PLASMID SYSTEM FOR PRODUCTION OF RECOMBINANT ADENOASSOCIATED VIRUS

1. CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 63/565,528, filed March 14, 2024, which is hereby incorporated by reference in its entirety.

2. SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted herewith and is hereby incorporated by reference in its entirety. Said .xml copy, created on March 12, 2025, is named 61491_114WO_SequenceListing, and is 288,737 bytes in size.

3. BACKGROUND

[0003] Advancements in genetic engineering have made gene therapies a reality, with recombinant Adeno-Associated Viruses (rAAV) at the forefront due to their low risk of insertional mutagenesis and ideal transduction profiles. However, therapeutic applications of rAAV are challenged by existing production methodologies which are largely inefficient, complex, and costly.

[0004] The conventional triple transfection method for rAAV production often yields inconsistent and low quantities of the virus, thereby raising issues of scalability - a critical factor for therapeutic applications serving large patient populations. Moreover, this method relies on the use of animal-derived materials, which can introduce the risk of contamination. The time-consuming nature of preparing the necessary large-scale cell cultures further exacerbates these challenges.

[0005] Another limiting factor is the purification process. Conventional methods often result in contaminants that require additional steps for removal, adding complexity to the process and reducing the yield. Furthermore, these methods are not cost-effective, presenting a major barrier to the large-scale production of rAAV for therapeutic use.

[0006] In light of these challenges, there is a need for a more efficient, scalable, and cost-effective solution for the production of rAAV. Overcoming these obstacles would unlock the full potential of rAAV as a delivery method for gene therapies, providing hope for patients and new avenues for research and development efforts in the field.

4. SUMMARY

[0007] The present disclosure provides an improved process of producing rAAV with better rAAV yields and purity. The method disclosed herein can be used to produce rAAV with a greater proportion of full rAAV capsids than empty or partially filled capsids and significant lower contaminants such as recombinant plasmid contaminants or host cell DNA (hcDNA) from the production process.

[0008] An aspect of the present disclosure includes a system for production of recombinant adeno-associated virus (rAAV) comprising: a first polynucleotide comprising a rep gene encoding a Rep protein, and a first promoter operably linked to the rep gene; a second polynucleotide comprising a helper virus gene, a third polynucleotide comprising (i) a capsid gene encoding a Capsid protein, a second promoter operably linked to the capsid gene; and (ii) an expression cassette comprising a transgene flanked by two inverted terminal repeats (ITRs).

[0009] In some embodiments, the first polynucleotide comprises a sequence encoding a Rep 78 protein, a Rep 68 protein, a Rep 52 protein, or a Rep 40 protein. In some embodiments, the first polynucleotide comprises two, three or four sequences, wherein each of the two, three, or four sequences encodes a Rep 78 protein, a Rep 68 protein, a Rep 52 protein, or a Rep 40 protein. In some embodiments, the first polynucleotide comprises sequences encoding a Rep 78 protein, a Rep 68 protein, a Rep 52 protein, and a Rep 40 protein.

[0010] In some embodiments, the first polynucleotide comprises sequences encoding AAV2 Rep 78, AAV2 Rep 68, AAV2 Rep 52, and AAV2 Rep 40 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV4 Rep 78, AAV4 Rep 68, AAV4 Rep 52, and AAV4 Rep 40 proteins. In some embodiments, the rep gene of the first polynucleotide comprises a sequence encoding a rep protein from an AAV serotype selected from: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, and AAV13. In some embodiments, the first polynucleotide comprises sequences encoding AAV10 Rep 78, AAV10 Rep 68, AAV10 Rep 52, and AAV10 Rep 40 proteins. In some embodiments, the first polynucleotide comprises sequences encoding: 30-50% of AAV2 and AAV10 Rep 78 proteins; 30-50% of AAV2 and AAV10 Rep 68 proteins; an AAV2 Rep 52 protein; and an AAV4 Rep 40 protein.

[0011] In some embodiments, the first promoter is from an AAV serotype that is different from the origin of the rep gene. In some embodiments, the first promoter is from an AAV serotype that is the same as the origin of the rep gene.

[0012] In some embodiments, the first promoter is a p5 promoter. In some embodiments, the p5 promoter is an AAV2 p5, AAV4 p5, or AAV10 p5 promoter. In some embodiments, the first polynucleotide further comprises a p19 promoter. In some embodiments, the p19 promoter is a AAV2 p19, AAV4 p19, or AAV10 p19 promoter. In some embodiments, the first promoter comprises a P7 promoter. In some embodiments, the first promoter comprises a p40 promoter.

[0013] In some embodiments, the p40 promoter is an AAV2 p40, AAV4 p40, or AAV10 p40 promoter. In some embodiments, the first polynucleotide is devoid of a p40 promoter. In some embodiments, the first polynucleotide comprises an inactivated p40 promoter.

[0014] In some embodiments, the first polynucleotide is devoid of: a p19 promoter or a p40 promoter, or both a p19 promoter and a p40 promoter. In some embodiments, the first polynucleotide comprises an inactivated p40 promoter. In some embodiments, the first polynucleotide comprises an inactivated p19 and p40 promoter.

[0015] In some embodiments, the first polynucleotide further comprises one or more additional promoters. In some embodiments, the one or more additional promoters is selected from: p19, CMV, SV40, Ef1a, UBC, p5, and a PGK promoter.

[0016] In some embodiments, the first polynucleotide comprises sequences encoding AAV2 Rep 78, AAV2 Rep 68, AAV2 Rep 52, and AAV2 Rep 40 proteins, wherein the first promoter is a AAV2 p5 promoter, and wherein the first polynucleotide further comprises a p19 promoter; the second polynucleotide comprises sequences encoding Adeno E2A, Adeno E4, and Adeno VA; and the third polynucleotide further comprises: a non-coding nucleotide sequence positioned between one of the two ITR sequences and the second promoter; and an AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and an AAV2 3' UTR sequence at the 3' end of the capsid coding sequence.

[0017] In some embodiments, the first polynucleotide comprises sequences encoding AAV4 Rep 78, AAV4 Rep 68, AAV4 Rep 52, and AAV4 Rep 40 proteins, wherein the first promoter is a AAV2 p5 promoter, and wherein the first polynucleotide further comprises a p19 promoter; the second polynucleotide comprises sequences encoding Adeno E2A, Adeno

E4, and Adeno VA; the second promoter of the third polynucleotide is a p41 promoter, and the third polynucleotide further comprises: a non-coding sequence positioned between one of the two ITR sequences and the second promoter; and a AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and AAV2 3' UTR sequence at the 3' end of the capsid coding sequence.

[0018] In some embodiments, the first polynucleotide comprises sequences encoding AAV10 Rep 78, AAV10 Rep 68, AAV10 Rep 52, and AAV10 Rep 40 proteins, wherein the first promoter is a AAV2 p5 promoter, and wherein the first polynucleotide further comprises a p19 promoter; the second polynucleotide comprises sequences encoding Adeno E2A, Adeno E4, and Adeno VA; the second promoter of the third polynucleotide is a p41 promoter, and the third polynucleotide further comprises: a non-coding sequence positioned between one of the two ITR sequences and the second promoter and a AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and AAV2 3' UTR sequence at the 3' end of the capsid coding sequence.

[0019] In some embodiments, the first polynucleotide comprises sequences encoding: AAV2 and AAV10 Rep 78 proteins; AAV2 and AAV10 Rep 68 proteins; an AAV2 Rep 52 protein; and an AAV4 Rep 40 protein, wherein the first promoter is an AAV2 p5 promoter, and wherein the first polynucleotide further comprises a p19 promoter; the second polynucleotide comprises coding sequences of Adeno E2A, Adeno E4, and Adeno VA; the second promoter of the third polynucleotide is a p41 promoter, and the third polynucleotide further comprises: a non-coding sequence positioned between one of the two ITR sequences and the second promoter and an AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and AAV2 3' UTR sequence at the 3' end of the capsid coding sequence.

[0020] In some embodiments, the first polynucleotide is devoid of a helper virus gene. In some embodiments, the first polynucleotide is devoid of adenovirus helper genes. In some embodiments, the first polynucleotide is devoid of a capsid gene.

[0021] In some embodiments, the first polynucleotide further comprises a nucleotide sequence encoding an additional protein. In some embodiments, the additional protein is from a Herpes simplex virus type 1 (HSV-1) or a bocavirus. In some embodiments, the additional protein is a helper virus gene selected from: NS2 and UL12.

[0022] In some embodiments, the first polynucleotide comprises sequences encoding: an AAV2 Rep 78 protein and an AAV2 Rep 68 protein; or a non-AAV2 Rep 78 protein and a non-AAV2 Rep 68 protein.

[0023] In some embodiments, the first promoter of the first polynucleotide is a p5 promoter. In some embodiments, the first polynucleotide is devoid of a p19 promoter. In some embodiments, the third polynucleotide comprises a promoter selected from: p19 promoter, CMV, SV40, Ef1a, UBC, p5, and a PGK promoter.

[0024] In some embodiments, the third polynucleotide further comprises a Rep 52 protein and a Rep 40 protein positioned between: two promoter sequences and downstream the 3'ITR sequence; or between the second promoter and the capsid protein.

[0025] In some embodiments, the Rep 52 protein is a non-AAV2 or an AAV2 Rep 52 protein; and wherein the Rep 40 protein is a non-AAV2 or an AAV2 Rep 40 protein.

[0026] In some embodiments, the two promoters are a p19 promoter and a p40 promoter.

[0027] In some embodiments, the first polynucleotide comprises sequences encoding a Rep 52 protein and a Rep 40 protein.

[0028] In some embodiments, the first polynucleotide or third polynucleotide is devoid of a p19 promoter.

[0029] In some embodiments, the third polynucleotide further comprises coding sequences of a Rep 78 protein and a Rep 68 protein positioned between: 2 promoters and downstream the 3' ITR sequence; or between the second promoter and the capsid protein.

[0030] In some embodiments, the Rep 78 protein is a non-AAV2 or an AAV2 Rep 78 protein; and wherein the Rep 68 protein is a non-AAV2 or an AAV2 Rep 68 protein.

[0031] In some embodiments, the first polynucleotide comprises sequences encoding a Rep 78 protein and a Rep 68 protein, optionally wherein the first polynucleotide comprises mutated coding sequences of Rep 52 and Rep 40 proteins with a mutated start codon.

[0032] In some embodiments, the first polynucleotide comprises sequences encoding an AAV2 Rep 78 protein and an AAV2 Rep 68 protein.

[0033] In some embodiments, the first promoter of the first polynucleotide is a p5 promoter.

[0034] In some embodiments, the first polynucleotide is devoid of a p19 promoter.

[0035] In some embodiments, the system further comprises a fourth polynucleotide comprising sequences encoding Rep 52 and Rep 40 proteins.

[0036] In some embodiments, the fourth polynucleotide comprises a p19 promoter.

[0037] In some embodiments, the fourth polynucleotide is smaller in size compared to the first polynucleotide.

[0038] In some embodiments, the fourth polynucleotide comprises a p19 promoter and a p40 promoter.

[0039] In some embodiments, the second polynucleotide comprises two helper virus genes from Adenovirus 5 or Adenovirus 2. In some embodiments, the second polynucleotide comprises two helper virus genes selected from Adeno E2A, Adeno E2B, Adeno E4, Adeno VA, Adeno L3 and Adeno L4. In some embodiments, the second polynucleotide comprises three helper virus genes selected from Adeno E2A, Adeno E2B, Adeno E4, Adeno VA, Adeno L3 and Adeno L4. In some embodiments, the second polynucleotide comprises Adeno E2A, Adeno E4, and Adeno VA. In some embodiments, the second polynucleotide comprises Adeno E2B, Adeno VA, Adeno L3, Adeno E2A, Adeno L4, and Adeno E4. In some embodiments, the second polynucleotide comprises E1 Open Reading Frame (ORF1), E4 Open Reading Frame (ORF2), ORF3, ORF4 and ORF 6/7.

[0040] In some embodiments, the second polynucleotide further comprises a helper virus gene selected from: NS2 and UL12.

[0041] In some embodiments, the second polynucleotide further comprises helper virus genes NS2 and UL12. In some embodiments, the second polynucleotide lacks Adeno E2B, Adeno VA, Adeno L3, or Adeno L4. In some embodiments, the second polynucleotide lacks two or more helper virus genes selected from Adeno E2B, Adeno VA, Adeno L3, and Adeno L4. In some embodiments, the second polynucleotide lacks Adeno E2B, Adeno VA, Adeno L3, and Adeno L4.

[0042] In some embodiments, the second polynucleotide has a length less than 14,000 nucleotides. In some embodiments, the second polynucleotide has a length less than 12,000 nucleotides. In some embodiments, the second polynucleotide has a length ranging from 9,000 to 13,000 nucleotides. In some embodiments, the second polynucleotide has a length ranging from 11,000 to 12,000 nucleotides.

[0043] In some embodiments, the third polynucleotide further comprises a 5' UTR nucleotide sequence between the second promoter and the capsid gene. In some embodiments, the 5' UTR sequence is a non-coding nucleotide sequence.

[0044] In some embodiments, the second promoter of the third polynucleotide is a p41 promoter, p40 promoter, CMV promoter, SV40 promoter, Ef1a promoter, TRE, UBC, or a PGK promoter.

[0045] In some embodiments, the 5'UTR sequence of the third polynucleotide has a length ranging from 150-400 nucleotides. In some embodiments, the 5'UTR sequence of the third polynucleotide has a length ranging from 100-400 nucleotides. In some embodiments, the 5'UTR sequence of the third polynucleotide has a length ranging from 200-400 nucleotides. In some embodiments, the capsid gene and the second promoter in the third polynucleotide are positioned outside of the two ITR sequences.

[0046] In some embodiments, the two ITR sequences are from an AAV serotype selected from: AAV1, AAV2, AAV3, AAV4, AA5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, and AAV13.

[0047] In some embodiments, the capsid gene and the second promoter in the third polynucleotide are positioned downstream of the 3'-ITR sequence. In some embodiments, the capsid gene and the second promoter in the third polynucleotide are positioned upstream of the 5'-ITR sequence.

[0048] In some embodiments, the third polynucleotide further comprises a non-coding nucleotide sequence positioned between one of the two ITR sequences and the second promoter.

[0049] In some embodiments, the non-coding nucleotide sequence of the third polynucleotide has a length ranging from 165-1300 nucleotides.

[0050] In some embodiments, the third polynucleotide comprises the AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and a AAV2 3' UTR sequence positioned at the 3' end of the capsid coding sequence.

[0051] In some embodiments, the third polynucleotide comprises the AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and a transcription termination sequence (polyA sequence) positioned at the 3' end of the capsid coding sequence.

[0052] In some embodiments, the third polynucleotide further comprises a AAV2 3' UTR sequence positioned at the 3' end of the capsid coding sequence.

[0053] In some embodiments, the AAV2 5' UTR sequence is positioned between the second promoter and the 5' end of the capsid coding sequence.

[0054] In some embodiments, the AAV2 3' UTR sequence is positioned at the 3' end of the capsid coding sequence.

[0055] v the third polynucleotide comprises the AAV2 5' UTR sequence positioned between the second promoter and the 5' end of the capsid coding sequence and a transcription termination sequence (e.g., polyA sequence) at the 3' end of the capsid coding sequence.

[0056] In some embodiments, the polyA sequence is a SV40 polyA sequence.

[0057] In some embodiments, the third polynucleotide further comprises a rep gene encoding a Rep protein.

[0058] In some embodiments, the third polynucleotide comprises sequences encoding one or more of: Rep 78 protein, Rep 68 protein, Rep 52 protein, and Rep 40 protein.

[0059] In some embodiments, the third polynucleotide comprises sequences encoding a Rep 52 protein and Rep 40 protein.

[0060] In some embodiments, the third polynucleotide comprises sequences encoding Rep 78, Rep 68, Rep 52, and Rep 40 proteins.

[0061] In some embodiments, the Capsid protein has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 100% amino acid sequence identity to the sequence of a AAV capsid protein of an AAV selected from the group consisting of: AAV9; Anc80L65; Anc80-55, Anc80-129, Anc80-156, Anc80-751, Anc80-1029, Anc80-1712, AAV2; AAV1; AAV6; AAV3; AAV LK03; AAV7; AAV8; AAV hu.37; AAV rh.10; AAV hu.68; AAV10; AAV5; AAV3-3; AAV4-4; AAV1-A; hu.46-A; hu.48-A; hu.44-A; hu.43-A; AAV6-A; hu.34-B; hu.47-B; hu.29-B; rh.63-B; hu.56-B; hu.45-B; rh.57-B; rh.35-B; rh.58-B; rh.28-B; rh.51-B; rh.19-B; rh.49-B; rh.52-B; rh.13-B; AAV2-B; rh.20-B; rh.24-B; rh.64-B; hu.27-B; hu.21-B; hu.22-B; hu.23-B; hu.7-C; hu.61-C; rh.56-C; hu. 9-C; hu.54-C; hu.53-C; hu.60-C; hu.55-C; hu.2-C; hu.1-C; hu.18-C; hu.3-C; hu.25-C; hu.15-C; hu.16-C; hu.11-C; hu.10-C; hu.4-C; rh.54-D; rh.48-D; rh.55-D; rh.62-D; AAV7-D; rh.52-E; rh.51-E; hu.39-E; rh.53-E; hu.37-E; rh.43-E; rh.50-E; rh.49-E; rh.61-E; hu.41-E; rh.64-E; rh74; hu.42-E; rh.57-E; rh.40-

E; hu.67-E; hu.17-E; hu.6-E; hu.66-E; rh.38-E; hu.32-F; AAV9/hu; hu.31-F; Anc80; Anc81; Anc82; Anc83; Anc84; Anc94; Anc113; Anc126; Anc127; Anc80L27; Anc80L59; Anc80L60; Anc80L62; Anc80L33; Anc80L36; Anc80L44; Anc80L1; Anc110; Anc80DI; CAPSID-1_AAV9_1000, Capsid-3_38181, and AAV9 (Capsid-2_MUT1-38181).

[0062] In some embodiments, the capsid protein is an AAV9 capsid variant protein, wherein the AAV9 capsid variant protein comprises a 6 to 12-mer peptide inserted into an AAV9 capsid protein.

[0063] In some embodiments, the first polynucleotide comprises sequences encoding Rep 78, Rep 68, Rep 52, and Rep 40 proteins, wherein the first promoter is a AAV2 p5 promoter, and wherein the first polynucleotide further comprises a p19 promoter; the second polynucleotide comprises sequences encoding Adeno E2A, Adeno E4, and Adeno VA; the second promoter of the third polynucleotide is a p41 promoter, and the third polynucleotide further comprises: a second non-coding sequence positioned between one of the two ITR sequences and the second promoter and a AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and AAV2 3' UTR at the 3' end of the capsid coding sequence.

