frameshift, one may also transplant from another serotype a portion of the *Cap* gene containing the coding sequence for an N terminal portion of AAP (e.g., residues 1-30, residues 1-28, residues 2-30, residues 2-28, etc.). In some embodiments, an AAV9 *Cap* gene portion coding for an AAV9 VP1/VP2 unique region (i.e., region common to VP1 and VP2 but absent in VP3), for example, a *cap9* sequence comprising nucleotides 18-151 of SEQ ID NO:14 is substituted for the corresponding region of *cap6*, to generate an engineered VP1 expression cassette expressing an engineered AAV6 VP1 with a portion of AAV9 VP1 (e.g., so as to remove the proteolytic site(s) as discussed above), as well as an engineered AAP6 whose N-terminal 1-30 amino acid residues are now identical to those of AAP9. In some embodiments, the AAP mutations are generated by the same method as described above for the AAV9 *Cap* gene transplant.

[0078] Additional examples of capsid proteins are described in the Examples below.

II. Rep Protein Expression Cassettes

[0079] In mammalian cells, the *Rep* gene is transcribed into a Rep78-encoding RNA under the control of the mammalian p5 promoter and a Rep52-encoding RNA under the mammalian p19 promoter situated within the Rep78 ORF (*see, e.g.*, Urabe et al., 2002, *supra*). The start codons for both Rep78 and Rep52 are ATG. It has been found that overexpression of Rep78 relative to Rep52 adversely impacts the yield of rAAV.

[0080] Failures of prior insect cell systems to produce rAAV with high yield have been attributed in part to the inability of these systems to achieve an optimal stoichiometry of the Rep proteins. In the present rAAV production systems, two separate expression cassettes are used for expressing Rep78 and Rep52. In some embodiments, the expression cassette for Rep78 and the expression cassette for Rep52 use insect promoters of the same or similar strengths; but the use of a weaker start codon (e.g., CTG, TTG, GTG, and ACG) for Rep78 helps achieve a desired Rep78:Rep52 protein ratio and high AAV yield. In certain embodiments, the two cassettes may use identical promoters such as those listed above for the capsid expression cassettes. In other embodiments, the Rep78 expression cassette has a canonical start codon (ATG) but uses a weaker promoter than the Rep52 expression cassette to achieve the desired stoichiometry.

[0081] In some embodiments, the Rep expression cassettes may contain additional regulatory elements such as those described above for the capsid protein expression cassettes.

[0082] In some embodiments, the coding sequences for the Rep proteins may be modified to further enhance AAV yield and potency. For example, the coding sequences may be codon-modified to increase mRNA stability, translation efficiency, or DNA vector stability in insect cells. To avoid production of Rep52 or any other by-product peptide from the Rep78 expression cassette, the Rep78 coding sequence may be mutated to remove the start codon for the embedded Rep52 ORF, any off-frame ATG sites, any undesired splice acceptor sites, any cryptic promoter sequences, and/or any destabilizing elements (*see, e.g.*, Smith et al., *supra*). Conversely, the Rep52 coding sequence may be trimmed to remove any Rep78 coding sequence upstream of the Rep52 start codon.

[0083] The use of separate expression cassettes for Rep78 and Rep52, together with the use of a suboptimal start codon for Rep78, surprisingly leads to high yield production of potent rAAV.

III. Production of rAAV in Insect Cells

[0084] Production of rAAV in insect cells can be performed as described previously. *See*, e.g., Urabe et al., 2002 and 2006, *supra*; Chen et al., *supra*; Smith et al., *supra*; Mietzsch et al., *supra*; WO 2007/046703, WO 2007/148971, WO 2009/104964, WO 2013/036118, and WO 2008/024998, all of which are incorporated herein by reference in their entirety.

[0085] The insect cells in the production methods of the present disclosure comprise the dual baculoviral helper vectors described herein. The insect cells may be, without limitation, a cultured cell line such as BTI-TN-5B1-4 derived from *Trichoplusia ni* (High Five™, Thermo Fisher Scientific, Carlsbad, CA), Sf9 cells or Sf21 cells (both of which are derived from *Spodoptera frugiperda*), Sf9 or TN368 cells with mammalian-type glycan profiles (GlycoBac), or Sf-RVN cells (MilliporeSigma), Sf9-13F12 cells (Rhadovirus-free cells; Ma et al., *Virology* (2019) 536:125-33).

[0086] The rAAV may comprise within its capsid an AAV vector containing a transgene of interest. The transgene may encode a reporter protein for detection using biochemical (luciferase, SEAP) or imaging (GFP, Venus, dTomato) techniques. The transgene may encode a therapeutic protein, including, without limitation, a chimeric antigen receptor (CAR), a C-peptide or insulin, collagen VII, IGF-I, lipoprotein lipase, fibrinogen, prothrombin, Factor V, Factor VIII, Factor IX, Factor XI, Factor XII, Factor XIII, von Willebrand factor, prekallikrein, high molecular weight kininogen (Fitzgerald factor), fibronectin, antithrombin III, heparin cofactor II, protein C, protein S, protein Z, protein Z-related protease inhibitor, plasminogen, alpha 2-antiplasmin, tissue plasminogen activator, urokinase, plasminogen activator inhibitor-1, plasminogen activator inhibitor-2, or any enzyme for treating a lysosome storage disease such as alpha-galactosidase A (for treating Fabry disease). The transgene may encode a sequence-specific binding protein (e.g., ZFP, TALE, TALEN, or dCas9). In some embodiments, the ZFP may be a ZFP transcription factor (e.g., a fusion protein in which a ZFP domain is fused to a transcription factor), a zinc finger nuclease (e.g., a fusion protein in which a ZFP domain is fused to a nuclease), or a ZFP base editor (e.g., a fusion protein in which a ZFP domain is fused to a nucleobase editor). The transgene may carry a sequence that can be incorporated into a specific site in the host genome (donor) by homologous recombination or non-homologous end joining. The transgene may encode an immunogenic protein for vaccination (e.g., a tumor antigen).

[0087] The AAV vector may comprise transcription regulatory elements (e.g., promoter and enhancer) that can direct and regulate expression of the transgene in human cells. The AAV vector may also comprise an AAV complete or partial inverted terminal repeat (ITR) on one or both ends of the transgene expression cassette. ITRs are required for packaging and viral integration into the host (human) genome. The AAV vector may be single-stranded or self-complementary.