[0064] In some embodiments,: the two ITR sequences are AAV2 ITR sequences.

[0065] In some embodiments, the first polynucleotide comprises sequences encoding a AAV2 Rep 78 protein, a AAV2 Rep 68 protein, a AAV2 Rep 52 protein, and a AAV2 Rep 40 protein.

[0066] In some embodiments, the first polynucleotide comprises sequences encoding Rep 78, Rep 68, Rep 52, and Rep 40 proteins, wherein the first promoter is an AAV2 p5 promoter; the second polynucleotide comprises sequences encoding Adeno E2A, Adeno E4, and Adeno VA; the second promoter of the third polynucleotide is selected from: a p41, p40, CMV, SV40, Ef1a, Ef1a core, PGK, CAG, UBC, CBh, or a TRE promoter; and the third polynucleotide further comprises: a non-coding sequence positioned between one of the two ITR sequences and the second promoter and a AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and AAV2 3' UTR at the 3' end of the capsid coding sequence.

[0067] In some embodiments, the first polynucleotide comprises sequences encoding Rep 78, Rep 68, Rep 52, and Rep 40 proteins, wherein the first promoter is a AAV2 p5 promoter; the second polynucleotide comprises sequences encoding Adeno E2A, Adeno E4, and Adeno

VA; . the second promoter of the third polynucleotide is a p41 promoter, and the third polynucleotide further comprises: a non-coding sequence positioned between one of the two ITR sequences and the second promoter and a AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and a transcription termination sequence (polyA sequence) at the 3' end of the capsid coding sequence.

[0068] In some embodiments, the first polynucleotide comprises sequences encoding: AAV2 Rep 78, AAV2 Rep 68, AAV2 Rep 52, and AAV2 Rep 40 proteins, and a UL12 or transthyretin (ttR) protein, and wherein the first promoter is an AAV2 p5 promoter.

[0069] In some embodiments, the second polynucleotide comprises sequences encoding Adeno E2A, Adeno E4, and Adeno VA.

[0070] In some embodiments, the second promoter of the third polynucleotide is selected from: a p41, p40, CMV, SV40, Ef1a, Ef1a core, PGK, CAG, UBC, CBh, or a TRE promoter.

[0071] In some embodiments, the first polynucleotide further comprises a coding sequence of UL12 or transthyretin(ttR).

[0072] In some embodiments, the first polynucleotide further comprises a coding sequence of transthyretin(ttR).

[0073] In some embodiments, the first polynucleotide is in a plasmid. In some embodiments, the second polynucleotide is in a plasmid. In some embodiments, the third polynucleotide is in a plasmid.

[0074] In some embodiments, the first polynucleotide is in a first plasmid, the second polynucleotide is in a second plasmid, and the third polynucleotide construct is in a third plasmid.

[0075] An aspect of the present disclosure includes a packaging cell comprising the system of the present disclosure.

[0076] A method of producing recombinant AAV, comprising: providing the system of of the present disclosure; introducing the first polynucleotide, the second polynucleotide and the third polynucleotide of the system into a cell; and culturing the cell under conditions suitable for recombinant AAV (rAAV) production.

[0077] In some embodiments, in step (b), a molar amount of the first polynucleotide introduced into the cell is at least twice of a molar amount of the second polynucleotide introduced into the cell.

[0078] In some embodiments, a molar amount of the first polynucleotide introduced into the cell is two to four times of a molar amount of the second polynucleotide introduced into the cell.

[0079] In some embodiments, a molar amount of the first polynucleotide introduced into the cell is at least three times of a molar amount of the second polynucleotide introduced into the cell.

[0080] In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least four times of a molar amount of the third polynucleotide introduced into the cell.

[0081] In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least five or six times of a molar amount of the third polynucleotide introduced into the cell.

[0082] In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least five times of a molar amount of the third polynucleotide introduced into the cell.

[0083] In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least ten times of a molar amount of the third polynucleotide introduced into the cell.

[0084] In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least fifteen times of a molar amount of the third polynucleotide introduced into the cell.

[0085] In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least twenty times of a molar amount of the third polynucleotide introduced into the cell.

[0086] In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least twenty-five times of a molar amount of the third polynucleotide introduced into the cell.

[0087] In some embodiments, the first polynucleotide comprises a molar amount ranging from about 5% to 95% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined.

[0088] In some embodiments, the first polynucleotide comprises a molar amount ranging from about 5% to about 35% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined.

[0089] In some embodiments, the first polynucleotide comprises a molar amount ranging from about 10% to about 90% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined.

[0090] In some embodiments, the second polynucleotide comprises a molar amount ranging from about 5% to 80% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined.

[0091] In some embodiments, the second polynucleotide comprises a molar amount of about 60% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined.

[0092] In some embodiments, the third polynucleotide comprises a molar amount ranging from about 1% to 90% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined.

[0093] In some embodiments, the third polynucleotide comprises a molar amount of about 70% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined.

[0094] In some embodiments, the system comprises a molar ratio of 6:3:1 of the second polynucleotide:first polynucleotide:third polynucleotide.

[0095] In some embodiments, the system comprises a molar ratio of 5:1:4 of the second polynucleotide:first polynucleotide:third polynucleotide.

[0096] In some embodiments, the system comprises a molar ratio of 1:2:7 of the second polynucleotide:first polynucleotide:third polynucleotide.

[0097] In some embodiments, culturing the cell under conditions suitable for recombinant AAV production comprises expanding the cell.

[0098] In some embodiments, the method comprises collecting the secreted recombinant AAV production expressed by the cell. In some embodiments, further comprising lysing the cell.

[0099] In some embodiments, further comprising purifying the lysed cell to collect rAAV particles.

[0100] In some embodiments, the collected rAAV particles comprise less than 5% of replication-competent adeno-associated virus particles (rcAAV).

[0101] In some embodiments, the collected rAAV particles comprise less than 1% of replication-competent adeno-associated virus particles (rcAAV).

[0102] An aspect of the present disclosure includes a recombinant adeno-associated virus (rAAV) produced by the method of the present disclosure, using the system of the present disclosure, or from the packaging cell of the present disclosure.

[0103] An aspect of the present disclosure includes a pharmaceutical composition comprising the recombinant adeno-associated virus (rAAV) of the present disclosure.

[0104] Another aspect of the present disclosure includes a system for production of recombinant adeno-associated virus (rAAV) comprising: a first polynucleotide comprising at least one rep gene encoding at least one functional Rep protein, and a first promoter operably linked to the at least one rep gene; a second polynucleotide comprising at least one helper virus gene, a third polynucleotide comprising (i) a capsid gene encoding a Capsid protein, a second promoter operably linked to the capsid gene and a non-coding sequence between the second promoter and the capsid gene; and (ii) an expression cassette comprising a transgene flanked by two inverted terminal repeats (ITRs).

[0105] In some embodiments, the second polynucleotide comprises at least two helper virus genes from Adenovirus 5 or Adenovirus 2. In some embodiments, the second polynucleotide comprises at least two helper virus genes selected from Adeno E2A, Adeno E2B, Adeno E4, Adeno VA, Adeno L3 and Adeno L4. In some embodiments, the second polynucleotide comprises at least three helper virus genes selected from Adeno E2A, Adeno E2B, Adeno E4, Adeno VA, Adeno L3 and Adeno L4. In some embodiments, the second polynucleotide comprises Adeno E2A, Adeno E4, and Adeno VA. In some embodiments, the the second polynucleotide comprising Adeno E2A, Adeno E4, and Adeno VA has a nucleotide sequence having at least 85% sequence identity to positions 1-9281 of SEQ ID NO: 3. In some

embodiments, the the second polynucleotide comprises Adeno E2B, Adeno VA, Adeno L3, Adeno E2A, Adeno L4, and Adeno E4.

[0106] In some embodiments, the Adeno E2B has a nucleotide sequence having at least 85% sequence identity to positions 1-699 of SEQ ID NO: 4. In some embodiments, the Adeno VA has a nucleotide sequence having at least 85% sequence identity to positions 773-932 and 1029-1102 of SEQ ID NO: 4. In some embodiments, the Adeno L3 has a nucleotide sequence having at least 85% sequence identity to positions 1103-1572 of SEQ ID NO: 4. In some embodiments, the Adeno E2A has a nucleotide sequence having at least 85% sequence identity to positions 1667-3257 of SEQ ID NO: 4. In some embodiments, the Adeno L4 has a nucleotide sequence having at least 85% sequence identity to positions 3286-5709 and 6393-7076 of SEQ ID NO: 4.

[0107] In some embodiments, the second polynucleotide comprises E1 Open Reading Frame (ORF), E4 Open Reading Frame (ORF) 2, ORF3, ORF4 and ORF 6/7. In some embodiments, the the second polynucleotide comprising E4 Open Reading Frame (ORF) 1 has a nucleotide sequence having at least 85% sequence identity to positions 9978-10362 of SEQ ID NO: 4. In some embodiments, the second polynucleotide comprising E4 Open Reading Frame (ORF) 2 has a nucleotide sequence having at least 85% sequence identity to positions 9538-9948 of SEQ ID NO: 4. In some embodiments the second polynucleotide comprising ORF3 has a nucleotide sequence having at least 85% sequence identity to positions 9191-9541 of SEQ ID NO: 4. In some embodiments, the second polynucleotide comprising ORF4 has a nucleotide sequence having at least 85% sequence identity to positions 8836-9180 of SEQ ID NO: 4. In some embodiments, the second polynucleotide comprising ORF 6/7 has a nucleotide sequence having at least 85% sequence identity to positions 7752-8915 of SEQ ID NO: 4.

[0108] In some embodiments, the second polynucleotide lacks Adeno E2B, Adeno VA, Adeno L3, or Adeno L4. In some embodiments, the second polynucleotide lacks two or more helper virus genes selected from Adeno E2B, Adeno VA, Adeno L3, and Adeno L4. In some embodiments, the second polynucleotide lacks Adeno E2B, Adeno VA, Adeno L3, and Adeno L4.

[0109] In some embodiments, the second polynucleotide has a length less than 14,000 nucleotides. In some embodiments, the second polynucleotide has a length less than 12,000 nucleotides. In some embodiments, the second polynucleotide has a length ranging from

9,000 to 13,000 nucleotides. In some embodiments, the second polynucleotide has a length ranging from 11,000 to 12,000 nucleotides.

[0110] In some embodiments, the second polynucleotide has a nucleotide sequence having at least 90% sequence identity to any one of SEQ ID NOs: 3-4. In some embodiments, the second polynucleotide has a nucleotide sequence having at least 95% sequence identity to any one of SEQ ID NOs: 3-4. In some embodiments, the second polynucleotide has a nucleotide sequence of any one of SEQ ID NOs: 3-4. In some embodiments, the second polynucleotide has a nucleotide sequence having at least 85% sequence identity to any one of SEQ ID Nos.: 3-4. In some embodiments, the second polynucleotide has a nucleotide sequence having at least 90% sequence identity to any one of SEQ ID Nos.: 3-4. In some embodiments, the second polynucleotide has a nucleotide sequence having at least 95% sequence identity to any one of SEQ ID Nos.: 3-4. In some embodiments, the second polynucleotide has a nucleotide sequence of any one of SEQ ID Nos.: 3-4.

[0111] In some embodiments, the second promoter of the third polynucleotide is a p41 promoter or a p40 promoter.

[0112] In some embodiments, the second promoter of the third polynucleotide has a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 11. In some embodiments, the second promoter of the third polynucleotide has a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 11. In some embodiments, the second promoter of the third polynucleotide has a nucleotide sequence of SEQ ID NO: 11.

[0113] In some embodiments, the second promoter of the third polynucleotide has a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 20. In some embodiments, the second promoter of the third polynucleotide has a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 20. In some embodiments, the second promoter of the third polynucleotide has a nucleotide sequence of SEQ ID NO: 20.

[0114] In some embodiments, the non-coding sequence of the third polynucleotide has a length ranging from 150-400 nucleotides. In some embodiments, the non-coding sequence of the third polynucleotide has a length ranging from 280-350 nucleotides. In some embodiments, the non-coding sequence of the third polynucleotide has a length ranging from 290-325 nucleotides. In some embodiments, the non-coding sequence of the third polynucleotide has a length ranging from 300-350 nucleotides. In some embodiments, the

non-coding sequence of the third polynucleotide has a length ranging from 200-300 nucleotides.

[0115] In some embodiments, the non-coding sequence of the third polynucleotide has a nucleotide sequence having at least 85% sequence identity to any one of SEQ ID NOs: 21-24. In some embodiments, the non-coding sequence of the third polynucleotide has a nucleotide sequence having at least 90% sequence identity to any one of SEQ ID NOs: 21-24. In some embodiments, the non-coding sequence of the third polynucleotide has a nucleotide sequence having at least 95% sequence identity to any one of SEQ ID NOs: 21-24. In some embodiments, the non-coding sequence of the third polynucleotide has a nucleotide sequence of any one of SEQ ID NOs: 21-24.

[0116] In some embodiments, the capsid gene and the second promoter in the third polynucleotide are positioned outside of the two ITR sequences. In some embodiments, the capsid gene and the second promoter in the third polynucleotide are positioned downstream of the 3'-ITR sequence. In some embodiments, the one of the two ITR sequences has a nucleotide sequence having at least 85% sequence identity to SEQ ID No: 82. In some embodiments, the one of the two ITR sequences has a nucleotide sequence having at least 90% sequence identity to SEQ ID No: 82.

[0117] In some embodiments, the one of the two ITR sequences has a nucleotide sequence having at least 95% sequence identity to SEQ ID No: 82. In some embodiments, the one of the two ITR sequences has a nucleotide sequence of SEQ ID No: 82.

[0118] In some embodiments, the third polynucleotide further comprises a second non-coding sequence positioned between one of the two ITR sequences and the second promoter. In some embodiments, the second non-coding sequence of the third polynucleotide has a nucleotide sequence having at least 85% sequence identity to any one of SEQ ID NOs: 75-81. In some embodiments, the non-coding sequence of the third polynucleotide has a nucleotide sequence having at least 90% sequence identity to any one of SEQ ID NOs: 75-81. In some embodiments, the non-coding sequence of the third polynucleotide has a nucleotide sequence having at least 95% sequence identity to any one of SEQ ID NOs: 75-81. In some embodiments, the non-coding sequence of the third polynucleotide has a nucleotide sequence of any one of SEQ ID NOs: 75-81.

[0119] In some embodiments, the second non-coding sequence of the third polynucleotide has a length ranging from 300-1100 nucleotides. In some embodiments, the second non-

coding sequence of the third polynucleotide has a length ranging from 350-1050 nucleotides. In some embodiments, the second non-coding sequence of the third polynucleotide has a length ranging from 350-800 nucleotides.

[0120] In some embodiments, the Capsid protein has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% amino acid sequence identity to the sequence of a AAV capsid protein of an AAV selected from the group consisting of: AAV9; Anc80L65; Anc80-55, Anc80-129, Anc80-156, Anc80-751, Anc80-1029, Anc80-1712, AAV2; AAV1; AAV6; AAV3; AAV LK03; AAV7; AAV8; AAV hu.37; AAV rh.10; AAV hu.68; AAV10; AAV5; AAV3-3; AAV4-4; AAV1-A; hu.46-A; hu.48-A; hu.44-A; hu.43-A; AAV6-A; hu.34-B; hu.47-B; hu.29-B; rh.63-B; hu.56-B; hu.45-B; rh.57-B; rh.35-B; rh.58-B; rh.28-B; rh.51-B; rh.19-B; rh.49-B; rh.52-B; rh.13-B; AAV2-B; rh.20-B; rh.24-B; rh.64-B; hu.27-B; hu.21-B; hu.22-B; hu.23-B; hu.7-C; hu.61-C; rh.56-C; hu. 9-C; hu.54-C; hu.53-C; hu.60-C; hu.55-C; hu.2-C; hu.1-C; hu.18-C; hu.3-C; hu.25-C; hu.15-C; hu.16-C; hu.11-C; hu.10-C; hu.4-C; rh.54-D; rh.48-D; rh.55-D; rh.62-D; AAV7-D; rh.52-E; rh.51-E; hu.39-E; rh.53-E; hu.37-E; rh.43-E; rh.50-E; rh.49-E; rh.61-E; hu.41-E; rh.64-E; rh74; hu.42-E; rh.57-E; rh.40-E; hu.67-E; hu.17-E; hu.6-E; hu.66-E; rh.38-E; hu.32-F; AAV9/hu; hu.31-F; Anc80; Anc81; Anc82; Anc83; Anc84; Anc94; Anc113; Anc126; Anc127; Anc80L27; Anc80L59; Anc80L60; Anc80L62; Anc80L33; Anc80L36; Anc80L44; Anc80L1; Anc110; Anc80DI; Capsid-1_AAV9_1000, Capsid-3_38181, and AAV9 (Capsid-2_MUT1-38181). In some embodiments, the capsid protein is a capsid protein of an AAV selected from the group consisting of: AAV9; Anc80L65; Anc80-55, Anc80-129, Anc80-156, Anc80-751, Anc80-1029, Anc80-1712, AAV2; AAV1; AAV6; AAV3; AAV LK03; AAV7; AAV8; AAV hu.37; AAV rh.10; AAV hu.68; AAV10; AAV5; AAV3-3; AAV4-4; AAV1-A; hu.46-A; hu.48-A; hu.44-A; hu.43-A; AAV6-A; hu.34-B; hu.47-B; hu.29-B; rh.63-B; hu.56-B; hu.45-B; rh.57-B; rh.35-B; rh.58-B; rh.28-B; rh.51-B; rh.19-B; rh.49-B; rh.52-B; rh.13-B; AAV2-B; rh.20-B; rh.24-B; rh.64-B; hu.27-B; hu.21-B; hu.22-B; hu.23-B; hu.7-C; hu.61-C; rh.56-C; hu. 9-C; hu.54-C; hu.53-C; hu.60-C; hu.55-C; hu.2-C; hu.1-C; hu.18-C; hu.3-C; hu.25-C; hu.15-C; hu.16-C; hu.11-C; hu.10-C; hu.4-C; rh.54-D; rh.48-D; rh.55-D; rh.62-D; AAV7-D; rh.52-E; rh.51-E; hu.39-E; rh.53-E; hu.37-E; rh.43-E; rh.50-E; rh.49-E; rh.61-E; hu.41-E; rh.64-E; rh74; hu.42-E; rh.57-E; rh.40-E; hu.67-E; hu.17-E; hu.6-E; hu.66-E; rh.38-E; hu.32-F; AAV9/hu; hu.31-F; Anc80; Anc81; Anc82; Anc83; Anc84; Anc94; Anc113; Anc126; Anc127; Anc80L27; Anc80L59; Anc80L60; Anc80L62; Anc80L33; Anc80L36; Anc80L44;

Anc80L1; Anc110; Anc80DI; Capsid-1_AAV9_1000, Capsid-3_38181, and AAV9 (Capsid-2_MUT1-38181).