IV. Pharmaceutical Compositions of rAAV

[0088] The rAAV capsids produced by the present methods can be formulated with a pharmaceutically acceptable carrier as a pharmaceutical composition. Formulations include, without limitation, suspensions in liquid or emulsified liquids. Pharmaceutically acceptable carriers include, for example, water, saline, dextrose, glycerol, sucrose, or the like, and combinations thereof. In addition, the composition may contain auxiliary substances, such as, wetting or emulsifying agents, pH buffering agents, stabilizing agents, or other reagents that enhance the effectiveness of the rAAV pharmaceutical composition.

[0089] The rAAV pharmaceutical composition may be delivered *in vivo* by administration to the patient, for example, by systemic administration (e.g., intravenous, intraperitoneal, intramuscular, subdermal, intrathecal, or intracranial infusion) or local injections. Alternatively, the rAAV can be delivered to cells *ex vivo*, such as cells explanted from a patient (e.g., lymphocytes, bone marrow aspirates, or tissue biopsy) or allogeneic cells (e.g., universal donor cells such as universal CAR T cells), followed by introduction of the treated cells into the patient, usually after selection for cells that have incorporated the rAAV vector.

[0090] Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Exemplary methods and materials are described below, although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention. In case of conflict, the present specification, including definitions, will control. Generally, nomenclature used in connection with, and techniques of, cell and tissue culture, molecular biology, virology, immunology, microbiology, genetics, analytical chemistry, synthetic organic chemistry, medicinal and pharmaceutical chemistry, and protein and nucleic acid chemistry and hybridization described herein are those well-known and

commonly used in the art. Enzymatic reactions and purification techniques are performed according to manufacturer's specifications, as commonly accomplished in the art or as described herein. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. Throughout this specification and embodiments, the words "have" and "comprise," or variations such as "has," "having," "comprises," or "comprising," will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers. All publications and other references mentioned herein are incorporated by reference in their entirety. Although a number of documents are cited herein, this citation does not constitute an admission that any of these documents forms part of the common general knowledge in the art.

[0091] In order that this invention may be better understood, the following examples are set forth. These examples are for purposes of illustration only and are not to be construed as limiting the scope of the invention in any manner.

EXAMPLES

Example 1: Production of AAV in Sf9 Insect Cells

[0092] Currently, helper systems used for Sf9 AAV manufacturing rely on leaky scanning, alternative splicing, and/or internal promoters to express all the Rep and capsid proteins required for AAV production (Smith et al., *supra*; Chen et al., *supra*). Since AAV capsids assemble based on the stoichiometry of the available capsid proteins (Berns and Parrish, *supra*), we hypothesized that exerting greater control over the amount of VP1 expressed during rAAV production could increase the amount of VP1 incorporated into rAAV capsids and thereby the viral potency.

[0093] To test this hypothesis, we cloned the coding sequences for individual capsid proteins into separate baculoviral vectors under the control of independent promoters. To allow greater control over the Rep proteins, we also cloned the coding sequences for individual Rep proteins into separate baculoviral vectors. In order to test various possible combinations of Rep and Cap coding sequences on different vectors, we constructed two versions of the baculoviral helper systems (**FIG. 5**).

[0094] In the first version, we paired the Rep78 expression cassette with the VP1 expression cassette and paired the Rep52 expression cassette with the VP2/VP3 expression cassette. In the

second version, we paired the Rep52 expression cassette with the VP1 expression cassette and paired the Rep78 expression cassette with the VP2/VP3 expression cassette. Both Rep expression cassettes were under the control of a polyhedrin promoter. Both the VP1 and VP2/3 expression cassettes were under the control of a p10 promoter. With the exception of the VP2/3 gene, which utilizes the same leaky scanning mechanism as in the natural context of AAV replication, none of the Rep coding sequences and the VP1 coding sequence in these helper vectors relies on leaky scanning or alternative splicing for expression. In order to reduce the number of baculoviral vectors required for rAAV production, we incorporated the coding sequence for the AAV minigenome into the Rep52-expressing vector in both versions (**FIG. 5**). For the present study, the AAV minigenome contained the expression cassette for alpha-galactosidase A (GLA) or zinc finger proteins (ZFP) (SBS#65918 and SBS#57890, whose coding sequences were separated by a nucleotide linker encoding a T2A self-cleaving peptide in the transgene expression cassette; *see* WO 2018/102665 and WO 2020/072677). A GLA-encoding rAAV may be useful in gene therapy for Fabry disease. The ZFP bound to and repressed the expression of the mouse microtubule-associated protein tau (*Mapt*) gene.

[0095] The coding sequences in the expression cassettes and the encoded polypeptide sequences are as follows. In the VP1 coding sequence, the ATG start codon for the native VP3 ORF was changed to GTG, resulting in an M-to-V substitution. In the Rep78 coding sequence, the ATG start codon for the native Rep58 ORF was changed to GTG, also resulting in an M-to-V substitution. For the studies herein, the Rep gene was derived from AAV2.

Generation of rAAV and Determination of Relative Yield

[0096] Naïve Sf9 insect cells were inoculated with baculovirus-infected insect cells (BIIC1 and BIIC2 for cells respectively infected by the two baculoviral vectors). The ratio of BIIC1:BIIC2:naïve Sf9 cells for the inoculation was 1:1:10000. Cell cultures were incubated for six days. The cells were then harvested, resuspended and freeze/thawed three times. The cell lysate was treated with benzonase. Cell debris was removed by centrifugation. AAV was concentrated by the addition of PEG and NaCl followed by incubation on ice and centrifugation of precipitated virus. Concentrated virus was resuspended and applied to a CsCl gradient followed by overnight ultracentrifugation. Banded AAV was collected and dialyzed. Then qPCR was performed to titer the vector genome (vg) content. rAAV6 (with a GLA-encoding transgene) and rAAV9 (with a ZFP-encoding transgene) tested herein were obtained by these

methods. Total AAV yield was calculated by multiplying the vg/mL by the total mL of the sample and expressed relative to the yield of the standard helper system 1, which expresses the AAV2 replicases and AAV6 capsid proteins and employs the one-vector system described in Smith et al., *supra*.

[0097] Additional rAAV serotypes, rAAV1, rAAV2, and rAAV3B, were obtained by the Version 2 method illustrated in **FIG. 5**. The VP1 in the rAAV1 and rAAV3B viruses contained an AAV2 PLA2 domain as described above. In the case of rAAV1, the VP1 and VP2 additionally contained an AAV9 transplant as described above to remove proteolytic sites. AAV2 and AAV3B did not appear susceptible to the proteolytic cleavage as observed with AAV1 and AAV6; and thus, their VP1/VP2 were not engineered to contain a transplanted AAV9 VP sequence). These three viruses also carried the same ZFP transgene as the rAAV9.