[0121] The system of any one of claims 0-89, wherein the Capsid protein is selected from VP1, VP2 and VP3. In some embodiments, the capsid protein is a capsid protein having a sequence selected from SEQ ID Nos: 25-162 and 212. In some embodiments, the capsid protein is Anc80. In some embodiments, the capsid protein is Anc80 having an amino acid sequence of SEQ ID NO: 105. In some embodiments, the capsid protein is Anc80L65. In some embodiments, the capsid protein is Anc80L65 having an amino acid sequence of SEQ ID NO: 115 or SEQ ID NO: 149.

[0122] In some embodiments, the capsid protein is AAV9 capsid protein or a modification thereof. In some embodiments, the capsid protein is AAV9 and has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% amino acid sequence identity to the sequence selected from: SEQ ID NOs: 61, 158, 159, and 163.

[0123] In some embodiments, the first polynucleotide is devoid of a helper virus gene. In some embodiments, the first polynucleotide is devoid of a capsid gene.

[0124] In some embodiments, the at least one rep gene of the first polynucleotide comprises a sequence encoding a functional Rep 78 protein, a Rep 68 protein, a Rep 52 protein, or a Rep 40 protein. In some embodiments, the at least one rep gene of the first polynucleotide comprises two, three or four sequences, wherein each of the two, three or four sequence encodes a functional Rep 78 protein, a Rep 68 protein, a Rep 52 protein, or a Rep 40 protein. In some embodiments, the first polynucleotide comprises sequences encoding a functional Rep 78 protein, a Rep 68 protein, a Rep 52 protein, and a Rep 40 protein. In some embodiments, the at least one rep gene of the first polynucleotide comprising the sequence encoding the functional Rep 78 protein has a nucleotide sequence having at least 85% sequence identity to positions 1-1866 of SEQ ID NOs: 6 or positions 117-1982 of SEQ ID NO: 7. In some embodiments, the at least one rep gene of the first polynucleotide comprising the sequence encoding the functional Rep 68 protein has a nucleotide sequence having at least 85% sequence identity to positions 1-1586 of SEQ ID NO: 6 or positions 117-1702 of SEQ ID NO: 7. In some embodiments, the at least one rep gene of the first polynucleotide comprising the sequence encoding the functional Rep 52 protein has a nucleotide sequence having at least 85% sequence identity to positions 673-1866 of SEQ ID NO: 6 or positions 789-1982 of SEQ ID NO: 7. In some embodiments, the at least one rep gene of the first

polynucleotide comprising the sequence encoding the functional Rep 40 protein has a nucleotide sequence having at least 85% sequence identity to positions 673-1611 of SEQ ID NO: 6 or positions 789-1727 of SEQ ID NO: 7.

[0125] In some embodiments, the first promoter of the first polynucleotide is a p5 promoter of AAV2 or a modification thereof. In some embodiments, the first promoter of the first polynucleotide has a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 2. In some embodiments, the first promoter of the first polynucleotide has a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 2.

[0126] In some embodiments, the first promoter of the first polynucleotide has a nucleotide sequence having at least 90% sequence identity to positions 2462-2577 of SEQ ID NO: 6 or positions 1-116 of SEQ ID NO: 7. In some embodiments, the first promoter of the first polynucleotide has a nucleotide sequence having at least 95% sequence identity to positions 2462-2577 of SEQ ID NO: 6 or positions 1-116 of SEQ ID NO: 7. In some embodiments, the first promoter of the first polynucleotide has a nucleotide sequence of positions 2462-2577 of SEQ ID NO: 6 or positions 1-116 of SEQ ID NO: 7.

[0127] In some embodiments, the first promoter of the first polynucleotide comprises a P5 promoter having a nucleotide sequence of any one of SEQ ID Nos.: 2, 8, and 9. In some embodiments, the at least one rep gene of the first polynucleotide comprises an internal P5 promoter or fragment thereof, wherein the first promoter is the internal P5 promoter or fragment thereof.

[0128] In some embodiments, the first polynucleotide comprises an internal p40 promoter or fragment thereof. In some embodiments, the internal p40 promoter or fragment thereof has a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 11. In some embodiments, the internal p40 promoter or fragment thereof has a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 11. In some embodiments, the internal p40 promoter or fragment thereof has a nucleotide sequence of SEQ ID NO: 11. In some embodiments, the first polynucleotide comprises an internal p19 promoter or fragment thereof. In some embodiments, the internal p19 promoter or fragment thereof has a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 10. In some embodiments, the internal p19 promoter or fragment thereof has a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 10.

[0129] In some embodiments, the internal p19 promoter or fragment thereof has a nucleotide sequence of SEQ ID NO: 10.

[0130] In some embodiments, the at least one rep gene of the first polynucleotide has a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 5. In some embodiments, the at least one rep gene of the first polynucleotide has a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 5. In some embodiments, the at least one rep gene of the first polynucleotide has a nucleotide sequence of SEQ ID NO: 5.

[0131] In some embodiments, the first polynucleotide has a nucleotide sequence having at least 90% sequence identity to positions 1-2577 or positions 1-1611 of SEQ ID NO: 6. In some embodiments, the first polynucleotide has a nucleotide sequence having at least 95% sequence identity to positions 1-2577 or positions 1-1611 of SEQ ID NO: 6. In some embodiments, the first polynucleotide has a nucleotide sequence of positions 1-2577 or positions 1-1611 of SEQ ID NO: 6.

[0132] In some embodiments, the first polynucleotide has a nucleotide sequence having at least 90% sequence identity to positions 1-1998 or positions 1-1982 of SEQ ID NO: 7. In some embodiments, the first polynucleotide has a nucleotide sequence having at least 95% sequence identity to positions 1-1998 or positions 1-1982 of SEQ ID NO: 7.

[0133] In some embodiments, the first polynucleotide has a nucleotide sequence of positions 1-1998 or positions 1-1982 of SEQ ID NO: 7. In some embodiments, the first polynucleotide has a nucleotide sequence having at least 85% sequence identity to any one of SEQ ID NOs: 6 and 7. In some embodiments, the first polynucleotide has a nucleotide sequence having at least 90% sequence identity to any one of SEQ ID NOs: 6 and 7. In some embodiments, the first polynucleotide has a nucleotide sequence having at least 95% sequence identity to any one of SEQ ID NOs: 6 and 7. In some embodiments, the first polynucleotide has a nucleotide sequence of SEQ ID NO: 6. In some embodiments, the first polynucleotide has a nucleotide sequence of SEQ ID NO: 7.

[0134] In some embodiments, the first polynucleotide is in a plasmid. In some embodiments, the second polynucleotide is in a plasmid. In some embodiments, the third polynucleotide is in a plasmid.

[0135] In some embodiments, the first polynucleotide is in a first plasmid, the second polynucleotide is in a second plasmid, and the third polynucleotide construct is in a third plasmid.

[0136] Another aspect of the present disclosure includes a packaging cell comprising the system disclosed herein.

[0137] Another aspect of the present disclosure includes a method of producing recombinant AAV, comprising: (a)providing the system described herein; (b)introducing the first polynucleotide, the second polynucleotide and the third polynucleotide of the system into a cell; and (c) culturing the cell under conditions suitable for recombinant AAV (rAAV) production. In some embodiments, in step (b), a molar amount of the first polynucleotide introduced into the cell is at least twice of a molar amount of the second polynucleotide introduced into the cell.

[0138] In some embodiments, a molar amount of the first polynucleotide introduced into the cell is two to four times of a molar amount of the second polynucleotide introduced into the cell. In some embodiments, a molar amount of the first polynucleotide introduced into the cell is three times of a molar amount of the second polynucleotide introduced into the cell. In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least four times of a molar amount of the third polynucleotide introduced into the cell. In some embodiments, a molar amount of the second polynucleotide introduced into the cell is five or six times of a molar amount of the third polynucleotide introduced into the cell. In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least five times of a molar amount of the third polynucleotide introduced into the cell. In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least ten times of a molar amount of the third polynucleotide introduced into the cell.

[0139] In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least fifteen times of a molar amount of the third polynucleotide introduced into the cell. In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least twenty times of a molar amount of the third polynucleotide introduced into the cell. In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least twenty-five times of a molar amount of the third polynucleotide introduced into the cell.

[0140] In some embodiments, the first polynucleotide comprises a molar amount ranging from about 35% to 95% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined. In some embodiments, the second polynucleotide comprises a molar amount ranging from about 5% to 65% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined. In some embodiments, the third polynucleotide comprises a molar amount ranging from about 1% to 60% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined.

[0141] In some embodiments, culturing the cell under conditions suitable for recombinant AAV production comprises expanding the cell.

[0142] In some embodiments, the method further comprises lysing the cell. In some embodiments, the method further comprises purifying the lysed cell to collect rAAV particles. In some embodiments, the collected rAAV particles comprise less than 5% of replication-competent adeno-associated virus particles (rcAAV).

[0143] In some embodiments, the collected rAAV particles comprise less than 1% of replication-competent adeno-associated virus particles (rcAAV).

[0144] Another aspect of the present disclosure includes a recombinant adeno-associated virus (rAAV) produced by the method described herein, using the system described herein, or from the packaging cell.

[0145] Another aspect of the present disclosure includes a pharmaceutical composition comprising the recombinant adeno-associated virus (rAAV).

5. BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0146] These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, and accompanying drawings, where:

[0147] FIG. 1A provides exemplary schematics of (i) standard 3-plasmid systems (used interchangeably as “3P control system” or “standard 3P system”), (ii) two plasmid 2P system (used interchangeably as “2P”), and (iii) split 3P system (used interchangeably as “S3P”).

[0148] FIG. 1B provides an exemplary schematic of a polynucleotide (a plasmid) including a capsid (cap), a transgene (gene of interest (GOI)) flanked by two ITR sequences, a p41

promoter, a non-coding sequence (e.g., 1st non-coding sequence) between the p41 promoter and the capsid gene, and a non-coding sequence (2nd non-coding sequence) between one ITR and the p41 non-coding sequence.

[0149] FIG. 2 provides an exemplary schematic of a split 3P system (S3P) according to one embodiment of the present disclosure.

[0150] FIG. 3 shows results (vector yield and % full capsid particles) from a plasmid ratio optimization screening at a small scale 250 ml volume using a split 3P system (S3P). AAV viral particles were produced using the S3P system with the plasmid ratios (rep:helper:capsid-transgene) of 5:1:0.2; 4:1:0.2; 3:1:0.2; 2:1:0.2; and 1:1:0.2.

[0151] FIG. 4 shows results (vector yield and % full capsid particles) from S3P system using one of two different helper plasmids (H-1 helper or H-2 helper) at a small scale 250 ml volume for each plasmid ratio tested. Two helper plasmids H-1 (SEQ ID NO: 3) and H-2 (SEQ ID NO: 4) of different sizes and designs were tested at various S3P molar ratios. The S3P plasmid with a smaller sized helper plasmid (H-1) resulted in an increase in crude vector titre and % full capsid particles compared to the larger sized helper plasmid (H-2).

[0152] FIG. 5 shows a 3L volume production of a S3P plasmid in a bioreactor with a 1:1:0.1 or 3:1:0.2 molar ratio tested. The S3P with a 1:1:1 molar ratio resulted in an increase in crude vector titre as compared to the 3:1:0.2 molar ratio tested. The S3P with a 3:1:0.2 molar ratio resulted in an increase in % full capsid particles as compared to the 3:1:0.2 molar ratio tested.

[0153] FIG. 6 shows results (vector yield and % full capsid particles) of a study testing the effect of helper plasmid size on rAAV production at a small scale 250 ml volume. Two helper plasmids (H-1 (SEQ ID NO: 3) and H-2 (SEQ ID NO: 4)) of different sizes were tested at various S3P molar ratios as specified in Example 4 (Experiment 5, Flask 1 to 18).

[0154] FIG. 7 shows a 3L volume production of a S3P plasmid system in a bioreactor compared to standard 3P systems. The S3P system resulted in an increase in crude vector titre and % full capsid particles as compared to the standard 3P system.

[0155] FIG. 8 shows a 50L volume production of a S3P plasmid system in a bioreactor compared to a S3P plasmid system tested in SF (shaken flask) 250 ml volumes and 3L volumes. The S3P system shows maintains yields and % full capsid particles across scales (SF, 3L, and 50L).

[0156] FIG. 9 shows a S3P plasmid system that includes helper H-1 or a H-3 Helper plasmid; and a rep plasmid encoding Rep 78, Rep 68, Rep 52, and Rep 40 proteins from an AAV2 serotype; and a cap-cargo (e.g., cap-GOI or cap-transgene) with a transgene flanked by two ITR sequences, a non-coding sequence between the 3' ITR and the promoter, and a AAV2 5' UTR sequence (non-coding sequence) between the promoter and capsid.

[0157] FIG. 10 shows a 4P plasmid system that includes a helper plasmid, a large rep plasmid that includes Rep 78 and Rep 68 proteins, a small rep plasmid that includes Rep 52 and Rep 40 proteins, and a cap-cargo with a transgene flanked by two ITR sequences, a non-coding sequence between the 3' ITR and the promoter, and a AAV2 5' UTR sequence (non-coding sequence) between the promoter and capsid.

[0158] FIG. 11 provides a S3P system that includes an exemplary split rep system, where a rep plasmid can include large rep proteins Rep 78 and Rep 68, and small rep proteins can be added to the cap-cargo. Alternatively, the cap-cargo can include large rep proteins and the small rep proteins can be in a separate plasmid.

[0159] FIG. 12 provides a capsid-cargo (CapCar) construct of FIG. 9 and FIG. 2, except the p41 promoter is switched out with a p40 promoter.

[0160] FIG. 13 provides a rep plasmid that includes Rep 78, Rep 68, Rep 52, and Rep 40 proteins alongside a UL12 protein and a EF1a promoter.

[0161] FIG. 14 provides a rep plasmid of FIG. 13 but switches out the EF1a promoter with a CMV promoter.

[0162] FIG. 15 provides a rep plasmid that includes Rep 78, Rep 68, Rep 52, and Rep 40 proteins alongside a UL12 protein and a EF1a promoter, and an NS2 protein and a CMV promoter.

[0163] FIG. 16 provides variations of rep plasmids used in Example 12. AAV2 Rep S-1 refers to an AAV2 Rep plasmid that has Rep 78, Rep 68, Rep52, and Rep40 with AAV2 Rep intron/exon sequences. AAV2 Rep S-2 (SEQ ID NO: 7) refers to an AAV2 Rep plasmid that has Rep 78, Rep 68, Rep52, and Rep40 sequences and also includes extraneous non-coding sequences, but does not have the AAV2 Rep intron/exon sequences. AAV2 Rep S-1 does not include these extraneous non-coding sequences. AAV2 Rep S-4 is a AAV2 Rep plasmid that has Rep 78, Rep 68, Rep52, and Rep40 sequences, AAV2 Rep intron/exon sequences, and additional extraneous non-coding sequences.

[0164] FIG. 17 represents molar ratios of helper:rep:capsid-transgene (GOI) plasmids in the S3P system that provided good AAV yields and full capsid production.

[0165] FIG. 18 provides an exemplary hybrid rep plasmid that includes an entire small rep (Rep 52 and Rep 40); a 3' end of Rep 78 and Rep 68, and 5' end of Rep 78 and Rep 68. This allows for mixing and matching rep proteins to optimize for performance.

[0166] FIG. 19 provides 3 exemplary Rep plasmids that include different Rep proteins alongside either a UL12 protein or tTR protein.

[0167] FIG. 20 provides a cap-cargo that in addition to the capsid, GOI, and ITR sequences, also include large or small rep proteins.

6. DETAILED DESCRIPTION

6.1. Definitions

[0168] "AAV" is adeno-associated virus and may be used to refer to the virus itself or derivatives thereof. The term covers all subtypes, serotypes and pseudotypes, and both naturally occurring and recombinant forms, except where required otherwise.

[0169] The term "AAV capsid protein" or simply "capsid protein" refers to a VP1, VP2, or VP3 capsid protein. In some embodiments, the AAV capsid protein is a wild type or modified capsid protein of AAV9; AAV2; AAV1; AAV6; AAV3; AAV LK03; AAV7; AAV8; AAV hu.37; AAV rh.10; AAV hu.68; AAV10; AAV5; AAV3-3; AAV4-4; AAV1-A; hu.46-A; hu.48-A; hu.44-A; hu.43-A; AAV6-A; hu.34-B; hu.47-B; hu.29-B; rh.63-B; hu.56-B; hu.45-B; rh.57-B; rh.35-B; rh.58-B; rh.28-B; rh.51-B; rh.19-B; rh.49-B; rh.52-B; rh.13-B; AAV2-B; rh.20-B; rh.24-B; rh.64-B; hu.27-B; hu.21-B; hu.22-B; hu.23-B; hu.7-C; hu.61-C; rh.56-C; hu. 9-C; hu.54-C; hu.53-C; hu.60-C; hu.55-C; hu.2-C; hu.1-C; hu.18-C; hu.3-C; hu.25-C; hu.15-C; hu.16-C; hu.11-C; hu.10-C; hu.4-C; rh.54-D; rh.48-D; rh.55-D; rh.62-D; AAV7-D; rh.52-E; rh.51-E; hu.39-E; rh.53-E; hu.37-E; rh.43-E; rh.50-E; rh.49-E; rh.61-E; hu.41-E; rh.64-E; rh74; hu.42-E; rh.57-E; rh.40-E; hu.67-E; hu.17-E; hu.6-E; hu.66-E; rh.38-E; hu.32-F; AAV9/hu; hu.31-F; Anc80; Anc81; Anc82; Anc83; Anc84; Anc94; Anc113; Anc126; Anc127; Anc80L27; Anc80L59; Anc80L60; Anc80L62; Anc80L65; Anc80L33; Anc80L36; Anc80L44; Anc80L1; Anc110; and Anc80DI. The modified capsid protein can be a VP1, VP2, or VP3 capsid protein with a targeting moiety (e.g. targeting peptide).

[0170] The terms "operably linked" and "operatively linked" refer to the functional relationship of the nucleic acid sequences with regulatory sequences of nucleotides, such as

promoters, enhancers, transcriptional and translational stop sites, and other signal sequences and indicates that two or more DNA segments are joined together such that they function in concert for their intended purposes. For example, operative linkage of nucleic acid sequences, typically DNA, to a regulatory sequence or promoter region refers to the physical and functional relationship between the DNA and the regulatory sequence or promoter such that the transcription of such DNA is initiated from the regulatory sequence or promoter, by an RNA polymerase that specifically recognizes, binds and transcribes the DNA.

[0171] The abbreviation "rAAV" refers to a recombinant adeno-associated viral particle composed of at least one AAV capsid protein and an encapsidated polynucleotide, sometimes referred to herein as a "genome". rAAV can include a genome that comprises a heterologous polynucleotide (i.e., a polynucleotide other than a wild-type AAV genome), such as a heterologous polynucleotide encoding a gene delivered to a mammalian cell. The heterologous nucleotide is sometimes referred to as a transgene.

[0172] The term "polynucleotide" refers to a biopolymer of nucleotide monomers that are covalently bonded in a chain. The nucleotide monomers are ribonucleotides, deoxyribonucleotides, or a combination thereof. Thus, this term includes, but is not limited to, single-, double-, or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases.

[0173] A polynucleotide or polypeptide has a certain percent "sequence identity" to another polynucleotide or polypeptide, meaning that, when aligned, that percentage of bases or amino acids are the same, and in the same relative position, when comparing the two sequences. Sequence identity can be determined in a number of different ways. To determine sequence identity, sequences can be aligned using various convenient methods and computer programs (e.g., BLAST, T-COFFEE, MUSCLE, MAFFT, etc.), available over the world wide web at sites including ncbi.nlm.nili.gov/BLAST, ebi.ac.uk/Tools/msa/tcoffee/, ebi.ac.uk/Tools/msa/muscle/, mafft.cbrc.jp/alignment/software/. See, e.g., Altschul et al. (1990), J. Mol. Bioi. 215:403-10.

[0174] The term "standard three-plasmid system" or "3P" as used herein refer to an rAAV production system using three different polynucleotides (e.g., plasmids) – the first polynucleotide comprising coding sequences of rep protein(s) and cap protein, the second polynucleotide comprising coding sequences of helper proteins and the third polynucleotide

comprising an expression cassette comprising a transgene (gene of interest (GOI)) flanked by two ITR sequences. The three plasmids are transfected into a host cell for production of AAVs. This is an AAV production method well known and commonly used in the field, e.g., as described in the US Pat No. 7,198,951, incorporated by reference in its entirety herein.

[0175] The term "two plasmid 2P system" or "2P" as used herein refers to a system using two polynucleotides (e.g., plasmids) – the first polynucleotide comprising coding sequences of helper proteins and rep protein(s) and the second polynucleotide comprising an expression cassette comprising a transgene (gene of interest (GOI)) flanked by two ITR sequences and a coding sequence of a cap protein. An example of the 2P system is illustrated in FIG. 1.

[0176] The term "split 3P system" or "S3P" as used herein refers to a system using three polynucleotides – the first polynucleotide comprising a coding sequence of rep protein(s), the second polynucleotide comprising a coding sequence of helper gene(s) and the third polynucleotide comprising an expression cassette comprising a transgene (gene of interest (GOI)) flanked by two ITR sequences. The three polynucleotides are separate molecules and can be cloned in plasmids, phagemids, cosmids, fosmids, bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), viral constructs, or any other polynucleotide vector known in the art. The third polynucleotide can further comprise a coding sequence of a capsid protein. The third polynucleotide can further comprise a non-coding sequence (e.g., 1st spacer or untranslated region (UTR)) between a promoter and the capsid protein. The third polynucleotide can further comprise a second non-coding sequence (e.g., 2nd spacer or stuffer) between an ITR and the promoter. One example of the split 3P system is illustrated in FIG. 1, but the split 3P system is not limited thereto.

[0177] The term "inverted terminal repeat" (or "ITR") refers to a polynucleotide sequence found at the ends of AAV genomes that form a hairpin, which contributes to the genome's ability to self-prime (allowing for primase-independent synthesis of the complementary second DNA strand) and provides for encapsidation of the genome into an AAV particle. An ITR can be a wild-type ITR or a variant thereof.

[0178] The term "non-coding sequence" or "spacer" or "stuffer" sequence, as used herein, refers to a non-coding nucleotide sequence that is non-naturally occurring, for example, when the non-coding sequence does not occur in a genomic sequence present in the genome of, e.g., a capsid protein and/or a gene of interest.

[0179] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the methods and compositions of matter belong. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the methods and compositions of matter, suitable methods and materials are described below. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

6.2. rAAV production system with three polynucleotides

[0180] One aspect of the present disclosure provides a system for production of recombinant adeno-associated virus (rAAV) comprising: a first polynucleotide comprising at least one rep gene encoding at least one functional Rep protein, and a first promoter operably linked to the at least one rep gene; a second polynucleotide comprising at least one helper virus gene, a third polynucleotide comprising (i) a capsid gene encoding a Capsid protein, a second promoter operably linked to the capsid gene and a non-coding sequence between the second promoter and the capsid gene; and (ii) an expression cassette comprising a transgene flanked by two inverted terminal repeat (ITR).

[0181] Another aspect of the present disclosure includes a system for production of recombinant adeno-associated virus (rAAV) comprising: a first polynucleotide comprising a rep gene encoding a Rep protein, and a first promoter operably linked to the rep gene; a second polynucleotide comprising a helper virus gene, a third polynucleotide comprising (i) a capsid gene encoding a Capsid protein, a second promoter operably linked to the capsid gene; and (ii) an expression cassette comprising a transgene flanked by two inverted terminal repeats (ITRs).

[0182] Another aspect of the present disclosure includes a system for production of recombinant adeno-associated virus (rAAV) comprising: a first polynucleotide comprising a rep gene encoding a Rep protein, and a first promoter operably linked to the rep gene; a second polynucleotide comprising a helper virus gene, a third polynucleotide comprising (i) a capsid gene encoding a Capsid protein, a second promoter operably linked to the capsid gene; and (ii) an expression cassette comprising a transgene flanked by two inverted terminal repeats (ITRs); and a fourth polynucleotide comprising a rep gene encoding a Rep protein,

and a third promoter operably linked to the rep gene, wherein the fourth polynucleotide is smaller or larger in size compared to the first polynucleotide.

[0183] In some embodiments, the rAAV production system is illustrated in any one of FIGs. 2, 9-11, 16. In some embodiments, the rAAV production system uses any of the polynucleotides illustrated in FIG. 1B, 2, 9-20.

6.2.1. First polynucleotide and Fourth polynucleotide

[0184] The rAAV production system disclosed herein can comprise a first polynucleotide comprising a rep gene encoding a Rep protein. In some embodiments, the first polynucleotide comprises a sequence encoding at least one Rep protein, at least two Rep proteins, at least three Rep proteins, at least four Rep proteins, or at least five Rep proteins.

[0185] The rAAV production system disclosed herein can optionally comprise a fourth polynucleotide comprising a rep gene encoding a Rep protein. In some embodiments, the first polynucleotide comprises a sequence encoding at least one Rep protein, at least two Rep proteins, at least three Rep proteins, at least four Rep proteins, or at least five Rep proteins. In some embodiments, the fourth polynucleotide comprises a sequence encoding at least one Rep protein, at least two Rep proteins, at least three Rep proteins, at least four Rep proteins, or at least five Rep proteins. In some embodiments, the first polynucleotide and the fourth polynucleotide encode one or more different Rep proteins. In some embodiments, the first polynucleotide and the fourth polynucleotide encode one or more same Rep proteins.

[0186] In some embodiments, the first polynucleotide is devoid of a helper virus gene.

[0187] In some embodiments, the first polynucleotide is devoid of adenovirus helper genes. In some embodiments, the first polynucleotide is devoid of a capsid gene.

[0188] In some embodiments, the fourth polynucleotide is devoid of a helper virus gene. In some embodiments, the fourth polynucleotide is devoid of adenovirus helper genes. In some embodiments, the fourth polynucleotide is devoid of a capsid gene.

6.2.1.1 Rep proteins

[0189] The AAV Rep protein acts as both a repressor and an activator to regulate AAV transcription during a productive infection. In some embodiments, the rep gene encodes four proteins for viral genome replication and packaging: Rep 78, Rep 68, Rep 52, and Rep 40. In some embodiments, the rep gene encodes two proteins for viral genome replication and

packaging: Rep 78 and Rep 68. In some embodiments, the rep gene encodes two proteins for viral genome replication and packaging: Rep 52 and Rep 40.

[0190] In some embodiments, the rep gene encodes at least one Rep protein. In some embodiments, the rep gene encodes at least two Rep proteins. In some embodiments, the rep gene encodes at least three Rep proteins. In some embodiments, the rep gene encodes at least four Rep proteins. In some embodiments, the rep gene encodes one, two, three or four proteins selected from: Rep 78, Rep 68, Rep 52, and Rep 40.

[0191] In some embodiments, the first polynucleotide comprises two, three or four sequences, wherein each of the two, three, or four sequences encodes a Rep 78 protein, a Rep 68 protein, a Rep 52 protein, or a Rep 40 protein.

[0192] In some embodiments, the Rep protein is a Rep 78 protein. In some embodiments, the Rep protein is a Rep 68 protein. In some embodiments, the Rep protein is Rep 52 protein. In some embodiments, the Rep protein is a Rep 40 protein. In some embodiments, the at least one rep gene of the first polynucleotide comprises a sequence encoding a Rep 78 protein, a Rep 68 protein, a Rep 52 protein, or a Rep 40 protein.

[0193] In some embodiments, the first polynucleotide comprises sequences encoding a Rep 78 protein, a Rep 68 protein, a Rep 52 protein, and a Rep 40 protein. In certain embodiments, the first polynucleotide comprises sequences encoding at least a Rep 78 protein. In certain embodiments, the first polynucleotide comprises sequences encoding at least a Rep 68 protein. In certain embodiments, the first polynucleotide comprises sequences encoding at least a Rep 52 protein. In certain embodiments, the first polynucleotide comprises sequences encoding at least a Rep 40 protein.

[0194] In certain embodiments, the first polynucleotide comprises sequences encoding at least one of a Rep 78 protein, a Rep 68 protein, a Rep 52 protein, and a Rep 40 protein. In certain embodiments, the first polynucleotide comprises sequences encoding at least two of a Rep 78 protein, a Rep 68 protein, a Rep 52 protein, and a Rep 40 protein. In certain embodiments, the first polynucleotide comprises sequences encoding at least three of a Rep 78 protein, a Rep 68 protein, a Rep 52 protein, and a Rep 40 protein.

[0195] In some embodiments, the rep gene comprises a gene encoding one or more Rep proteins selected from: a Rep 52 protein, a Rep 40 protein, a Rep 68 protein, and a Rep 78 protein.

[0196] In certain embodiments, the first polynucleotide comprises a sequence encoding at a Rep 78 protein and a Rep 68 protein. In some embodiments, the first polynucleotide comprises a sequence encoding a Rep 52 protein, and a sequence encoding a Rep 40 protein. In certain embodiments, the first polynucleotide comprises three sequences, wherein each sequence encodes a Rep 78 protein, Rep 68 protein, Rep 52 protein, or Rep 40 protein. In some embodiments, the first polynucleotide comprises a sequence encoding a Rep 52 protein, a sequence encoding a Rep 40 protein, a sequence encoding a Rep 78 protein, and a sequence encoding a Rep 68 protein. In some embodiments, the first polynucleotide comprises sequences encoding a Rep 78 protein, a Rep 68 protein, a Rep 52 protein, and a Rep 40 protein.

[0197] In certain embodiments, the first polynucleotide comprises a rep gene encoding the following Rep proteins: Rep 78 protein, Rep 68 protein, Rep 52 protein, and Rep 40 protein.

[0198] In some embodiments, the rep gene comprises a gene encoding a Rep 52 protein, a gene encoding a Rep 40 protein, a gene encoding a Rep 68 protein, or a gene encoding a Rep 78 protein.

[0199] In some embodiments, the rep gene comprises a gene encoding a Rep 52 protein, a gene encoding a Rep 40 protein, a gene encoding a Rep 68 protein, and a gene encoding a Rep 78 protein.

[0200] In some embodiments, the Rep gene of the first polynucleotide comprises a sequence encoding a rep protein from an AAV serotype selected from: AA1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, and AAV13. In some embodiments, the Rep protein is a non-AAV2 Rep protein. In some embodiments, the Rep protein is an AAV1 Rep protein, an AAV2 Rep protein, an AAV3 Rep protein, an AAV4 Rep protein, an AAV5 Rep protein, an AAV6 Rep protein, an AAV7 Rep protein, an AAV8 Rep protein, an AAV9 Rep protein, an AAV10 Rep protein, an AAV11 Rep protein, an AAV12 Rep protein, or an AAV13 Rep protein.

[0201] In some embodiments, the Rep 78 protein is an AAV1 Rep 78 protein, an AAV2 Rep 78 protein, an AAV3 Rep 78 protein, an AAV4 Rep 78 protein, an AAV5 Rep 78 protein, an AAV6 Rep 78 protein, an AAV7 Rep 78 protein, an AAV8 Rep 78 protein, an AAV9 Rep 78 protein, an AAV10 Rep 78 protein, an AAV11 Rep 78 protein, an AAV12 Rep 78 protein, or an AAV13 Rep 78 protein.

[0202] In some embodiments, the Rep 68 protein is an AAV1 Rep 68 protein, an AAV2 Rep 68 protein, an AAV3 Rep 68 protein, an AAV4 Rep 68 protein, an AAV5 Rep 68 protein, an AAV6 Rep 68 protein, an AAV7 Rep 68 protein, an AAV8 Rep 68 protein, an AAV9 Rep 68 protein, an AAV10 Rep 68 protein, an AAV11 Rep 68 protein, an AAV12 Rep 68 protein, or an AAV13 Rep 68 protein.

[0203] In some embodiments, the Rep 52 protein is an AAV1 Rep 52 protein, an AAV2 Rep 52 protein, an AAV3 Rep 52 protein, an AAV4 Rep 52 protein, an AAV5 Rep 52 protein, an AAV6 Rep 52 protein, an AAV7 Rep 52 protein, an AAV8 Rep 52 protein, an AAV9 Rep 52 protein, an AAV10 Rep 52 protein, an AAV11 Rep 52 protein, an AAV12 Rep 52 protein, or an AAV13 Rep 52 protein.

[0204] In some embodiments, the Rep 40 protein is an AAV1 Rep 40 protein, an AAV2 Rep 40 protein, an AAV3 Rep 40 protein, an AAV4 Rep 40 protein, an AAV5 Rep 40 protein, an AAV6 Rep 40 protein, an AAV7 Rep 40 protein, an AAV8 Rep 40 protein, an AAV9 Rep 40 protein, an AAV10 Rep 40 protein, an AAV11 Rep 40 protein, an AAV12 Rep 40 protein, or an AAV13 Rep 40 protein.

[0205] In some embodiments, the first polynucleotide comprises sequences encoding AAV1 Rep 78, AAV1 Rep 68, AAV1 Rep 52, and AAV1 Rep40 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV2 Rep78, AAV2 Rep68, AAV2 Rep52, and AAV2 Rep 40 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV3 Rep 78, AAV3 Rep 68, AAV3 Rep 52, and AAV3 Rep 40 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV4 Rep 78, AAV4 Rep68, AAV4 Rep 52, and AAV4 Rep 40 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV5 Rep 78, AAV5 Rep 68, AAV5 Rep 52, and AAV5 Rep 40 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV6 Rep 78, AAV6 Rep 68, AAV6 Rep 52, and AAV6 Rep 40 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV7 Rep 78, AAV7 Rep 68, AAV7 Rep 52, and AAV7 Rep 40 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV8 Rep 78, AAV8 Rep 68, AAV8 Rep 52, and AAV8 Rep 40 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV9 Rep 78, AAV9 Rep 68, AAV9 Rep 52, and AAV9 Rep 40 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV10 Rep 78, AAV10 Rep 68, AAV10 Rep

52, and AAV10 Rep 40 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV11 Rep 78, AAV11 Rep 68, AAV11 Rep 52, and AAV11 Rep 40 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV12 Rep 78, AAV12 Rep 68, AAV12 Rep 52, and AAV12 Rep 40 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV13 Rep 78, AAV13 Rep 68, AAV13 Rep 52, and AAV13 Rep 40 proteins.

[0206] In some embodiments, the first polynucleotide comprises sequences encoding AAV1 Rep 78 and AAV1 Rep 68 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV2 Rep 78 and AAV2 Rep 68 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV3 Rep 78 and AAV3 Rep 68 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV4 Rep 78 and AAV4 Rep 68 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV5 Rep 78 and AAV5 Rep 68 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV6 Rep 78 and AAV6 Rep 68 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV7 Rep 78 and AAV7 Rep 68 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV8 Rep 78 and AAV8 Rep 68 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV9 Rep 78 and AAV9 Rep 68 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV10 Rep 78 and AAV10 Rep 68 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV11 Rep 78 and AAV11 Rep 68 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV12 Rep 78 and AAV12 Rep 68 proteins. In some embodiments, the first polynucleotide comprises sequences encoding AAV13 Rep 78 and AAV13 Rep 68 proteins.

[0207] In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV1 Rep 78. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV2 Rep 78. In some embodiments, the

first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV3 Rep 78. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV4 Rep 78. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV5 Rep 78. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV6 Rep 78. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV7 Rep 78. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV8 Rep 78. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV9 Rep 78. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV10 Rep 78. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about

60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV11 Rep 78. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV12 Rep 78. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV13 Rep 78.

[0208] In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV1 Rep 68. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV2 Rep 68. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV3 Rep 68. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV4 Rep 68. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV5 Rep 68. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an

AAV6 Rep 68. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV7 Rep 68. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV8 Rep 68. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV9 Rep 68. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV10 Rep 68. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV11 Rep 68. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV12 Rep 68. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV13 Rep 68.

[0209] In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV1 Rep 52. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%,

about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV2 Rep 52. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV3 Rep 52. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV4 Rep 52. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV5 Rep 52. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV6 Rep 52. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV7 Rep 52. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV8 Rep 52. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV9 Rep 52. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about

95%, about 97%, or 100% of an AAV10 Rep 52. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV11 Rep 52. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV12 Rep 52. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV13 Rep 52.