[0098] In the studies, the AAV genomes included ITR (inverted terminal repeats) from AAV2 and the insect cells expressed AAV2 replicases.

[0099] The capsid protein sequences of the rAAV6, rAAV9, rAAV1, rAAV2, and rAAV3B viruses herein are shown below. The capsid proteins of rAAV6 contained sequences from AAV2 and AAV9 as described in detail above. The capsid proteins of rAAV9, rAAV1, and rAAV3B also were engineered to contain sequences from other AAV serotypes, as indicated below.

Determination of rAAV6 Potency

[00100] rAAV6 obtained by the method described above in this Example was applied to HepG2 cells at a multiplicity of infection (MOI or virus/cell based on the vg titer) of 900K. The cells were incubated for five days. The tissue culture supernatant was collected and assayed for GLA enzyme activity by measuring the level of a fluorescent reporter 4-methylumbelliferyl (4-MU) released from a GLA substrate. Enzymatic activity was calculated relative to activity with an rAAV sample produced by standard helper system 1.

Determination of rAAV9 Potency

[00101] rAAV9 obtained by the method described above in this Example was applied to mouse cortical neurons at an MOI of 30K and incubated for six days. The cells were harvested for total RNA and used to generate cDNA. The cDNA was analyzed by qPCR for expression of mouse *Mapt* mRNA and zinc finger protein (ZFP) mRNA encoded by the AAV transgene. After normalizing the mRNA expression to the host cell RNA, potency was calculated relative to the

rAAV9 produced by the AAV9 standard helper system (*infra*). The potency of the rAAV1, rAAV2, and rAAV3B viruses can be tested in the same manner as well.

Sequences of Rep and Capsid Proteins

[00102] The amino acid and coding sequences for the rAAV6, rAAV1, rAAV2, rAAV3B, and rAAV9 viruses tested herein are shown below.

rAAV6 VP1 coding sequence:

```

ACGGCTGCCGACGGTTATCTACCCGATTGGCTCGAGGACAACCTCTCTGA
GGGCATTTCGCGAGTGGTGGGACCTCAAACCTGGAGCCCCGAAACCCAAAG
CCAACCAGCAAAAGCAGGACGACGGCCGGGGTCTGGTGCTTCTGGGTAC
AAGTACCTCGGACCCCTTCAACGGACTCGACAAGGGAGAGCCGGTCAACGA
GGCAGACGCCGCGGCCCTCGAGCACGACAAAGCCTACGACCGGCAGCTCG
ACAGCGGAGACAACCCGTACCTCAAGTACAACCACGCCGACGCGGAGTTT
CAGGAGCGCCTTCAAGAAGATACGTCTTTTGGGGGCAACCTCGGGCGAGC
AGTCTTCCAGGCCAAGAAGAGGGTTCTCGAACCTTTTGGTCTGGTTGAGG
AAGGTGCTAAGACCGCTCCTGGAAAGAAACGTCCGGTAGAGCAGTCGCCA
CAAGAGCCAGACTCCTCCGCGGGTATTGGCAAATCGGGTGACAGCCCCGC
TAAAAAGAGACTCAATTTCCGGTCAGACTGGCGACACAGAGTCAGTCCCAG
ACCCTCAACCAATCGGAGAACCTCCCGCAGCCCCCTCAGGTGTGGGATCT
CTTACAGTGGCTTCAGGCGGTGGCGCACCAGTGGCAGACAATAACGAAGG
CGCCGACGGAGTGGGTAATGCCTCAGGAAATTGGCATTGCGATTCCACAT
GGCTGGGCGACAGAGTCATCACCACCAGCACCCGAACATGGGCCTTGCCC
ACCTATAACAACCACCTCTACAAGCAAATCTCCAGTGCTTCAACGGGGGC
CAGCAACGACAACCACTACTTCGGCTACAGCACCCCTGGGGGTATTTTG
ATTTCAACAGATTCCACTGCCATTTCTCACCACGTGACTGGCAGCGACTC
ATCAACAACAATTGGGGATTCCGGCCCAAGAGACTCAACTTCAAGCTCTT
CAACATCCAAGTCAAGGAGGTACGACGAATGATGGCGTCACGACCATCG
CTAATAACCTTACCAGCACGGTTCAAGTCTTCTCGGACTCGGAGTACCAG
TTGCCGTACGTCTCCTCGGCTCTGCGCACCAAGGGCTGCCCTCCCTCCGTCCC
GGCGGACGTGTTTCATGATTCCGCAGTACGGCTACCTAACGCTCAACAATG
GCAGCCAGGCAGTGGGACGGTCATCCTTTTACTGCCCTGGAATATTTCCCA
TCGCAGATGCTGAGAACGGCAATAACTTTACCTTACGCTACACCTTCGA
GGACGTGCCTTTCCACAGCAGCTACGCGCACAGCCAGAGCCTGGACCGGC
TGATGAATCCTCTCATCGACCAGTACCTGTATTACCTGAACAGAACTCAG
AATCAGTCCGGAAGTGCCCAAAACAAGGACTTGCTGTTTAGCCGGGGGTC
TCCAGCTGGCATGTCTGTTTCAGCCCCAAAACCTGGCTACCTGGACCTGTT
ACCGGCAGCAGCGCGTTTCTAAAACAAAACAGACAACAACAACAGCAAC
TTTACCTGGACTGGTGCTTCAAAATATAACCTTAATGGGCGTGAATCTAT
AATCAACCCTGGCACTGCTATGGCTCACACAAAAGACGACAAAGACAAGT
TCTTTCCCATGAGCGGTGTCATGATTTTTTGAAAGGAGAGCGCCGGAGCT
TCAAACTACTGCATTGGACAATGTCATGATCACAGACGAAGAGGAAATCAA
AGCCACTAACCCCGTGGCCACCGAAAGATTTGGGACTGTGGCAGTCAATC
TCCAGAGCAGCAGCACAGACCCTGCGACCGGAGATGTGCATGTTATGGGA
GCCTTACCTGGAATGGTGTGGCAAGACAGAGACGTATATCTGCAGGGTCC
TATTTGGGCCAAAATTCCTCACACGGATGGACACTTTCACCCGTCTCCTC
TCATGGGCGGCTTTGGACTTAAGCACCCGCCTCCTCAGATCCTCATCAA