[0210] In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV1 Rep 40. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV2 Rep 40. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV3 Rep 40. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV4 Rep 40. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV5 Rep 40. In some embodiments, the first polynucleotide comprises

sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV6 Rep 40. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV7 Rep 40. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV8 Rep 40. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV9 Rep 40. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV10 Rep 40. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV11 Rep 40. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV12 Rep 40. In some embodiments, the first polynucleotide comprises sequences encoding about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, or 100% of an AAV13 Rep 40.

[0211] In some embodiments, the first polynucleotide comprises sequences encoding about 50% of an AAV2 Rep 78 protein, about 50% of an AAV10 Rep 78 protein, about 50% of an

AAV2 Rep 68 protein, about 50% of an AAV10 Rep 68 protein, an AAV2 Rep 52 protein, and an AAV4 Rep 40 protein.

[0212] In some embodiments, the first polynucleotide has a nucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, or at least 90% sequence identity to positions 1-2577 or positions 1-1611 of SEQ ID NO: 6. In some embodiments, the first polynucleotide has a nucleotide sequence having at least 95% sequence identity to positions 1-2577 or positions 1-1611 of SEQ ID NO: 6. In some embodiments, the first polynucleotide has a nucleotide sequence of positions 1-2577 or positions 1-1611 of SEQ ID NO: 6.

[0213] In some embodiments, the first polynucleotide has a nucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, or at least 90% sequence identity to positions 1-1998 or positions 1-1982 of SEQ ID NO: 7. In some embodiments, the first polynucleotide has a nucleotide sequence having at least 95% sequence identity to positions 1-1998 or positions 1-1982 of SEQ ID NO: 7. In some embodiments, the first polynucleotide has a nucleotide sequence of positions 1-1998 or positions 1-1982 of SEQ ID NO: 7.

[0214] In some embodiments, the first polynucleotide has a nucleotide sequence having at least 85% sequence identity to any one of SEQ ID NOs: 6 and 7. In some embodiments, the first polynucleotide has a nucleotide sequence having at least 90% sequence identity to any one of SEQ ID NOs: 6 and 7. In some embodiments, the first polynucleotide has a nucleotide sequence having at least 95% sequence identity to any one of SEQ ID NOs: 6 and 7. In some embodiments, the first polynucleotide has a nucleotide sequence of SEQ ID NO: 6. In some embodiments, the first polynucleotide has a nucleotide sequence of SEQ ID NO: 7.

[0215] In some embodiments, the Rep proteins of the system are split into two polynucleotides. In such embodiments, the system comprises a fourth polynucleotide (e.g., a 4 polynucleotide system). In certain embodiments, the first polynucleotide comprises sequences encoding a Rep 78 and a Rep 68 protein from an AAV serotype selected from: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, and AAV13.

[0216] In certain embodiments, a fourth polynucleotide comprises sequences encoding a Rep 52 protein and a Rep 40 protein. In certain embodiments, the fourth polynucleotide comprises sequences encoding a Rep 52 and a Rep 40 protein from an AAV serotype selected from:

AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, and AAV13. In certain embodiments, the first polynucleotide comprises mutated coding sequences of Rep 52 and Rep 40 proteins with a mutated start codon.

[0217] In certain embodiments, the first polynucleotide comprises sequences encoding a Rep 52 and a Rep 40 protein from an AAV serotype selected from: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, and AAV13. In certain embodiments, a fourth polynucleotide comprises sequences encoding a Rep 78 protein and a Rep 68 protein. In certain embodiments, the fourth polynucleotide comprises sequences encoding a Rep 78 and a Rep 68 protein from an AAV serotype selected from: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, and AAV13. In certain embodiments, the first polynucleotide comprises mutated coding sequences of Rep 78 and Rep 68 proteins with a mutated start codon. In certain embodiments, the first polynucleotide comprises mutated coding sequences of Rep 78 and Rep 68 proteins with a mutated start codon and an inactivated p19 promoter. In certain embodiments, the first polynucleotide comprising a mutated coding sequences of Rep 78 and Rep 68 proteins with a mutated start codon and an inactivated p19 promoter, does not comprise Rep 52 and Rep 40 proteins.

[0218] In some embodiments, the fourth polynucleotide is smaller in size compared to the first polynucleotide. In some embodiments, the fourth polynucleotide is larger in size compared to the first polynucleotide.

[0219] In certain embodiments, the rep gene encoding at least one Rep protein comprises a nucleotide sequence having at least 65% sequence identity to SEQ ID NO: 5, 6, or 7. In certain embodiments, the rep gene encoding at least one Rep protein comprises a nucleotide sequence having at least 70% sequence identity to SEQ ID NO: 5, 6, or 7. In certain embodiments, the rep gene encoding at least one Rep protein comprises a nucleotide sequence having at least 75% sequence identity to SEQ ID NO: 5, 6, or 7. In certain embodiments, the rep gene encoding at least one Rep protein comprises a nucleotide sequence having at least 80% sequence identity to SEQ ID NO: 5, 6, or 7. In certain embodiments, the rep gene encoding at least one Rep protein comprises a nucleotide sequence having at least 85% sequence identity to SEQ ID NO: 5, 6, or 7. In certain embodiments, the rep gene encoding at least one Rep protein comprises a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 5, 6, or 7. In certain

embodiments, the rep gene encoding at least one Rep protein comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 5, 6, or 7. In certain embodiments, the rep gene encoding at least one Rep protein comprises a nucleotide sequence having at least 97% sequence identity to SEQ ID NO: 5, 6, or 7. In certain embodiments, the rep gene encoding at least one Rep protein comprises a nucleotide sequence having at least 99% sequence identity to SEQ ID NO: 5, 6, or 7. In certain embodiments, the rep gene encoding at least one Rep protein comprises a nucleotide sequence of SEQ ID NO: 5, 6, or 7.

[0220] In some embodiments, the at least one rep gene comprises the sequence encoding the Rep 78 protein has a nucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to positions 1-1866 of SEQ ID NOs: 6 or positions 117-1982 of SEQ ID NO: 7.

[0221] In some embodiments, the at least one rep gene comprising the sequence encoding the Rep 68 protein has a nucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to positions 1-1586 of SEQ ID NO: 6 or positions 117-1702 of SEQ ID NO: 7.

[0222] In some embodiments, the at least one rep gene comprising the sequence encoding the Rep 52 protein has a nucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to positions 673-1866 of SEQ ID NO: 6 or positions 789-1982 of SEQ ID NO: 7.

[0223] In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to any one of SEQ ID NOs: 8-32. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 8. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence having at least

65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 9. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 10. In some embodiments, the rep gene is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 11. In some embodiments, is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 12. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 13. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 14. In some embodiments, the rep gene is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 15. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 16. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 17. In some embodiments, the at least one rep gene is a rep

protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 18. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 19. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 20. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 21. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 22. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 23. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 24. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 25. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence

identity, or at least 95% sequence identity to SEQ ID NO: 26. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 27. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 28. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 29. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 30. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 31. In some embodiments, the at least one rep gene is a rep protein comprising an amino acid sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 32.

6.2.1.2 First promoters

[0224] Some aspects of the present system include the first polynucleotide comprising a first promoter operably linked to the rep gene.

[0225] In some embodiments, the first promoter is selected from the following promoters: p5, p7, p19, p40, p41, UBC, CMV, SV40, Ef1a, and a PGK promoter.

[0226] In some embodiments, the rep gene is under the control of or operably linked to p5 or p19 promoter. In some embodiments, the rep gene is under the control of or operably linked to p5 and pl9 promoters. In some embodiments, the rep gene is under the control of or

operably linked to p5, pl9, and p40 promoters. In order for a promoter to be "functional", the promoter must drive expression of a protein.

6.2.1.2.1 P5 Promoter

[0227] In some embodiments, the p5 promoter is located upstream or downstream of the open reading frame (ORF) of the rep gene. In some embodiments, the p5 promoter is located within the ORF of the rep gene. In some embodiments, the p5 promoter originates from a naturally occurring or mutated AAV serotype. In certain embodiments, the p5 promoter originates from an AAV serotype selected from: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, or AAV13 serotype. In some embodiments, the first promoter is an AAV1 p5, AAV2 p5, AAV3 p5, AAV4 p5, AAV6 p5, AAV7 p5, AAV8 p5, AAV9 p5, AAV10 p5, AAV11 p5, AAV12 p5, or AAV13 p5 promoter.

[0228] In certain embodiments, the p5 promoter comprises a nucleotide sequence of positions 17 through 133 of the nucleotide sequence of the rep gene (SEQ ID NO: 5).

[0229] In some embodiments, the p5 promoter comprises a nucleotide sequencing having at least 80% sequence identity to any one of SEQ ID NOs: 47-48. In some embodiments, the p5 promoter comprises a nucleotide sequencing having at least 85% sequence identity to SEQ ID NOS: 47-48. In some embodiments, the p5 promoter comprises a nucleotide sequencing having at least 90% sequence identity to SEQ ID NOS: 47-48. In some embodiments, the p5 promoter comprises a nucleotide sequencing having at least 95% sequence identity to SEQ ID NOS: 47-48. In some embodiments, the p5 promoter comprises a nucleotide sequencing having at least 97% sequence identity to SEQ ID NOS: 47-48. In some embodiments, the p5 promoter comprises a nucleotide sequencing having at least 99% sequence identity to SEQ ID NOS: 47-48. In some embodiments, the p5 promoter comprises a nucleotide sequencing of SEQ ID NOS: 47-48.

[0230] In some embodiments, the p5 promoter induces expression of Rep 78 and Rep 68 proteins. Rep 78 and Rep 68 can be two alternative splice variants (Rep 78 comprises an intron that is excised in Rep 68). In some embodiments, the pl9 promoter induces expression of Rep 52 and Rep 40 proteins. Rep 52 and Rep 40 can be alternative splice variants (Rep 52 comprises an intron that is excised in Rep 40). The four Rep proteins are known to be involved in replication and packaging of the viral genome, and are, therefore, useful in rAAV production. In some embodiments, the first polynucleotide comprising one or more rep

proteins comprises a p19 promoter and a p40 promoter. In some embodiments, the first polynucleotide comprises a p5 promoter, a p19 promoter, and a p40 promoter.

6.2.1.2.2 P19 promoter

[0231] In some embodiments, the first promoter of the first polynucleotide comprises a p19 promoter. In some embodiments, the first polynucleotide further comprises a p19 promoter.

[0232] In some embodiments, the p19 is located upstream or downstream of the ORF of the rep gene. In some embodiments, the p19 promoter is located within the ORF of the rep gene. In some embodiments, the p19 promoter originates from a naturally occurring or mutated AAV serotype. In certain embodiments, the p19 promoter originates from an AAV2 serotype.

[0233] In certain embodiments, the p19 promoter originates from an AAV serotype selected from: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, or AAV13 serotype. In some embodiments, the p19 promoter is an AAV1 p19, AAV2 p19, AAV3 p19, AAV4 p19, AAV6 p19, AAV7 p19, AAV8 p19, AAV9 p19, AAV10 p19, AAV11 p19, AAV12 p19, or AAV13 p19 promoter.

[0234] In certain embodiments, the p19 promoter comprises a nucleotide sequence of positions 543 through 664 of the nucleotide sequence of the Rep gene (SEQ ID NO: 5).

[0235] In some embodiments, the p19 promoter comprises a nucleotide sequencing having at least 65% sequence identity to any one of SEQ ID NOs: 49-50. In some embodiments, the p19 promoter comprises a nucleotide sequencing having at least 70% sequence identity to any one of SEQ ID NOs: 49-50. In some embodiments, the p19 promoter comprises a nucleotide sequencing having at least 75% sequence identity to any one of SEQ ID NOs: 49-50. In some embodiments, the p19 promoter comprises a nucleotide sequencing having at least 80% sequence identity to any one of SEQ ID NOs: 49-50. In some embodiments, the p19 promoter comprises a nucleotide sequencing having at least 85% sequence identity to SEQ ID NO: 49. In some embodiments, the p19 promoter comprises a nucleotide sequencing having at least 90% sequence identity to any one of SEQ ID NOs: 49-50. In some embodiments, the p19 promoter comprises a nucleotide sequencing having at least 95% sequence identity to any one of SEQ ID NOs: 49-50. In some embodiments, the p19 promoter comprises a nucleotide sequencing having at least 97% sequence identity to any one of SEQ ID NOs: 49-50. In some embodiments, the p19 promoter comprises a nucleotide sequencing of any one of SEQ ID NOs: 49-50.

[0236] In some embodiments, the first polynucleotide is devoid of a 19 promoter.

6.2.1.2.3 P40 promoter

[0237] In some embodiments, the first promoter of the first polynucleotide comprises a p40 promoter. In some embodiments, the first polynucleotide further comprises a p40 promoter.

[0238] In some embodiments, the p40 promoter is located upstream or downstream of the ORF of the rep gene. In some embodiments, the p40 promoter is located within the ORF of the rep gene. In some embodiments, the p40 promoter originates from a naturally occurring or mutated AAV serotype. In certain embodiments, the p40 promoter originates from an AAV2 serotype.

[0239] In certain embodiments, the p40 promoter originates from an AAV serotype selected from: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, or AAV13 serotype. In some embodiments, the p40 promoter is an AAV1 p40, AAV2 p40, AAV3 p40, AAV4 p40, AAV6 p40, AAV7 p40, AAV8 p40, AAV9 p40, AAV10 p40, AAV11 p40, AAV12 p40, or AAV13 p40 promoter.

[0240] In certain embodiments, the p40 promoter comprises a nucleotide sequence of positions 1512 through 1693 of the nucleotide sequence of the Rep gene (SEQ ID NO: 5).

[0241] In some embodiments, the p40 promoter comprises a nucleotide sequencing having at least 80% sequence identity to SEQ ID NO: 51. In some embodiments, the p40 promoter comprises a nucleotide sequencing having at least 85% sequence identity to SEQ ID NO: 51. In some embodiments, the p40 promoter comprises a nucleotide sequencing having at least 90% sequence identity to SEQ ID NO: 51. In some embodiments, the p40 promoter comprises a nucleotide sequencing having at least 95% sequence identity to SEQ ID NO: 51. In some embodiments, the p40 promoter comprises a nucleotide sequencing having at least 97% sequence identity to SEQ ID NO: 51. In some embodiments, the p40 promoter comprises a nucleotide sequencing of SEQ ID NO: 51.

[0242] In some embodiments, the first polynucleotide is devoid of a p40 promoter. In some embodiments, the first polynucleotide is devoid of a p40 and p19 promoter.

6.2.1.2.4 P19 promoter

[0243] In some embodiments, the first polynucleotide comprises a p19 promoter. In some embodiments, the p19 promoter comprises a nucleotide sequence having at least 85%

sequence identity, at least 90% sequence identity, or 95% sequence identity to SEQ ID NO: 50. In some embodiments, the p19 promoter comprises a nucleotide sequence of SEQ ID NO: 50.

6.2.1.3 Additional promoters

[0244] In some embodiments, the first polynucleotide comprises, in addition to the first promoter, one or more additional promoters is selected from: p19, CMV, SV40, Ef1a, UBC, p5, CBh, Tre, PGK, CAG, and a p7 promoter. In some embodiments, the one or more additional promoters is a p19 promoter. In some embodiments, the one or more additional promoters is a CMV promoter. In some embodiments, the one or more additional promoters is a SV40 promoter. In some embodiments, the one or more additional promoters is a Ef1a promoter. In some embodiments, the one or more additional promoters is a UBC promoter. In some embodiments, the one or more additional promoters is a p5 promoter. In some embodiments, the one or more additional promoters is a p7 promoter. In some embodiments, the one or more additional promoters is a CBh promoter. In some embodiments, the one or more additional promoters is a TRE promoter. In some embodiments, the one or more additional promoters is a CAG promoter. In some embodiments, the one or more additional promoters is a CBh promoter. In some embodiments, the one or more additional promoters is a TRE promoter. In some embodiments, the one or more additional promoters is a p7 promoter. A list of promoters that can be used in the first polynucleotide can be found in Table 32.

6.2.1.4 Additional proteins

[0245] In some embodiments, the first polynucleotide further comprises a nucleotide sequence encoding an additional protein.

[0246] In certain embodiments, the additional protein is from: a Herpes simplex virus type 1 (HSV-1) and a bocavirus. In some embodiments, the additional protein is a helper virus gene selected from: NS2 and UL12. In some embodiments, the additional protein is NS2. In some embodiments, the additional protein is UL12.

[0247] In some embodiments, the first polynucleotide further comprises a coding sequence of UL12 or transthyretin (ttr). In some embodiments, the first polynucleotide further comprises a coding sequence of transthyretin (ttR). In some embodiments, the first polynucleotide further comprises a nucleotide sequence of rtTA3. In some embodiments, the rtTA3 activate the TRE

promoter in the presence of doxycycline/tetracycline. In some embodiments, the rtTA3 has a nucleotide sequence of SEQ ID NO.: 42. In some embodiments, the first polynucleotide further comprises a nucleotide sequence of tTA (TetR). In some embodiments,the tTA has a nucleotide sequence of SEQ ID NO: 43.

[0248] In some embodiments, the first polynucleotide comprises sequences encoding: AAV2 Rep78, AAV2 Rep68, AAV2 Rep52, and AAV2 Rep40 proteins, and a UL12 or transthyretin (ttR) protein.

6.2.2. Second polynucleotide

6.2.2.1 Helper Virus Genes

[0249] The rAAV production system disclosed herein can comprise a second polynucleotide comprising a helper virus gene. In some embodiments, the second polynucleotide comprises two helper virus genes from Adenovirus 5 or Adenovirus 2.

[0250] In some embodiments, the helper virus gene is an Adenovirus gene or Herpesvirus gene. AAVs are generally replication-deficient, requiring the presence of a helper virus or helper virus functions (e.g., herpes simplex virus (HSV) and/or adenovirus (AdV)) in order to replicate within an infected cell. For example, in some embodiments, AAVs require adenoviral El A, E2A, E4, and/or VA RNA genes in order to replicate within a host cell.

[0251] In some embodiments, the helper virus gene is from adenovirus genes and/or their variants. In some embodiments, helper virus gene from adenovirus are one or more of E2A (e.g., E2A DNA Binding Protein (DBP)), E4 (e.g., E4 Open Reading Frame (ORF) 2, ORF3, ORF4, ORF6/7), VA, and/or variants thereof.

[0252] In some embodiments, the helper virus gene comprises an Adenovirus 5 or Adenovirus 2 gene. In some embodiments, the helper virus gene comprises an Adenovirus 5 gene. In some embodiments, the helper virus gene comprises an Adenovirus 2 gene. In some embodiments, the helper virus gene is selected from: E2A DNA Binding Protein (DBP) gene, E4 Open Reading Frame (ORF) 2, ORF3, ORF4 and ORF 6/7. In some embodiments, helper virus gene from adenovirus is E2A (e.g., E2A DNA Binding Protein (DBP)). In some embodiments, helper virus gene from adenovirus is E4 (e.g., E4 Open Reading Frame (ORF) 2, ORF3, ORF4, ORF6/7).

[0253] In some embodiments, the second polynucleotide comprises two helper virus genes selected from Adeno E2A, Adeno E2B, Adeno E4, Adeno VA, Adeno L3 and Adeno L4. In

some embodiments, the second polynucleotide comprises three helper virus genes selected from Adeno E2A, Adeno E2B, Adeno E4, Adeno VA, Adeno L3 and Adeno L4.In some embodiments, the second polynucleotide comprises Adeno E2A, Adeno E4, and Adeno VA.

[0254] In certain embodiments, the second polynucleotide comprising Adeno E2A, Adeno E4, and Adeno VA has a nucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity, or at least 99% sequence identity to positions 1-9281 of SEQ ID NO: 3. In certain embodiments, the second polynucleotide comprising Adeno E2A, Adeno E4, and Adeno VA has a nucleotide sequence of positions 1-9281 of SEQ ID NO: 3.

[0255] In certain embodiments, the second polynucleotide comprises Adeno E2B, Adeno VA, Adeno L3, Adeno E2A, Adeno L4, and Adeno E4. In certain embodiments, the Adeno E2B has a nucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity, or at least 99% sequence identity to positions 1-699 of SEQ ID NO: 4. In certain embodiments, the Adeno E2B has a nucleotide sequence of positions 1-699 of SEQ ID NO: 4.

[0256] In certain embodiments, the Adeno VA has a nucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity, or at least 99% sequence identity to positions 773-932 and 1029-1102 of SEQ ID NO: 4. In certain embodiments, the Adeno VA has a nucleotide sequence of positions 773-932 and 1029-1102 of SEQ ID NO: 4.

[0257] In certain embodiments, the Adeno L3 has a nucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity, or at least 99% sequence identity to positions 1103-1572 of SEQ ID NO: 4. In certain embodiments, the Adeno L3 has a nucleotide sequence of positions 1103-1572 of SEQ ID NO: 4.

[0258] In certain embodiments, the Adeno E2A has a nucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least

95% sequence identity, or at least 99% sequence identity to positions 1667-3257 of SEQ ID NO: 4. In certain embodiments, the Adeno E2A has a nucleotide sequence of positions 1667-3257 of SEQ ID NO: 4.

[0259] In certain embodiments, the Adeno L4 has a nucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity, or at least 99% sequence identity to positions 3286-5709 and 6393-7076 of SEQ ID NO: 4. In certain embodiments, the Adeno L4 has a nucleotide sequence of positions 3286-5709 and 6393-7076 of SEQ ID NO: 4.

[0260] In certain embodiments, the second polynucleotide comprises E1 Open Reading Frame (ORF), E4 Open Reading Frame (ORF) 2, ORF3, ORF4 and ORF 6/7.

[0261] In certain embodiments, the second polynucleotide comprising E4 Open Reading Frame (ORF) 1 has a nucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity, or at least 99% sequence identity to positions 9978-10362 of SEQ ID NO: 4. In certain embodiments, the second polynucleotide comprising E4 Open Reading Frame (ORF) 1 has a nucleotide sequence of positions 9978-10362 of SEQ ID NO: 4.

[0262] In certain embodiments, the second polynucleotide comprising E4 Open Reading Frame (ORF) 2 has a nucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity, or at least 99% sequence identity to positions 9538-9948 of SEQ ID NO: 4. In certain embodiments, the second polynucleotide comprising E4 Open Reading Frame (ORF) 2 has a nucleotide sequence of positions 9538-9948 of SEQ ID NO: 4.

[0263] In certain embodiments, the second polynucleotide comprising ORF3 has a nucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity, or at least 99% sequence identity to positions 9191-9541 of SEQ ID NO: 4. In certain embodiments, the second polynucleotide comprising ORF3 has a nucleotide sequence of positions 9191-9541 of SEQ ID NO: 4.

[0264] In certain embodiments, the second polynucleotide comprising ORF4 has a nucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity, or at least 99% sequence identity to positions 8836-9180 of SEQ ID NO: 4. In certain embodiments, the second polynucleotide comprising ORF4 has a nucleotide sequence of positions 8836-9180 of SEQ ID NO: 4.

[0265] In certain embodiments, the second polynucleotide comprising ORF 6/7 has a nucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity, or at least 99% sequence identity to positions 7752-8915 of SEQ ID NO: 4. In certain embodiments, the second polynucleotide comprising ORF 6/7 has a nucleotide sequence of positions 7752-8915 of SEQ ID NO: 4.

[0266] In some embodiments, the second polynucleotide has a nucleotide sequence having at least 90% sequence identity to any one of SEQ ID NOs: 3-4. In some embodiments, the second polynucleotide has a nucleotide sequence having at least 95% sequence identity to any one of SEQ ID NOs: 3-4. In some embodiments, the second polynucleotide has a nucleotide sequence of any one of SEQ ID NOs: 3-4.

[0267] In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 65% sequence identity to SEQ ID NO: 1. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 70% sequence identity to SEQ ID NO: 1. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 75% sequence identity to SEQ ID NO: 1. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 80% sequence identity to SEQ ID NO: 1. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 85% sequence identity to SEQ ID NO: 1. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 1. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 1. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 97% sequence identity to SEQ ID NO: 1. In certain embodiments, the at least one helper virus gene comprises a nucleotide

sequence having at least 99% sequence identity to SEQ ID NO: 1. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence of SEQ ID NO: 1.

[0268] In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 65% sequence identity to SEQ ID NO: 3. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 70% sequence identity to SEQ ID NO: 3. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 75% sequence identity to SEQ ID NO: 3. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 80% sequence identity to SEQ ID NO: 3. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 85% sequence identity to SEQ ID NO: 3. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 3. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 3. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 97% sequence identity to SEQ ID NO: 3. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 99% sequence identity to SEQ ID NO: 3. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence of SEQ ID NO: 3.

[0269] In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 65% sequence identity to SEQ ID NO: 4. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 70% sequence identity to SEQ ID NO: 4. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 75% sequence identity to SEQ ID NO: 4. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 80% sequence identity to SEQ ID NO: 4. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 85% sequence identity to SEQ ID NO: 4. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 4. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 4. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence having at least 97% sequence identity to SEQ ID NO: 4. In certain embodiments, the at least one helper virus gene comprises a nucleotide

sequence having at least 99% sequence identity to SEQ ID NO: 4. In certain embodiments, the at least one helper virus gene comprises a nucleotide sequence of SEQ ID NO: 4.

[0270] In certain embodiments, the second polynucleotide has a nucleotide sequence of any one of SEQ ID Nos.: 3, and 4.

[0271] In certain embodiments, the second polynucleotide lacks Adeno E2B, Adeno VA, Adeno L3, or Adeno L4. In certain embodiments, the second polynucleotide lacks two or more helper virus genes selected from Adeno E2B, Adeno VA, Adeno L3, and Adeno L4. In certain embodiments, the second polynucleotide lacks Adeno E2B, Adeno VA, Adeno L3, and Adeno L4.

[0272] In some embodiments, the second polynucleotide further comprises a helpervirus gene selected from NS2 and UL12. In certain embodiments, the second polynucleotide further comprises helper virus gene NS2. In certain embodiments, the second polynucleotide further comprises helper virus gene UL12. In certain embodiments, the second polynucleotide further comprises helper virus gene NS2 and UL12.

6.2.2.2 Helper plasmid size

[0273] In some embodiments, the second polynucleotide has a length less than 16,000 nucleotides, less than 15,000 nucleotides, less than 14,000 nucleotides, less than 13,000 nucleotides, less than 10,000 nucleotides, less than 9,000 nucleotides, or less than 8,000 nucleotides.

[0274] In some embodiments, the second polynucleotide has a length less than 14,000 nucleotides. In some embodiments, the second polynucleotide has a length less than 13,000 nucleotides. In some embodiments,the second polynucleotide has a length less than 12,000 nucleotides.

[0275] In some embodiments, the second polynucleotide has a length ranging from 9,000 to 16,000 nucleotides. In some embodiments, the second polynucleotide has a length ranging from 8,000 to 14,000 nucleotides. In some embodiments, the second polynucleotide has a length ranging from 9,000 to 14,000 nucleotides. In some embodiments, the second polynucleotide has a length ranging from 13,000 to 14,000 nucleotides. In some embodiments, the second polynucleotide has a length ranging from 11,000 to 12,000 nucleotides.

6.2.3. Third polynucleotide

[0276] Some aspects of the present system include a third polynucleotide comprising (i) a capsid gene encoding a Capsid protein, a second promoter operably linked to the capsid gene; and (ii) an expression cassette comprising a transgene flanked by two inverted terminal repeat (ITR).

[0277] In some embodiments, the third polynucleotide comprises a non-coding sequence between the second promoter and the capsid gene.

[0278] In some embodiments, the third polynucleotide comprises a second non-coding sequence positioned between one of the two ITR sequences and the second promoter. In some embodiments, the second promoter is operably linked to the capsid gene.

6.2.3.1 AAV Capsids

[0279] The third polynucleotide can comprise a capsid gene encoding any of the Capsid proteins known in the art or a modification thereof. The Capsid protein can be a naturally occurring VP1, VP2, and/or VP3 capsid protein or a non-naturally occurring VP1, VP2, and/or VP3 capsid protein. The non-naturally occurring VP1, VP2, and/or VP3 capsid protein includes a capsid protein generated by biological or chemical alteration or in silico design, or variation of a naturally occurring AAV capsid protein.

[0280] Accordingly, in some embodiments, the AAV capsid protein includes, but is not limited to, a capsid protein of various AAV serotypes (e.g., AAV1, AAV2, AAV3B, AAV5, AAV6, AAV8, and AAV9) or a variant thereof. A non-naturally occurring VP1, VP2, or VP3 capsid protein further includes an artificial capsid protein created by in silico design or synthesis. An artificial capsid protein includes, but is not limited to, AAV capsid proteins disclosed in PCT/US2014/060163, USP9695220, PCT/US2016/044819, PCT/US2018/032166, PCT/US2019/031851, and PCT/US2019/047546, which are incorporated herein by reference in their entireties.

[0281] In some embodiments, the AAV capsid protein is the capsid protein of AAV9 (Genbank Ace. No: AAS99264.1), AAV1 (Genbank Ace. No: AAD27757.1), AAV2 (Genbank Ace. No: AAC03780.1), AAV3 (Genbank Ace. No: AAC55049.1), AAV3b (Genbank Ace. No: AF028705.1), AAV4 (Genbank Ace. No: AAC58045.1), AAV5 (Genbank Ace. No: AAD13756.1), AAV6 (Genbank Ace. No: AF028704.1), AAV7 (Genbank Ace. No: AAN03855.1), AAV 8 (Genbank Ace. No: AAN03857.1), AAV10

(Genbank Ace. No: AAT46337.1), AAVrh10 (Genbank Ace. No: AY243015.1), AAV11 (Genbank Ace. No: AAT46339.1), AAV12 (Genbank Ace. No: ABI16639.1), or AAV13 (Genbank Ace. No: ABZ10812.1), AAVpol (Genbank Ace. No: FJ688147.1). In certain embodiments, the AAV capsid protein is the capsid protein of AAV9 (Genbank Ace. No: AAS99264.1).

[0282] The AAV capsid protein can be VP1 capsid protein having a sequence selected from: (AAV1 (AAD27757)), (AAV2 (AAC03780)), (AAV3 (AAC55049)), (AAV5 (AAD13756)), (AAV6 (AAB95450)), (AAV7 (AF513851_2)), (AAV8 (AF513852_2)), (AAV9 (AAS99264)), (AAV10 (AAT46337)), (AAV hu.68), (Anc80) CAPSID-1_AAV9_1000, Anc80L65, Anc80L65 liver off, Anc80L65 Deco1, Anc80L65 Liver Off Deco1, AAV9 (Capsid-2_MUT1-38181), Capsid-3_38181, AAV9 (Capsid-4_MUT1-40049), AAV9 (Capsid-5_MUT1-38170), AAV9 (Capsid-6_MUT1-39374, AAV9 (Capsid-7_MUT1-20169, AAV9 (Capsid-8_MUT1-49911, AAV9 (Capsid-9_MUT1-38249, AAV9 (Capsid-10_MUT1-38219, AAV9 (Capsid-11_MUT1-26512, and AAV9. The AAV capsid protein can be a VP2 or VP3 protein having a part of one of the sequences. For example, VP2 protein can have a sequence corresponding to amino acids 138 to 736 of AAV9 VP1 and VP3 protein can have a sequence corresponding to amino acids 138 to 736 of AAV9 VP1 protein.

[0283] The AAV capsid protein can be VP1 capsid protein having any member sequence of the ancestral AAV library selected from (AAV1 (AAD27757)), (AAV2 (AAC03780)), (AAV3 (AAC55049)), (AAV5 (AAD13756)), (AAV6 (AAB95450)), (AAV7 (AF513851_2)), (AAV8 (AF513852_2)), (AAV9 (AAS99264)), (AAV10 (AAT46337)), (AAV hu.68), (Anc80) CAPSID-1_AAV9_1000, Anc80L65, Anc80L65 liver off, Anc80L65 Deco1, Anc80L65 Liver Off Deco1, AAV9 (Capsid-2_MUT1-38181), Capsid-3_38181, AAV9 (Capsid-4_MUT1-40049, AAV9 (Capsid-5_MUT1-38170), AAV9 (Capsid-6_MUT1-39374, AAV9 (Capsid-7_MUT1-20169, AAV9 (Capsid-8_MUT1-49911, AAV9 (Capsid-9_MUT1-38249, AAV9 (Capsid-10_MUT1-38219, AAV9 (Capsid-11_MUT1-26512, and AAV9. The AAV capsid protein can be a VP2 or VP3 protein having a part of one of the sequences. For example, VP2 protein can have a sequence corresponding to amino acids 138 to 736 of AAV9 VP1 and VP3 protein can have a sequence corresponding to amino acids 138 to 736 of AAV9 VP1 protein. When a SEQ ID NO for a library sequence is used in this disclosure, it refers to a sequence of any one member of the library.

[0284] In some embodiments, the AAV capsid protein is a liver-toggle mutant described in WO2019/217911, which is incorporated by reference in its entirety herein.

[0285] In some embodiments, the AAV capsid protein is a capsid protein (VP1, VP2 or VP3) of an AAV variant selected from the group consisting of: AAV2; AAV1; AAV6; AAV3; AAV LK03; AAV7; AAV8; AAV hu.37; AAV rh.10; AAV9; AAV hu.68; AAV10; AAV5; AAV3-3; AAV4-4; AAV1-A; hu.46-A; hu.48-A; hu.44-A; hu.43-A; AAV6-A; hu.34-B; hu.47-B; hu.29-B; rh.63-B; hu.56-B; hu.45-B; rh.57-B; rh.35-B; rh.58-B; rh.28-B; rh.51-B; rh.19-B; rh.49-B; rh.52-B; rh.13-B; AAV2-B; rh.20-B; rh.24-B; rh.64-B; hu.27-B; hu.21-B; hu.22-B; hu.23-B; hu.7-C; hu.61-C; rh.56-C; hu. 9-C; hu.54-C; hu.53-C; hu.60-C; hu.55-C; hu.2-C; hu.1-C; hu.18-C; hu.3-C; hu.25-C; hu.15-C; hu.16-C; hu.11-C; hu.10-C; hu.4-C; rh.54-D; rh.48-D; rh.55-D; rh.62-D; AAV7-D; rh.52-E; rh.51-E; hu.39-E; rh.53-E; hu.37-E; rh.43-E; rh.50-E; rh.49-E; rh.61-E; hu.41-E; rh.64-E; hu.42-E; rh.57-E; rh.40-E; rh74; hu.67-E; hu.17-E; hu.6-E; hu.66-E; rh.38-E; hu.32-F; AAV9/hu; hu.31-F; Anc80L27; Anc80L59; Anc80L60; Anc80L62; Anc80L65; Anc80L33; Anc80L36; Anc80L44; Anc80L1; Anc80-55, Anc80-129, Anc80-156, Anc80-751, Anc80-1029, Anc80-1712; Anc110; Anc80DI; CAPSID-1_AAV9_1000, Capsid-3_38181, and AAV9 (Capsid-2_MUT1-38181). In some embodiments, the reference AAV capsid protein is a capsid protein of any member protein of an ancestral AAV library selected from: Anc80; Anc81; Anc82; Anc83; Anc84; Anc94; Anc113; Anc126; and Anc127.

[0286] In some embodiments, the AAV capsid protein is a protein having a sequence selected from SEQ ID Nos: 90-120. In some embodiments, the AAV capsid protein is a protein having a sequence selected from SEQ ID Nos: 90-120. In some embodiments, the AAV capsid protein is a protein having a VP2 (corresponding to amino acids 138 to 736 of AAV9 VP1) or VP3 portion (corresponding to amino acids 138 to 736 of AAV9 VP1) of the protein having a sequence selected from SEQ ID NOs: 90-120.

[0287] In some embodiments, the AAV capsid protein is a capsid protein of the AAV variant modified to include one or more liver-toggle mutations described in WO2019/217911. In some embodiments, the AAV capsid protein comprises (1) an alanine (A) or glycine (G) amino acid residue at an amino acid position corresponding to position 266 in Anc80 VP1 and/or (2) a lysine (K) or arginine (R) amino acid residue at an amino acid position corresponding to position 168 in Anc80 VP1. In some embodiments, the AAV capsid protein comprises (i) an alanine (A) amino acid residue at an amino acid position corresponding to

position 266 in Anc80 VP1 and/or b) a lysine (K) amino acid residue at an amino acid position corresponding to position 168 in Anc80 VP1. In some embodiments, the AAV capsid protein comprises (ii) an alanine (A) amino acid residue at an amino acid position corresponding to position 267 in AAV9 VP1 protein and/or a threonine (T) amino acid residue at an amino acid position corresponding to position 269 in AAV9 VP1.