```

AACACGCCTGTTCTGCGAATCCTCCGGCAGAGTTTTCGGCTACAAAGTT
 TGCTTCATTCATCACCCAGTATTCCACAGGACAAGTGAGCGTGGAGATTG
 AATGGGAGCTGCAGAAAGAAAACAGCAAACGCTGGAATCCCGAAGTGCAG
 TATACATCTAACTATGCAAAATCTGCCAACGTTGATTTCACTGTGGACAA
 CAATGGACTTTTATACTGAGCCTCGCCCCATTGGCACCCGTTACCTCACCC
 GTCCCCTGTAA (SEQ ID NO: 15)

rAAV6 VP1 polypeptide:

XAADGYLPDW LEDNLSEGIR EWWDLKPGAP KPKANQQKQD DGRGLVLPGY KYLGPFNGLD
 KGEPVNEADA AALEHDKAYD RQLDSGDNPY LKYNHADA EF QERLQEDTSF GGNLGRAVFQ
 AKKRVLEPFG LVEEGAKTAP GKRRPVEQSP QEPDSSAGIG KSGAQPAKKR LNFQQTGDTE
 SVPDPQPIGE PPAAPSGVGS LTVASGGGAP VADNNEGADG VGNASGNWHC DSTWLGDRVI
 TTSTRTWALP TYNHLYKQI SSASTGASND NHYFGYSTPW GYFDNRFHC HFSRPDWQRL
 INNNWGFRPK RLNFKLFNIQ VKEVTTNDGV TTIANNLTST VQVFSDEYQ LPYVLGSAHQ
 GCLPPFPADV FMIPQYGYLT LNNGSQAVGR SSFYCLEYFP SQMLRTGNF TFSYTFEDVP
 FHSSYAHSQS LDRLMNPLID QYLYYLNRTQ NQSGSAQNKD LLFSRGSFAG MSVQPKNWLP
 GPCYRQQRVS KTKTDNNNSN FTWTGASKYN LNGRESIINP GTAMASHKDD KDKFFPMMSGV
 MIFGKESAGA SNTALDNVMI TDEEEIKATN PVATERFGTV AVNLQSSSTD PATGDVHVMG
 ALPGMVWQDR DVYLQGPWA KIPHTDGHFH PSPLMGGFGL KHPPQILIK NTPVPANPPA
 EFSATKFASF ITQYSTGQVS VEIEWELQKE NSKRWNPEVQ YTSNYAKSAN VDFTVDNNGL
 YTEPRPIGTR YLTRPL (SEQ ID NO:16; X = M or T)

rAAV6 VP2/VP3 coding sequence:

ACGGCTCCTGGAAAGAAACGTCCGGTAGAGCAGTCGCCACAAGAGCCAGA
 CTCCTCCGCGGGTATTGGCAAATCGGGTGACAGCCCGCTAAAAAGAGAC
 TCAATTTTCGGTCAGACTGGCGACACAGAGTCAGTCCCAGACCCTCAACCA
 ATCGGAGAACCTCCCGCAGCCCCCTCAGGTGTGGGATCTCTTACAATGGC
 TTCAGGCGGTGGCGCACCAAGTGGCAGACAATAACGAAGGCGCCGACGGAG
 TGGGTAATGCCTCAGGAAATTGGCATTGCGATTCCACATGGCTGGGCGAC
 AGAGTCATCACCACCAGCACCCGAACATGGGCCTTGCCACCTATAACAA
 CCACCTCTACAAGCAAATCTCCAGTGCTTCAACGGGGGCCAGCAACGACA
 ACCACTACTTTCGGCTACAGCACCCCTGGGGGTATTTTGATTTCAACAGA
 TTCCACTGCCATTTCTCACCACGTGACTGGCAGCGACTCATCAACAACAA
 TTGGGGATTCCGGCCCAAGAGACTCAACTTCAAGCTCTTCAACATCCAAG
 TCAAGGAGGTCACGACGAATGATGGCGTCACGACCATCGCTAATAACCTT
 ACCAGCACGGTTCAAGTCTTCTCGGACTCGGAGTACCAGTTGCCGTACGT
 CCTCGGCTCTGCGCACCAAGGGCTGCCTCCCTCCGTTCCCGGCGGACGTGT
 TCATGATTCCGCGAGTACGGCTACCTAACGCTCAACAATGGCAGCCAGGCA
 GTGGGACGGTCATCCTTTTACTGCCTGGAATATTTCCCATCGCAGATGCT
 GAGAACGGGCAATAACTTTACCTTACGCTACACCTTCGAGGACGTGCCTT
 TCCACAGCAGCTACGCGCACAGCCAGAGCCTGGACCGGCTGATGAATCCT
 CTCATCGACAGTACCTGTATTACCTGAACAGAACTCAGAATCAGTCCGG
 AAGTGCCCAAAACAAGGACTTGCTGTTTAGCCGGGGGTCTCCAGCTGGCA
 TGTCTGTTTCAGCCCAAAACTGGCTACCTGGACCTGTTACCGGCAGCAG
 CGCGTTTCTAAAACAAAAACAGACAACAACAGCAACTTTACCTGGAC
 TGGTGCTTCAAAATATAACCTTAATGGGCGTGAATCTATAATCAACCCTG
 GCACTGCTATGGCCTCACACAAAGACGACAAAGACAAGTTCTTTCCCATG
 AGCGGTGTCATGATTTTTGGAAAGGAGAGCGCCGGAGCTTCAAACTGC
 ATTGGACAATGTCATGATCACAGACGAAGAGGAAATCAAAGCCACTAACC

CCGTGGCCACCGAAAGATTTGGGACTGTGGCAGTCAATCTCCAGAGCAGC
 AGCACAGACCCTGCGACCGGAGATGTGCATGTTATGGGAGCCTTACCTGG
 AATGGTGTGGCAAGACAGAGACGTATATCTGCAGGGTCTTATTTGGGCCA
 AAATTCCTCACACGGATGGACACTTTCACCCGTCTCTCTCATGGGCGGC
 TTTGGACTTAAGCACCCGCCTCCTCAGATCCTCATCAAAAAACACGCCTGT
 TCCTGCGAATCCTCCGGCAGAGTTTTCGGCTACAAAGTTTGCTTCATTCA
 TCACCCAGTATTCCACAGGACAAGTGAGCGTGGAGATTGAATGGGAGCTG
 CAGAAAGAAAACAGCAAACGCTGGAATCCCGAAGTGCAGTATACATCTAA
 CTATGCAAAATCTGCCAACGTTGATTTCACTGTGGACACAATGGACTTT
 ATACTGAGCCTCGCCCCATTGGCACCCGTTACCTCACCCGTCCCCTGTAA
 (SEQ ID NO:17)

rAAV6 VP2 polypeptide sequence:

XAPGKKRPVE QSPQEPDSSA GIGKSGAQPA KKRLNFGQTG DTESVPDPQP IGEPPAAPSG
 VGSLTMASGG GAPVADNNEG ADGVGNASGN WHCDSTWLGD RVITTSTRTW ALPTYNNHLY
 KQISSASTGA SNDNHYFGYS TPWGYFDFNR FHCHFSPRDW QRLINNNWGF RPKRLNFKLF
 NIQVKEVTN DGVTTIANNL TSTVQVFSDS EYQLPYVLGS AHQGCLPPFP ADVFMIPQYG
 YLTLNNGSQA VGRSSFYCLE YFPSQMLRTG NNFTFSYTFE DVPFHSSYAH SQSLDRLMNP
 LIDQYLYLN RTQNQSGSAQ NKDLLFSRGS PAGMSVQPKN WLPGPCYRQQ RVSKTKTDNN
 NSNFTWTGAS KYNLNGRESI INPGTAMASH KDDKDKFFPM SGVMIFGKES AGASNTALDN
 VMITDEEEIK ATNPVATERF GTVAVNLQSS STDPATGDVH VMGALPGMVW QDRDVYLQGP
 IWAKIPHTDG HFHPSPLMGG FGLKHPPPQI LIKNTVPAN PPAEFSATKF ASFITQYSTG
 QVSVEIEWEL QKENS KRWN EVQYTSNYAK SANVDFTVDN NGLYTEPRPI GTRYLTRPL
 (SEQ ID NO:18; X = M or T)

rAAV6 VP3 polypeptide sequence:

MASGGGAPVA DNNEGADGVG NASGNWHCDS TWLGDRVITT STRTWALPTY NNHLYKQISS
 ASTGASNDNH YFGYSTPWGY FDFNRFHCHF SPRDWQRLIN NNWGFPRPKRL NFKLFNIQVK
 EVTTNDGVTT IANNLTSTVQ VFSDEYQLP YVLGSAHQGC LPPFPADVEM IPQYGYLTIN
 NGSQAVGRSS FYCLEYFPSQ MLRTGNNTF SYTFEDVPFH SSYAHQSOLD RLMNPLIDQY
 LYYLNRTQNQ SGSAQNKDLL FSRGSPAGMS VQPKNWLP GP CYRQQRVSKT KTDNNNSNFT
 WTGASKYNLN GRESIINPGT AMASHKDDK KFFPMSGVMI FGKESAGASN TALDNVMITD
 EEEIKATNPV ATERFGTVAV NLQSSSTDPA TGDVHVMGAL PGMVWQDRDV YLQGPWAKI
 PHTDGHFHP PLMGGFGLKH PPPQILIKNT PVPANPPAEF SATKFASFIT QYSTGQVSVE
 IEWELQKENS KRWNPEVQYT SNYAKSANVD FTVDNNGLYT EPRPIGTRYL TRPL
 (SEQ ID NO:19)

rAAV1 VP1/VP2/VP3 polypeptide sequences (transplanted AAV2 PLA2 domain is shown in box; transplanted AAV9 VP2/VP3 proteolytic resistant domain is italicized and boldfaced; the first amino acids of VP2 and VP3 (T138 and V203, respectively, in SEQ ID NO:24) are boldfaced and underlined):

TAADGYLPDW LEDNLSEGIR EWWDLKPGAP KPKANQQKQD DGRGLVLPGY KYLGPFNGLD
 KGEPVNEADA AALEHDKAYD RQLDSGDNPY LKYNHADA EF QERLQEDTSF GGNLGRAVFQ
 AKKRVLPLEG LVEEAKTAP GKRPVEQSP QEPDSS**AGIG KSGAQPAKKR LNFQGTGDTE**
SVPDPQPIGE PPAAPSGVGS LTVASGGGAP VADNNEGADG VGNASGNWHC DSTWLGD RVI
 TTSTRTWALP TYNNHLYKQI SSASTGASND NHYFGYSTPW GYFDFNRFHC HFSPRDWQRL

INNNWGFRPK RLNFKLFNIQ VKEVTNDGV TTIANNLTST VQVFSSEYQ LPYVLGSAHQ
 GCLPPFPADV FMIPQYGYLT LNNGSQAVGR SSFYCLEYFP SQMLRTGNNF TFSYTFEEVP
 FHSSYAHSQS LDRLMNPLID QLYYLNRTQ NQSGSAQNKD LLFSRGPAG MSVQPKNWLP
 GPCYRQQRVS KTKTDNNNSN FTWTGASKYN LNGRESIINP GTAMASHKDD EDKFFPMSGV
 MIFGKESAGA SNTALDNVMI TDEEEIKATN PVATERFGTV AVNFQSSSTD PATGDVHAMG
 ALPGMVWQDR DVYLQGPPIWA KIPHTDGHFH PSPLMGGFGL KNPPPQILIK NTPVPANPPA
 EFSATKFASF ITQYSTGQVS VEIEWELQKE NSKRWNPEVQ YTSNYAKSAN VDFTVDNNGL
 YTEPRPIGTR YLTRPL (SEQ ID NO:24)

rAAV2 VP1/VP2/VP3 polypeptide sequences (the first amino acids of VP2 and VP3 (T138 and V203, respectively, in SEQ ID NO:25) are boldfaced and underlined):

TAADGYLPDW LEDTLSEGIR QWWKLKPGPP PPKPAERHKD DSRGLVLPGY KYLGPFNGLD
 KGEPVNEADA AALEHDKAYD RQLDSGDNPY LKYNHADA EF QERLKEDTSF GGNLGRAVFQ
 AKKRILEPLG LVEEPV**K**TAP GKRPVEHSP VEPDSSSGTG KAGQQPARKR LNFGQTGDAD
 SVPDPQPLGQ PPAAPSGLGT NT**V**ATGSGAP VADNNEGADG VGNSSGNWHC DSTWLGDRI
 TTSTRTWALP TYNNHLYKQI SSQSGASNDN HYFGYSTPWG YFDFNRFHCH FSPRDWQRLI
 NNNWGFRPKR LNFKLFNIQV KEVTQNDGTT TIANNLTSTV QVFTDSEYQL PYVLGSAHQG
 CLPPFPADV MVPQYGYLTL NNGSQAVGRS SFYCLEYFPS QMLRTGNNFT FSYTFEDVFP
 HSSYAHSQSL DRLMNPLIDQ YLYYLSRTNT PSGTTTQSRL QFSQAGASDI RDQSRNWLP
 PCYRQQRVSK TSADNNNSEY SWTGATKYHL NGRDSLVPNG PAMASHKDDE EKFFPQSGVL
 IFGKQGSSEKT NVDIEKVMIT DEEEIIRTTN VATEQYGSVS TNLQRGNRQA ATADVNTQGV
 LPMGMVWQDR DVYLQGPPIWA IPHTDGHFH SPLMGGFGL HPPPQILIKN TFPVPANPSTT
 FSAKFASFI TQYSTGQVS EIEWELQKEN SKRWNPFIQY TSNYNKSVNV DFTVDTNGVY
 SEPRPIGTRY LTRNL (SEQ ID NO:25)

rAAV3B VP1/VP2/VP3 polypeptide sequences (transplanted AAV2 PLA2 domain is shown in box; the first amino acids of VP2 and VP3 (T138 and V203, respectively, in SEQ ID NO:26) are boldfaced and underlined):