[0288] In some embodiments, the AAV capsid protein comprises one or more modifications as described in WO2019/217911 or WO 2021/050614, which are both incorporated by reference herein in their entireties. In some embodiments, the AAV capsid protein comprises one or more modifications as described in PCT Application No. PCT/US2022/015842, which is herein incorporated by reference in its entirety.

[0289] In some embodiments, the capsid protein comprises an amino acid sequence having at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity, at least 96% sequence identity, at least 97% sequence identity, at least 98% sequence identity, or at least 99% sequence identity to any one of SEQ ID Nos: 90-120 or a fragment thereof. at least In some embodiments, the AAV capsid protein is a capsid protein having a sequence selected from SEQ ID Nos: 90-120 or a fragment thereof.

[0290] In some embodiments, the AAV capsid protein is a liver-toggle mutant of a capsid protein of an AAV variant selected from the group consisting of : AAV2; AAV1; AAV6; AAV3; AAV LK03; AAV7; AAV8; AAV hu.37; AAV rh.10; AAV9; AAV hu.68; AAV10; AAV5; AAV3-3; AAV4-4; AAV1-A; hu.46-A; hu.48-A; hu.44-A; hu.43-A; AAV6-A; hu.34-B; hu.47-B; hu.29-B; rh.63-B; hu.56-B; hu.45-B; rh.57-B; rh.35-B; rh.58-B; rh.28-B; rh.51-B; rh.19-B; rh.49-B; rh.52-B; rh.13-B; AAV2-B; rh.20-B; rh.24-B; rh.64-B; hu.27-B; hu.21-B; hu.22-B; hu.23-B; hu.7-C; hu.61-C; rh.56-C; hu. 9-C; hu.54-C; hu.53-C; hu.60-C; hu.55-C; hu.2-C; hu.1-C; hu.18-C; hu.3-C; hu.25-C; hu.15-C; hu.16-C; hu.11-C; hu.10-C; hu.4-C; rh.54-D; rh.48-D; rh.55-D; rh.62-D; AAV7-D; rh.52-E; rh.51-E; hu.39-E; rh.53-E; hu.37-E; rh.43-E; rh.50-E; rh.49-E; rh.61-E; hu.41-E; rh.64-E; rh74; hu.42-E; rh.57-E; rh.40-E; hu.67-E; hu.17-E; hu.6-E; hu.66-E; rh.38-E; hu.32-F; AAV9/hu; hu.31-F; Anc80; Anc81; Anc82; Anc83; Anc84; Anc94; Anc113; Anc126; Anc127; Anc80L27; Anc80L59; Anc80L60; Anc80L62; Anc80L65; Anc80L33; Anc80L36; Anc80L44; Anc80L1; Anc80-55, Anc80-129, Anc80-156, Anc80-751, Anc80-1029, Anc80-1712, Anc110; Anc80DI, and CAPSID-1_AAV9_1000, Capsid-3_38181, and AAV9 (Capsid-2_MUT1-38181).

[0291] In some embodiments, the capsid protein comprises an unmodified or modified rAAV9 capsid protein. In some embodiments, the rAAV comprises a VP1, VP2 and/or VP3 capsid protein comprising an amino acid sequence having at least 90%, 95%, 97%, 98%, 99% or 100% sequence identity to the corresponding protein(s) in AAV2; AAV1; AAV6; AAV3; AAV LK03; AAV7; AAV8; AAV hu.37; AAV rh.10; AAV9; AAV hu.68; AAV10; AAV5; AAV3-3; AAV4-4; AAV1-A; hu.46-A; hu.48-A; hu.44-A; hu.43-A; AAV6-A; hu.34-B; hu.47-B; hu.29-B; rh.63-B; hu.56-B; hu.45-B; rh.57-B; rh.35-B; rh.58-B; rh.28-B; rh.51-B; rh.19-B; rh.49-B; rh.52-B; rh.13-B; AAV2-B; rh.20-B; rh.24-B; rh.64-B; hu.27-B; hu.21-B; hu.22-B; hu.23-B; hu.7-C; hu.61-C; rh.56-C; hu. 9-C; hu.54-C; hu.53-C; hu.60-C; hu.55-C; hu.2-C; hu.1-C; hu.18-C; hu.3-C; hu.25-C; hu.15-C; hu.16-C; hu.11-C; hu.10-C; hu.4-C; rh.54-D; rh.48-D; rh.55-D; rh.62-D; AAV7-D; rh.52-E; rh.51-E; hu.39-E; rh.53-E; hu.37-E; rh.43-E; rh.50-E; rh.49-E; rh.61-E; hu.41-E; rh.64-E; rh74; hu.42-E; rh.57-E; rh.40-E; hu.67-E; hu.17-E; hu.6-E; hu.66-E; rh.38-E; hu.32-F; AAV9/hu; hu.31-F; Anc80; Anc81; Anc82; Anc83; Anc84; Anc94; Anc113; Anc126; Anc127; Anc80L27; Anc80L59; Anc80L60; Anc80L62; Anc80L65; Anc80L33; Anc80L36; Anc80L44; Anc80L1; Anc80-55; Anc80-129; Anc80-156; Anc80-751; Anc80-1029; Anc80-1712; Anc110; Anc80DI; or Capsid-1_AAV9_1000. In some embodiments, the AAV capsid protein a capsid protein of Capsid-1_AAV9_1000. In some embodiments, the AAV capsid protein a capsid protein of Capsid-1_AAV9_1000 with a liver-toggle mutant. In some embodiments, the AAV capsid protein a capsid protein of Capsid-3_38181, and AAV9 (Capsid-2_MUT1-38181). In some embodiments, the AAV capsid protein a capsid protein of AAV9 (Capsid-2_MUT1-38181). In some embodiments, the AAV capsid protein a capsid protein of AAV9 (Capsid-2_MUT1-38181) with a liver-toggle mutant.

[0292] In some embodiments, the AAV capsid protein comprises a modified AAV capsid protein comprising at least one liver-toggle mutation as compared to a reference capsid protein.

[0293] In some embodiments, the reference capsid protein is a VP1, VP2 and/or VP3 protein. In some embodiments, the reference AAV capsid protein is a capsid protein having any one of SEQ ID NOs:25-162 and 212 or a fragment thereof. In some embodiments, the reference capsid protein is a VP1, VP2 and/or VP3 protein.

6.2.3.2 Second promoter

[0294] Aspects of the present system includes the third polynucleotide comprising a second promoter operably linked to the capsid gene.

[0295] In some embodiments, the promoter of the third polynucleotide drives expression of the capsid protein.

[0296] In some embodiments, the promoter of the third polynucleotide is a p41 promoter, p40 promoter, CMV promoter, SV40 promoter, Ef1a promoter, TRE promoter, UBC promoter, or a PGK promoter. In some embodiments, the promoter of the third polynucleotide has a nucleotide sequence of any one of SEQ ID Nos: 33-54. In some embodiments, the promoter of the third polynucleotide has a nucleotide sequence of any one of SEQ ID Nos: 121-122.

[0297] In some embodiments, the promoter is a p40 promoter. In certain embodiments, the p40 promoter comprises a nucleotide sequencing having at least 80% sequence identity to SEQ ID NO: 51. In certain embodiments, the p40 promoter comprises a nucleotide sequencing having at least 85% sequence identity to SEQ ID NO: 51. In certain embodiments, the p40 promoter comprises a nucleotide sequencing having at least 90% sequence identity to SEQ ID NO: 51. In certain embodiments, the p40 promoter comprises a nucleotide sequencing having at least 95% sequence identity to SEQ ID NO: 51. In certain embodiments, the p40 promoter comprises a nucleotide sequencing having at least 97% sequence identity to SEQ ID NO: 51. In certain embodiments, the p40 promoter comprises a nucleotide sequencing of SEQ ID NO: 51.

[0298] In some embodiments, the p40 promoter originates from an AAV serotype. In certain embodiments, the p40 promoter originates from an AAV2 serotype.

[0299] In certain embodiments, the p40 promoter originates from an AAV serotype selected from: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, or AAV13 serotype. In some embodiments, the p40promoter is an AAV1 p40, AAV2 p40, AAV3 p40, AAV4 p40, AAV6 p40, AAV7 p40, AAV8 p40, AAV9 p40, AAV10 p40, AAV11 p40, AAV12 p40, or AAV13 p40 promoter.

[0300] In some embodiments, the promoter is a p41 promoter. In certain embodiments, the p41 promoter comprises a nucleotide sequencing having at least 80% sequence identity to SEQ ID NO: 52. In certain embodiments, the p41 promoter comprises a nucleotide

sequencing having at least 85% sequence identity to SEQ ID NO: 52. In certain embodiments, the p41 promoter comprises a nucleotide sequencing having at least 90% sequence identity to SEQ ID NO: 20. In certain embodiments, the p41 promoter comprises a nucleotide sequencing having at least 95% sequence identity to SEQ ID NO: 52. In certain embodiments, the p41 promoter comprises a nucleotide sequencing having at least 97% sequence identity to SEQ ID NO: 52. In certain embodiments, the p41 promoter comprises a nucleotide sequencing of SEQ ID NO: 52.

[0301] In some embodiments, the p41 promoter originates from an AAV serotype. In certain embodiments, the p41 promoter originates from an AAV2 serotype. In certain embodiments, the p41 promoter originates from an AAV5 serotype.

[0302] In certain embodiments, the p41 promoter originates from an AAV serotype selected from: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, or AAV13 serotype. In some embodiments, the p40promoter is an AAV1 p41, AAV2 p41, AAV3 p41, AAV4 p41, AAV6 p41, AAV7 p41, AAV8 p41, AAV9 p41, AAV10 p41, AAV11 p41, AAV12 p41, or AAV13 p41 promoter.

[0303] In some embodiments, the promoter of the third polynucleotide is a CMV promoter. In some embodiments, the promoter of the third polynucleotide is a SV40 promoter. In some embodiments, the promoter of the third polynucleotide is a Ef1a promoter. In some embodiments, the promoter of the third polynucleotide is a TRE promoter. In some embodiments, the promoter of the third polynucleotide is a UBC promoter. In some embodiments, the promoter of the third polynucleotide is a PGK promoter. In some embodiments, the promoter of the third polynucleotide is a CAG promoter. In some embodiments, the promoter of the third polynucleotide is a CBh promoter. In some embodiments, the promoter of the third polynucleotide is a rTA (TetR) promoter. In some embodiments, the promoter of the third polynucleotide is a p5 promoter. In some embodiments, the promoter of the third polynucleotide is a p19 promoter. In some embodiments, the promoter of the third polynucleotide is a p40 promoter. In some embodiments, the promoter of the third polynucleotide is a p41 promoter. In some embodiments, the promoter of the third polynucleotide is a TRE promoter. In some embodiments, the promoter of the third polynucleotide is a p7 promoter. A list of promoters that can be used in the third polynucleotide can be found in Table 32.

6.2.3.3 Non-coding sequence between promoter and capsid

[0304] The third polynucleotide of the present system can further comprise a non-coding sequence. The non-coding sequence is positioned between the second promoter of the third polynucleotide and the capsid gene (1st non-coding nucleotide sequence ("1st spacer") in FIG. 1B).

[0305] In some embodiments, the non-coding sequence is a 5' untranslated region (UTR) sequence. In some embodiments, the non-coding sequence is a 3' UTR sequence. In some embodiments, the third polynucleotide comprises a 5' UTR and a 3' UTR sequence.

[0306] In certain embodiments, the non-coding sequence is positioned between the 3' end of the second promoter and the 5' end of the capsid gene.

[0307] In some embodiments, the third polynucleotide comprises a AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and AAV2 3' UTR at the 3' end of the capsid coding sequence.

[0308] In some embodiments, the non-coding sequence comprises a contiguous nucleotide sequence. In some embodiments, the non-coding sequence is a polynucleotide fragment of 100-400 nucleotides long. In some embodiments, the non-coding sequence is a polynucleotide fragment of 150-350 nucleotide long. In some embodiments, the non-coding sequence is a polynucleotide fragment of 150-300 nucleotides long. In some embodiments, the non-coding sequence is a polynucleotide fragment of 150-280 nucleotides long. In some embodiments, the non-coding sequence is a polynucleotide fragment of 150-260 nucleotides long.

[0309] In certain embodiments, the non-coding sequence comprises a nucleotide sequence having a length ranging from 150-350 nucleotides. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having a length ranging from 290-350 nucleotides. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having a length ranging from 300-350 nucleotides. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having a length ranging from 310-350 nucleotides. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having a length ranging from 290-350 nucleotides. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having a length ranging from 280-325 nucleotides. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having a

length ranging from 290-325 nucleotides. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having a length ranging from 300-325 nucleotides. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having a length ranging from 310-325 nucleotides. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having a length ranging from 290-325 nucleotides. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having a length of at least 280 nucleotides, at least 285 nucleotides, at least 290 nucleotides, at least 295 nucleotides, at least 300 nucleotides, at least 305 nucleotides, at least 310 nucleotides, at least 315 nucleotides, at least 320 nucleotides, at least 325 nucleotides, at least 330 nucleotides, at least 335 nucleotides, at least 340 nucleotides, at least 345 nucleotides, at least 350 nucleotides, at least 355 nucleotides, or at least 360 nucleotides. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having a length of at least 300 nucleotides, at least 310 nucleotides, at least 312 nucleotides, at least 314 nucleotides, at least 316 nucleotides, at least 318 nucleotides, at least 320 nucleotides, at least 322 nucleotides, at least 324 nucleotides, at least 326 nucleotides, at least 328 nucleotides, or at least 330 nucleotides.

[0310] In certain embodiments, the non-coding sequence comprises a fragment of a nucleotide sequence of SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 65% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 70% sequence identity to SEQ ID NO: 5. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 75% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 80% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 85% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 55.

[0311] In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 65% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 70% sequence identity to

SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 75% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 80% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 85% sequence identity to SEQ ID NO: 21. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 90% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 95% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 97% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 98% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 99% sequence identity to SEQ ID NO: 21. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 100% sequence identity to SEQ ID NO: 55.

[0312] In certain embodiments, the non-coding sequence sequence comprises a fragment of a nucleotide sequence of SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 65% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 70% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 75% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 80% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 85% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 55.

[0313] In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 65% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 70% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide

sequence having less than 75% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 80% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 85% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 90% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 95% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 97% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 98% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 99% sequence identity to SEQ ID NO: 55. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 100% sequence identity to SEQ ID NO: 55.

[0314] In certain embodiments, the non-coding sequence comprises a fragment of a nucleotide sequence of SEQ ID NO: 56. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 65% sequence identity to SEQ ID NO: 56. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 70% sequence identity to SEQ ID NO: 56. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 75% sequence identity to SEQ ID NO: 56. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 80% sequence identity to SEQ ID NO: 56. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 85% sequence identity to SEQ ID NO: 56. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 56. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 56.

[0315] In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 65% sequence identity to SEQ ID NO: 56. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 70% sequence identity to SEQ ID NO: 56. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 75% sequence identity to SEQ ID NO: 56. In certain embodiments,

the non-coding sequence comprises a nucleotide sequence having less than 80% sequence identity to SEQ ID NO: 56. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 85% sequence identity to SEQ ID NO: 56. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 90% sequence identity to SEQ ID NO: 56. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 95% sequence identity to SEQ ID NO: 23. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 97% sequence identity to SEQ ID NO: 56. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 98% sequence identity to SEQ ID NO: 56. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 99% sequence identity to SEQ ID NO: 56. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 100% sequence identity to SEQ ID NO: 56.

[0316] In certain embodiments, the non-coding sequence comprises a fragment of a nucleotide sequence of SEQ ID NO: 57. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 65% sequence identity to SEQ ID NO: 57. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 70% sequence identity to SEQ ID NO: 57. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 75% sequence identity to SEQ ID NO: 57. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 80% sequence identity to SEQ ID NO: 57. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 85% sequence identity to SEQ ID NO: 57. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 57. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 57.

[0317] In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 65% sequence identity to SEQ ID NO: 57. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 70% sequence identity to SEQ ID NO: 57. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 75% sequence identity to SEQ ID NO: 57. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 80% sequence

identity to SEQ ID NO: 57. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 85% sequence identity to SEQ ID NO: 57. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 90% sequence identity to SEQ ID NO: 57. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 95% sequence identity to SEQ ID NO: 57. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 97% sequence identity to SEQ ID NO: 57. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 98% sequence identity to SEQ ID NO: 57. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 99% sequence identity to SEQ ID NO: 57. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having less than 100% sequence identity to SEQ ID NO: 57.

[0318] In certain embodiments, the non-coding sequence comprises a fragment of a nucleotide sequence of SEQ ID NO: 58. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 65% sequence identity to SEQ ID NO: 57. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 70% sequence identity to SEQ ID NO: 58. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 75% sequence identity to SEQ ID NO: 58. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 80% sequence identity to SEQ ID NO: 58. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 85% sequence identity to SEQ ID NO: 58. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 58. In certain embodiments, the non-coding sequence comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 58.

[0319] In some embodiments, the non-coding sequence positioned between the promoter and the capsid protein allows for expression of the capsid protein.

6.2.3.1 Non-coding sequence between ITR and promoter

[0320] In some embodiments, the third polynucleotide further comprises a second non-coding sequence (e.g., stuffer sequence or 2nd spacer sequence) positioned between one of the two ITR sequences and the second promoter (2nd non-coding sequence ("2nd spacer") in FIG. 1B).

[0321] In some embodiments, the non-coding sequence between the 3'ITR and promoter prevents reverse packaging due to being over a size packaging limit of AAV. In some embodiments, the size packaging limit is 4 kb or more, 4.5 kb or more, 4.7 kb or more, or 5 kg or more.

[0322] In some embodiments, the third polynucleotide comprises a promoter operably linked to the transgene and positioned between the two ITR sequences. In some embodiments, the promoter operably linked to the transgene is distinct from the second promoter of the third polynucleotide. In some embodiments, the promoter operably linked to the transgene is distinct from the first promoter of the first polynucleotide. In some embodiments, the second non-coding sequence is positioned between the second promoter of the third polynucleotide and the capsid gene.

[0323] In some embodiments, the second non-coding sequence of the third polynucleotide has a nucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity, or at least 99% sequence identity to any one of SEQ ID NOs: 59-67.

[0324] In some embodiments, the non-coding sequence of the third polynucleotide has a nucleotide sequence of any one of SEQ ID NOs: 59-67.