LAADGYLPDW LEDNLSEGIR EWWALKPGVP QPKANQQHQD NRRG**GLVLPGY** KYLGPFNGLD
 KGEPVNEADA AALEHDKAYD RQLDSGDNPY LKYNHADA EF QERL**Q**EDTSF GGNLGRAVFQ
 AKKRILEPLG LVEEAA**K**TAP GKRPVDQSP QEPDSSSGVG KSGKQPARKR LNFGQTGDSE
 SVPDPQPLGE PPAAPTSLSG NT**V**ASGGGAP VADNNEGADG VGNSSGNWHC DSQWLGDRI
 TTSTRTWALP TYNNHLYKQI SSQSGASNDN HYFGYSTPWG YFDFNRFHCH FSPRDWQRLI
 NNNWGFRPKK LSFKLFNIQV KEVTQNDGTT TIANNLTSTV QVFTDSEYQL PYVLGSAHQG
 CLPPFPADV MVPQYGYLTL NNGSQAVGRS SFYCLEYFPS QMLRTGNNFQ FSYTFEDVFP
 HSSYAHSQSL DRLMNPLIDQ YLYYLNRTQG TTSGTTNQSR LLFSQAGPQS MSLQARNWLP
 GPCYRQQRSL KTANDNNNSN FPWTAASKYH LNGRDSLVPN GPAMASHKDD EEKFFPMHGN
 LIFGKEGTTA SNAELDNVMI TDEEEIIRTTN PVATEQYGTV ANNLSQSSNTA PTTRTVNDQG
 ALPGMVWQDR DVYLQGPPIWA KIPHTDGHFH PSPLMGGFGL KHPPPQIMIK NTPVPANPPT
 TFSPAKFASF ITQYSTGQVS VEIEWELQKE NSKRWNPEIQ YTSYNKSVN VDFTVDTNGV
 YSEPRPIGTR YLTRNL (SEQ ID NO:26)

rAAV9 VP1/VP2/VP3 polypeptide sequences (transplanted AAV2 PLA2 domain is shown in box; the first amino acids of VP2 and VP3 (T138 and V203, respectively, in SEQ ID NO:27) are boldfaced and underlined):

LAADGYLPDW	LEDNLSEGIR	EWALKPGAP	QPKANQQHQD	NARG	LVLPGY	KYLGPENGLD
KGEPVNEADA	AALEHDKAYD	RQLDSGDNPY	LKYNHADAEF	QERI	KEDTSF	GNLGRAVFQ
AKKRLLEPLG	LVEEAAK T AP	GKKRPVEQSP	QEPDSSAGIG	KSGAQPAKKR	LNFGQTGDTE	
SVPDPQPIGE	PPAAPSGVGS	LT V ASGGGAP	VADNNEGADG	VGSSSGNWHC	DSQWLGDRI	
TTSTRTWALP	TYNHLYKQI	SNSTSGGSSN	DNAYFGYSTP	WGYFDFNRFH	CHFSPRDWQR	
LINNNWGFRP	KRLNFKLENI	QVKEVTDNNG	VKTIANNLTS	TVQVFTDSY	QLPYVLGSAH	
EGCLPPFPAD	VFMIPQYGYL	TLNDGSQAVG	RSSFYCLEYF	PSQMLRTGNN	FQFSYEFENV	
PFHSSYAHSQ	SLDRLMNPLI	DQYLYYLSKT	INGSGQNQQT	LKFSVAGPSN	MAVQGRNYIP	
GPSYRQQRVS	TTVTQNNNSE	FAWPGASSWA	LNGRNSLMNP	GPAMASHKEG	EDRFFPLSGS	
LIFGKQGTGR	DNVDADKVM	TNEEEIKTTN	PVATESYQGV	ATNHQSAQAQ	AQTGWVQNGG	
ILPGMVWQDR	DVYLQGP	KIPHTDGNFH	PSPLMGGFGM	KHPPPPQILIK	NTPVPADPPT	
AFNKDKLNSF	ITQYSTGQVS	VEIEWELQKE	NSKRWNPEIQ	YTSNYYKSNN	VEFAVNTEGV	
YSEPRPIGTR	YLTRNL	(SEQ ID NO:27)				

AAV2 Rep78 coding sequence:

CTGGCGGGGTTTTACGAGATTGTGATTAAAGGTCCCCAGCGACCTTGACGA
GCATCTGCCCCGCATTTCTGACAGCTTTGTGAACTGGGTGGCCGAGAAGG
AGTGGGAGTTGCCGCCAGATTCTGACTTGGATCTGAATCTGATTGAGCAG
GCACCCCTGACCGTGGCCGAGAAGCTGCAGCGCGACTTTCTGACGGAGTG
GCGCCGTGTGAGTAAGGCCCGGAGGCCCTTTTCTTTGTGCAATTTGAGA
AGGGAGAGAGCTACTTCCACTTACACGTGCTCGTGGAAACACCGGGGTG
AAATCCTTAGTTTTGGGACGTTTCTGAGTCAGATTCGCGAAAACTGAT
TCAGAGAATTTACCGCGGGATCGAGCCGACTTTGCCAACTGGTTCGCGG
TCACAAAGACCAGAAACGGCGCCGGAGGCGGGAACAAGGTGGTGGACGAG
TGCTACATCCCCAATTACTTGCTCCCCAAAACCCAGCCTGAGCTCCAGTG
GGCGTGGACTAATTTAGAACAGTATTTAAGCGCCTGTTTGAATCTCACGG
AGCGTAAACGGTTGGTGGCGCAGCATCTGACGCACGTGTCGACAGACGAG
GAGCAGAACAAAGAGAATCAGAATCCCAATTCTGACGCGCCGGTGATCAG
ATCAAAAACCTTCAGCCAGGTACGTGGAGCTGGTGGGTGGCTCGTGGACA
AGGGGATTACCTCGGAGAAGCAGTGGATCCAGGAGGACCAGGCCTCATAC
ATCTCCTTCAATGCGGCCTCCAACCTCGCGGTCCCAATCAAGGCTGCCTT
GGACAATGCGGGAAAGATTATGAGCCTGACTAAAACCGCCCCGACTACC
TGGTGGGCCAGCAGCCCGTGGAGGACATTTCCAGCAATCGGATTTATAAA
ATTTTGGAACTAAACGGGTACGATCCCCAATATGCGGCTTCCGTCTTTCT
GGGATGGGCCACGAAAAAGTTTCGGCAAGAGGAACACCATCTGGCTGTTTG
GGCCTGCAACTACCGGGAAGACCAACATCGCGGAGGCCATAGCCCACACT
GTGCCCTTCTACGGGTGCGTAAACTGGACCAATGAGAACTTTCCCTTCAA
CGACTGTGTCGACAAGATGGTGATCTGGTGGGAGGAGGGAAGATGACCG
CCAAGGTGCGTGGAGTGGCCAAAGCCATTTCTCGGAGGAAGCAAGGTGCGC
GTGGACCAGAAATGCAAGTCTCGGCCAGATAGACCCGACTCCCGTGAT
CGTCACCTCCAACACCAACATGTGCGCCGTGATTGACGGAACTCAACGA
CCTTCGAACACCAGCAGCCGTGCAAGACCGGATGTTCAAATTTGAACTC
ACCCGCCGTCTGGATCATGACTTTGGGAAGGTACCAAGCAGGAAGTCAA
AGACTTTTTCCGGTGGGCAAAGGATCACGTGGTTGAGGTGGAGCATGAAT
TCTACGTCAAAAAGGGTGGAGCCAAGAAAAGACCCGCCCCAGTGACGCA
GATATAAGTGAGCCCCAACGGGTGCGCGAGTCAGTTGCGCAGCCATCGAC
GTCAGACGCGGAAGCTTCGATCAACTACGCAGACAGGTACCAAAAACAAAT
GTTCTCGTCACGTGGGCATGAATCTGATGCTGTTTCCCTGCAGACAATGC
GAGAGAATGAATCAGAATTCAAATATCTGCTTCACTCACGGACAGAAAGA
CTGTTTAGAGTGCTTTCCCGTGTGAGAATCTCAACCCGTTTCTGTCGTCA

AAAAGGCGTATCAGAACTGTGCTACATTCATCATATCATGGGAAAGGTG
 CCAGACGCTTGCACTGCCTGCGATCTGGTCAATGTGGATTGGATGACTG
 CATCTTTGAACAATAA (SEQ ID NO:20)

AAV2 Rep78 polypeptide:

XAGFYEIVIK VPSDLDEHLP GISDSFVNWV AEKEWELPPD SLDLNLIEQ APLTVAEKLQ
 RDFLTEWRRV SKAPEALFFV QFEKGESYFH LHVLEVETGV KSLVLGRFLS QIREKLIQRI
 YRGIEPTLPN WFAVTKTRNG AGGKNKVDE CYIPNYLLPK TQPELQWAWT NLEQYLSACL
 NLTERKRLVA QHLTHVSQTQ EQNKENQNP SDAPVIRSKT SARYVELVGW LVDKGITSEK
 QWIQEDQASY ISFNAASNSR SGIKAALDNA GKIMSLTKTA PDYLVGQQPV EDISSNRIYK
 ILELNGYDPQ YAASVFLGWA TKKFGKRNTI WLFGPATTGK TNIAEIAIHT VPFYGCNVWT
 NENFPFNDVC DKMVIWEEG KMTAKVVESA KAILGGSKVR VDQKCKSSAQ IDPTFVIVTS
 NTNMCVIDG NSTTFEHQQP LQDRMFKFEL TRRLDHDFGK VTKQEVKDFE RWAKDHVVEV
 EHEFYVKKGK AKKRPA PSDA DISEPKRVRE SVAQPSTSDA EASINYADRY QNKCSRHVGM
 NLMLFPCRQC ERMNQNSNIC FTHGQKDCLE CFPVSESQPV SVVKKAYQKL CYIHHIMGKV
 PDACTACDLV NVDLDDCIFE Q (SEQ ID NO:21; X = M or L)

AAV2 Rep52 coding sequence:

ATGGAGCTGGTCGGGTGGCTCGTGGACAAGGGGATTACCTCGGAGAAGCA
 GTGGATCCAGGAGGACCAGGCCTCATACATCTCCTTCAATGCGGCCTCCA
 ACTGCGGTGCCAAATCAAGGCTGCCTTGGACAATGCGGGAAAGATTATG
 AGCCTGACTAAAACCGCCCCGACTACCTGGTGGGCCAGCAGCCCGTGGA
 GGACATTTCCAGCAATCGGATTTATAAAATTTTGAACATAACGGGTACG
 ATCCCCAATATGCGGCTTCCGTCTTTCTGGGATGGGCCACGAAAAAGTTC
 GGCAAGAGGAACACCATCTGGCTGTTTGGGCCTGCAACTACCGGGAAGAC
 CAACATCGCGGAGGCCATAGCCACACTGTGCCCTTCTACGGGTGCGTAA
 ACTGGACCAATGAGAACTTTCCCTTCAACGACTGTGTGACAAGATGGTG
 ATCTGGTGGGAGGAGGGGAAGATGACCGCAAGGTCGTGGAGTCGGCCAA
 AGCCATTCTCGGAGGAAGCAAGGTGCGCGTGGACCAGAAATGCAAGTCCT
 CGGCCCAGATAGACCCGACTCCCGTGATCGTCACCTCCAACACCAACATG
 TGCGCCGTGATTGACGGGAACCAACGACCTTCAACACCAAGCAGCCGTT
 GCAAGACCGGATGTTCAAATTTGAACACACCGCCGCTGCGATCATGACT
 TTGGGAAGGTCACCAAGCAGGAAGTCAAAGACTTTTTCCGGTGGGCAAG
 GATCACGTGGTTGAGGTGGAGCATGAATTCTACGTCAAAAAGGGTGGAGC
 CAAGAAAAGACCCGCCCCCAGTGACGAGATATAAGTGAGCCCAAACGGG
 TGCGCGAGTCAGTTGCGCAGCCATCGACGTCAGACGCGGAAGCTTCGATC
 AACTACGACAGAGGTACCAAAACAAATGTTCTCGTCACGTGGGCATGAA
 TCTGATGCTGTTCCCTGCAGACAATGCGAGAGAATGAATCAGAATTCAA
 ATATCTGCTTCACTCACGGACAGAAAGACTGTTTAGAGTGCTTTCCCGTG
 TCAGAAATCTCAACCCGTTTCTGTGCTCAAAAAGGCGTATCAGAACTGTG
 CTACATTCATCATATCATGGGAAAGGTGCCAGACGCTTGCACTGCCTGCG
 ATCTGGTCAATGTGGATTGGATGACTGCATCTTTGAACAATAA
 (SEQ ID NO:22)

AAV2 Rep52 polypeptide:

MELVGWLVDK GITSEKQWIQ EDQASYISFN AASNSRSQIK AALDNAGKIM SLTKTAPDYL
 VGQQPVEDIS SNRIYKILEL NGYDPQYAAS VFLGWATKKF GKRNTIWLFG PATTGKTNIA
 EAIHTVFPY GCVNWTNENF PFNDVCVKM IWWEEGKMTA KVVESAKAIL GGSKVRVDQK

CKSSAQIDPT PVIIVTSNTNM CAVIDGNSTT FEHQQLQDR MFKFELTRRL DHDFGKVTQK
 EVKDFFRWAK DHVVEVEHEF YVKKGGAKKR PAPSDADISE PKRVRESVAQ PSTSDAEASI
 NYADRYQNK C SRHVGMLML FPCRQCERMN QNSNICFTHG QKDCLECFPV SESQPVSVVK
 KAYQKLCYIH HIMGKVPDAC TACDLVNVDL DDCIFEQ (SEQ ID NO:23)

Results

[00103] We tested the ability of each version in **FIG. 5** to produce rAAV6 and the potency of the resulting rAAV with respect to standard helper system 1 and standard helper system 2, which differs from standard helper system 1 in that its capsid expression cassettes use the AAV6/2/9 hybrid sequences described above. Surprisingly, the pairing of the Rep and Cap genes had a significant impact on both yield and potency. Version 1 had an approximately two-fold reduction in yield and eight-fold increase in potency, whereas Version 2 had an approximately four-fold increase in yield and five-fold increase in potency (**Table 1**). Comparison of the capsid ratios showed that the Version 1 rAAV6 had very little VP2 and a VP1:VP3 ratio similar to that observed with wildtype AAV (1:10). Recombinant AAV produced by the Version 2 system had a similar capsid ratio to that observed with standard helper system 2 (**Table 1**).

Table 1 Characterization of rAAV6*

Helper	Relative Yield (N=3)	Potency (N=3)	Capsid ratio		
			VP1	VP2	VP3
Standard helper system 1	1.0	1.0	1	2	66
Standard helper system 2	3.8	3.1	1	1.6	24
Version 1	0.5	8.5	1	0.005	14
Version 2	4.1	5.2	1	1.4	26

*The yield and potency numbers are averages of three independent production lots. The capsid ratios are from a single production lot.

[00104] For rAAV9, Version 1 had an approximately three-fold reduction in yield and similar potency, whereas Version 2 had an approximately four-fold increase in yield and two-fold decrease in potency, as compared to standard helper system 1 (**Table 2**). Comparison of the capsid ratios showed that the Version 1 rAAV9 had half as much VP2 as the AAV9 standard helper system and a VP1:VP3 ratio one half of that observed with wildtype AAV (1:10). Recombinant AAV9 produced by the Version 2 system had a similar capsid ratio to that observed with rAAV9 provided by the AAV9 standard helper system (**Table 2**). The AAV9

standard helper system was the same as the AAV6 standard helper system 2, except that instead of producing AAV6/2/9 hybrid capsid proteins, it produced hybrid AAV9 capsid proteins in which the VP1 protein was engineered to contain an AAV2 PLA2 domain as described above.

Table 2 Characterization of rAAV9*

Helper	Relative Yield (N=6)	Potency (N=6)	Capsid ratio		
			VP1	VP2	VP3
AAV9 standard helper	1.0	1.0	1	1.7	18
Version 1	0.3	0.9	1	0.8	20
Version 2	4.0	0.5	1	1.5	26

*The yield and potency numbers and capsid ratios are from six dependent production lots.

[00105] Additional serotypes rAAV1, rAAV2 and rAAV3B were successfully produced by the Version 2 method of **FIG. 5**.

[00106] These data suggest that the pairing of Rep and Cap expression cassettes on a helper vector influences the yield and potency of produced rAAV. Furthermore, the data demonstrate that the Rep and Cap proteins can be successfully expressed from two separate baculoviral vectors and help produce infectious rAAV at high yield. Lastly, these data demonstrate that the production system described herein is broadly applicable to different AAV serotypes and is not restricted to the more extensively tested AAV6 and AAV9 versions.

Table 3 List of Sequences

SEQ ID NO	Description
1	AAV6 VP1 amino acid sequence
2	AAV6 proteolytic site
3	AAV6 VP1/VP2 fragment
4	AAV9 VP1/VP2 fragment
5	AAV6 VP1 fragment
6	AAV2 VP1 fragment
7	AAV6/2/9 VP1 amino acid sequence
8	AAP6 residues 1-30
9	AAP9 residues 1-30
10	AAP6 amino acid sequence
11	AAV9 VP1 amino acid sequence
12	AAV9 VP1 fragment
13	Partial AAV6 <i>Cap</i> nucleotide sequence
14	Partial AAV9 <i>Cap</i> nucleotide sequence
15	VP1 coding sequence of rAAV6 in Example 1
16	VP1 polypeptide sequence of rAAV6 in Example 1
17	VP2/VP3 coding sequence of rAAV6 in Example 1
18	VP2 polypeptide sequence of rAAV6 in Example 1
19	VP3 polypeptide sequence of rAAV6 in Example 1
20	AAV2 Rep78 coding sequence in Example 1
21	AAV2 Rep78 polypeptide sequence in Example 1
22	AAV2 Rep52 coding sequence in Example 1
23	AAV2 Rep52 polypeptide sequence in Example 1
24	VP1/VP2/VP3 polypeptide sequences of rAAV1 in Example 1
25	VP1/VP2/VP3 polypeptide sequences of rAAV2 in Example 1
26	VP1/VP2/VP3 polypeptide sequences of rAAV3B in Example 1
27	VP1/VP2/VP3 polypeptide sequences of rAAV9 in Example 1
28-73	Sequences in figures