[0325] In some embodiments, the second non-coding sequence of the third polynucleotide has a length ranging from 150-1800 nucleotides. In some embodiments, the second non-coding sequence of the third polynucleotide has a length ranging from 350-1050 nucleotides. In some embodiments, the second non-coding sequence of the third polynucleotide has a length ranging from 150-800 nucleotides. In some embodiments, the second non-coding sequence of the third polynucleotide has a length ranging from 150-1700 nucleotides. In some embodiments, the second non-coding sequence of the third polynucleotide has a length ranging from 150-1600 nucleotides. In some embodiments, the second non-coding sequence of the third polynucleotide has a length ranging from 150-1500 nucleotides. In some embodiments, the second non-coding sequence of the third polynucleotide has a length ranging from 150-1400 nucleotides. In some embodiments, the second non-coding sequence of the third polynucleotide has a length ranging from 150-1300 nucleotides. In some embodiments, the second non-coding sequence of the third polynucleotide has a length ranging from 150-1200 nucleotides. In some embodiments, the second non-coding sequence

of the third polynucleotide has a length ranging from 150-1100 nucleotides. In some embodiments, the second non-coding sequence of the third polynucleotide has a length ranging from 150-1000 nucleotides. In some embodiments, the second non-coding sequence of the third polynucleotide has a length ranging from 150-900 nucleotides. In some embodiments, the second non-coding sequence of the third polynucleotide has a length ranging from 150-800 nucleotides. In some embodiments, the second non-coding sequence of the third polynucleotide has a length ranging from 150-700 nucleotides. In some embodiments, the second non-coding sequence of the third polynucleotide has a length ranging from 150-600 nucleotides.

[0326] In some embodiments, the second non-coding sequence of the third polynucleotide has a length of less than 1700 nucleotides, less than 1600 nucleotides, less than 1500 nucleotides, less than 1400 nucleotides, less than 1300 nucleotides, less than1200 nucleotides, less than 1100 nucleotides, less than 1000 nucleotides, less than 900 nucleotides, less than 800 nucleotides, less than 700 nucleotides, less than 600 nucleotides, less than 500 nucleotides, less than 400 nucleotides, less than 300 nucleotides, less than 200 nucleotides, less than 160 nucleotides, less than 150 nucleotides, or less than 100 nucleotides.

6.2.3.2 ITR sequences

[0327] In some embodiments, the capsid gene and the second promoter in the third polynucleotide are positioned outside of the two ITR sequences.

[0328] In some embodiments, the capsid gene and the second promoter in the third polynucleotide are positioned upstream of the 5'-ITR sequence. In some embodiments, the capsid gene and the second promoter in the third polynucleotide are positioned downstream of the 3'-ITR sequence.

[0329] In some embodiments, the two ITR sequences are from an AAV serotype selected from: AAV1, AAV2, AAV3, AAV4, AA5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, and AAV13. In some embodiments, the two ITR sequences are from an AAV1 serotype. In some embodiments, the two ITR sequences are from an AAV2 serotype. In some embodiments, the two ITR sequences are from an AAV3 serotype. In some embodiments, the two ITR sequences are from an AAV4 serotype. In some embodiments, the two ITR sequences are from an AAV5 serotype. In some embodiments, the two ITR sequences are from an AAV6 serotype. In some embodiments, the two ITR sequences are from an AAV7 serotype. In some embodiments, the two ITR sequences are from an AAV8 serotype. In some

embodiments, the two ITR sequences are from an AAV9 serotype. In some embodiments, the two ITR sequences are from an AAV10 serotype. In some embodiments, the two ITR sequences are from an AAV11 serotype. In some embodiments, the two ITR sequences are from an AAV12 serotype. In some embodiments, the two ITR sequences are from an AAV13 serotype.

[0330] In some embodiments, wherein the capsid gene and the second promoter in the third polynucleotide are positioned downstream of the 3' ITR sequence. In some embodiments, wherein the capsid gene and the second promoter in the third polynucleotide are positioned upstream o the 5' ITR sequence.

[0331] In some embodiments, at least one of the two ITR sequences has a nucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to any SEQ ID NO: 81. In some embodiments, the at least one of the two ITR sequences has a nucleotide sequence of SEQ ID NO: 82. In some embodiments, each of the two ITR sequences has a nucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to SEQ ID NO: 82. In some embodiments, each of the two ITR sequences has a nucleotide sequence of SEQ ID NO: 82.

[0332] In some embodiments, the 5'-ITR sequence comprises a nucleotide sequence of any one of SEQ ID Nos: 68-74. In some embodiments, the 3'-ITR sequence comprises a nucleotide sequence of any one of SEQ ID Nos: 75-81.

6.2.3.3 Poly A

[0333] In some embodiments, the third polynucleotide further comprises a polyA transcription termination sequence.

[0334] In some embodiments, the third polynucleotide further comprises the AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and a transcription termination sequence (polyA sequence) positioned at the 3' end of the capsid coding sequence. In some embodiments, the third polynucleotide further comprises a AAV2 3' UTR sequence positioned at the 3' end of the capsid coding sequence. In some embodiments, the AAV2 5' UTR sequence is positioned between the second promoter and the 5' end of the

capsid coding sequence. In some embodiments, the AAV2 3' UTR sequence is positioned at the 3' end of the capsid coding sequence.

In some embodiments, the polyA sequence is a SV40 polyA sequence.

6.2.3.4 Rep protein

[0335] In some embodiments, the third polynucleotide further comprises a rep gene encoding a rep protein.

[0336] In some embodiments, the third polynucleotide comprises one, two, three, or four sequences, each one, two, three, or four sequence encoding a Rep 78 protein, a Rep 68 protein, a Rep 52 protein, or a Rep 40 protein. In certain embodiments, the third polynucleotide comprises sequences encoding one or more of: Rep78 protein, Rep68 protein, Rep52 protein, and Rep40 protein.

[0337] In some embodiments, the third polynucleotide comprises sequences encoding a Rep 52 and a Rep 40 protein. In some embodiments, the third polynucleotide comprises sequences encoding a Rep 52 protein. In some embodiments, the third polynucleotide comprises sequences encoding a Rep 40 protein. In some embodiments, the third polynucleotide comprises sequences encoding a Rep 78 protein. In some embodiments, the third polynucleotide comprises sequences encoding a Rep 68 protein. In some embodiments, the third polynucleotide comprises sequences encoding Rep78, Rep68, Rep52, and Rep40 proteins.

[0338] In some embodiments, the Rep 52 protein is from an AAV serotype selected from: AAV1, AAV2, AAV3, AAV4, AA5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, and AAV13. In some embodiments, the Rep 40 protein is from an AAV serotype selected from: AAV1, AAV2, AAV3, AAV4, AA5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, and AAV13. In some embodiments, the Rep 78 protein is from an AAV serotype selected from: AAV1, AAV2, AAV3, AAV4, AA5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, and AAV13. In some embodiments, the Rep 68 protein is from an AAV serotype selected from: AAV1, AAV2, AAV3, AAV4, AA5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, and AAV13.

[0339] In some embodiments, the third polynucleotide further comprises a Rep 52 protein and a Rep 40 protein positioned between two promoter sequences and downstream the 3' ITR sequence. In some embodiments, the two promoters are a p19 promoter and a p40 promoter.

[0340] In some embodiments, the third polynucleotide further comprises a Rep 52 and Rep 40 protein positioned between the second promoter and the capsid protein.

[0341] In some embodiments, the third polynucleotide further comprises a Rep 78 protein and a Rep 68 protein positioned between two promoter sequences and downstream the 3' ITR sequence. In some embodiments, the two promoters are a p19 promoter and a p40 promoter.

[0342] In some embodiments, the third polynucleotide further comprises a Rep 78 and Rep 68 protein positioned between the second promoter and the capsid protein.

6.2.3.5 Expression Cassette

[0343] The third polynucleotide further comprises an expression cassette comprising a transgene flanked by inverted terminal repeat (ITR) sequences.

[0344] In some embodiments, the transgene is flanked by one or more inverted terminal repeat (ITR) sequences (e.g., one or more AAV ITRs). In some embodiments, the transgene is flanked by two ITR sequences. In some embodiments, the expression cassette comprises one or more transgenes flanked by ITR sequences. In some embodiments, the expression cassette comprises one or more transgenes flanked by homology arm sequences. In some embodiments, the expression cassette comprises one or more transgenes flanked by homology arm sequences and ITRs.

[0345] In some embodiments, the expression cassette further comprises a promoter operably linked to the transgene between the two ITRs.

[0346] In some embodiments, the expression cassette comprises one or more heterologous nucleic acid sequences encoding a reporter gene (e.g., a fluorescent or luminescent reporter). In some embodiments, the expression cassette comprises a sequence encoding one or more biomarkers. In some embodiments, the expression cassette comprises one or more transcription termination sequences (e.g., a polyA sequence). In some embodiments, expression cassette comprises one or more promoter sequences. In some embodiments, the expression cassette comprises one or more enhancer sequences. In some embodiments, expression cassette comprises one or more intron sequences.

[0347] In some embodiments, a transgene is a gene chosen to improve one or more signs and/or symptoms of a disease, disorder, or condition. In some embodiments, a transgene may integrate into a host cell genome through use of the third polynucleotide as described herein. In some embodiments, transgenes are functional versions of disease associated genes (i.e., gene isoform(s) which are associated with a disease, disorder or condition) found in a host cell. In some embodiments, transgenes are an optimized version of disease-associated genes found in a host cell (e.g., codon optimized or expression-optimized variants). In some embodiments, transgenes are variants of disease-associated genes found in a host cell (e.g., functional gene fragment or variant thereof). In some embodiments, a transgene is a gene that causes expression of a peptide that is normally expressed in one or more healthy tissues. In some embodiments, a transgene is a gene that causes expression of a peptide that is normally expressed in liver cells. In some embodiments, a transgene is a gene that causes expression of a peptide that is normally expressed in muscle cells. In some embodiments, a transgene is a gene that causes expression of a peptide that is normally expressed in central nervous system cells. In some embodiments, a transgene comprises a gene that causes expression of a peptide that is not normally expressed in one or more healthy tissues (e.g, peptide expressed ectopically). In some embodiments, a transgene is a gene that causes expression of a peptide that is ectopically expressed in one or more healthy tissues (e.g., liver, muscle, central nervous system (CNS)). In some embodiments, a transgene is a gene that causes expression of a peptide that is ectopically expressed in one or more healthy tissues and normally expressed in one or more healthy tissues (e.g., liver, muscle, central nervous system (CNS)).

[0348] In some embodiments, a transgene comprises a gene encoding a functional protein.

[0349] The transgene can be, for example, a reporter gene (e.g., beta-lactamase, beta-galactosidase (LacZ), alkaline phosphatase, thymidine kinase, green fluorescent polypeptide (GFP), chloramphenicol acetyltransferase (CAT), or luciferase, or fusion polypeptides that include an antigen tag domain such as hemagglutinin or Myc), or a therapeutic gene (e.g., genes encoding hormones or receptors thereof, growth factors or receptors thereof, differentiation factors or receptors thereof, immune system regulators (e.g., cytokines and interleukins) or receptors thereof, enzymes, RNAs (e.g., inhibitory RNAs or catalytic RNAs), or target antigens (e.g., oncogenic antigens, autoimmune antigens). In some embodiments, the modified rAAV comprises an expressible polynucleotide encoding a therapeutic tRNA, miRNA, gene editing guide RNA, or RNA-editing guide RNA.

[0350] In some embodiments, the transgene can be selected depending, at least in part, on the particular disease or deficiency being treated. Simply by way of example, gene transfer or gene therapy can be applied to the treatment of hemophilia, retinitis pigmentosa, cystic fibrosis, leber congenital amaurosis, lysosomal storage disorders, inborn errors of metabolism (e.g., inborn errors of amino acid metabolism including phenylketonuria, inborn errors of organic acid metabolism including propionic acidemia, inborn errors of fatty acid metabolism including medium-chain acyl-CoA dehydrogenase deficiency (MCAD)), cancer, achromatopsia, cone-rod dystrophies, macular degenerations (e.g., age-related macular degeneration), lipopolypeptide lipase deficiency, familial hypercholesterolemia, spinal muscular atrophy, Duchenne's muscular dystrophy, Alzheimer's disease, Parkinson's disease, obesity, inflammatory bowel disorder, diabetes, congestive heart failure, hypercholesterolemia, hearing loss, coronary heart disease, familial renal amyloidosis, Marfan's syndrome, fatal familial insomnia, Creutzfeldt-Jakob disease, sickle-cell disease, Huntington's disease, fronto-temporal lobar degeneration, Usher syndrome, lactose intolerance, lipid storage disorders (e.g., Niemann-Pick disease, type C), Batten disease, choroideremia, glycogen storage disease type II (Pompe disease), ataxia telangiectasia (Louis-Bar syndrome), congenital hypothyroidism, severe combined immunodeficiency (SCID), and/or amyotrophic lateral sclerosis (ALS). A transgene also can be, for example, an immunogen that is useful for immunizing a subject (e.g., a human, an animal (e.g., a companion animal, a farm animal, an endangered animal). For example, immunogens can be obtained from an organism (e.g., a pathogenic organism) or an immunogenic portion or component thereof (e.g., a toxin polypeptide or a by-product thereof). By way of example, pathogenic organisms from which immunogenic polypeptides can be obtained include viruses (e.g., picornavirus, enteroviruses, orthomyxovirus, reovirus, retrovirus), prokaryotes (e.g., Pneumococci, Staphylococci, Listeria, Pseudomonas), and eukaryotes (e.g., amebiasis, malaria, leishmaniasis, nematodes). It would be understood that the methods described herein and compositions produced by such methods are not to be limited by any particular transgene.

6.2.4. Polynucleotides

[0351] In some embodiments of the systems of the present disclosure, the polynucleotide are circular polynucleotides. In some embodiments, the polynucleotide provided herein (e.g., the first polynucleotide, second polynucleotide, and the third polynucleotide) are in plasmids.

[0352] In certain embodiments, the first polynucleotide is a circular polynucleotide. In certain embodiments, the second polynucleotide is a circular polynucleotide. In certain embodiments, the third polynucleotide is a circular polynucleotide. In certain embodiments, the fourth polynucleotide is a circular polynucleotide.

[0353] In some embodiments, circular polynucleotide has a closed confirmation with no free ends. Non-limiting examples of circular polynucleotide include plasmids. In some embodiments, the first polynucleotide is in a plasmid. In some embodiments, the second polynucleotide is a plasmid. In some embodiments, the third polynucleotide is in a plasmid. In some embodiments, the fourth polynucleotide is in a plasmid.

[0354] In some embodiments, the first polynucleotide is a linear polynucleotide. In some embodiments, the first polynucleotide is a linear double-stranded polynucleotide. In some embodiments, the first polynucleotide is a linear single-stranded polynucleotide.

[0355] In some embodiments, the second polynucleotide is a linear polynucleotide. In some embodiments, the second polynucleotide is a linear double-stranded polynucleotide. In some embodiments, the second polynucleotide is a linear single-stranded polynucleotide. In some embodiments, the third polynucleotide is a linear polynucleotide. In some embodiments, the third polynucleotide is a linear double-stranded polynucleotide. In some embodiments, the third polynucleotide is a linear single-stranded polynucleotide. In some embodiments, the first polynucleotide, second polynucleotide, and the third polynucleotides are linear polynucleotides. In some embodiments, the fourth polynucleotide is a linear polynucleotide. In some embodiments, the fourth polynucleotide is a linear double-stranded polynucleotide. In some embodiments, the first polynucleotide, second polynucleotide, third polynucleotide, and fourth polynucleotide are linear polynucleotides.

6.3. Host cells

[0356] In some embodiments, the polynucleotides of the systems are transfected or transformed into a host cell. The present disclosure provides a host cell comprising one or more of the polynucleotides disclosed herein. The host cell can be a prokaryotic cell or eukaryotic cell. In some embodiments, the host cell is a mammalian cell or a yeast cell. In some embodiments, the host cell is a cancer cell line. In some embodiments, the host cell is HEK293, CHO, Cos-7, VPC 2.0, or NSO.

[0357] In some embodiments, all the components required for the host cell to package and generate a recombinant AAV are provided to the host cell in trans. In some embodiments, any one or more of the required components (e.g., an expression cassette, rep sequences, cap sequences, and/or helper functions) are provided by a stable host cell which has been engineered to contain one or more of the required components. In some embodiments, such a stable host cell contains the required component(s) under the control of an inducible promoter. In some embodiments, the required component(s) is under the control of a constitutive promoter.

[0358] In some embodiments, the host cell comprises the first polynucleotide, the second polynucleotide, and the third polynucleotide described herein. In some embodiments, the first polynucleotide, second polynucleotide, and the third polynucleotide are transiently transduced to the host cell.

6.4. Methods of Producing rAAV

[0359] In another aspect, the present disclosure provides a method of producing recombinant rAAV. In some embodiments, the method comprises obtaining a system comprising a first polynucleotide, a second polynucleotide, and a third polynucleotide described herein; transfecting a packaging cell with the first polynucleotide, the second polynucleotide, and the third polynucleotide; and culturing the packaging cell under conditions suitable for recombinant AAV (rAAV) production. In some embodiments, culturing the cell under conditions suitable for recombinant AAV production comprises expanding the cell. In some embodiments, the method comprises collecting the secreted recombinant AAV production expressed by the cell.

[0360] In some embodiments, culturing the packaging cell under conditions suitable for recombinant AAV production comprises expanding the packaging cell.

[0361] In some embodiments, the method further comprises lysing the packaging cell. rAAV vector particles of the present disclosure can be harvested from rAAV production cultures by lysis of the host cells of the production culture or by harvest of the spent media from the production culture, provided the cells are cultured under conditions known in the art to cause release of rAAV particles into the media from intact cells, as described in U.S. Pat. No. 6,566,118, which is hereby incorporated by reference in its entirety. Suitable methods of lysing cells are also known in the art and include for example multiple freeze/thaw cycles,

sonication, micro-fluidization, and treatment with chemicals, such as detergents and/or proteases.

[0362] In some embodiments, the method further comprises purifying the lysed cells to collect a quantity of rAAV particles. In some embodiments, purifying the lysed cells comprises clarifying the harvest cells to remove host cell debris. In some embodiments, the lysed cells are clarified by filtration through a series of depth filters including, for example, a grade DOHC Millipore Millistak+HC Pod Filter, a grade A1HC Millipore Millistak+HC Pod Filter, and a 0.2 μm Filter Opticap XL10 Millipore Express SHC Hydrophilic Membrane filter. Clarification can also be achieved by a variety of other standard techniques known in the art, such as, centrifugation or filtration through any cellulose acetate filter of 0.2 μm or greater pore size known in the art. Still other suitable depth filters, e.g., in the range of about 0.045 μm to about 0.2 μm or other filtration techniques may be used.

[0363] In some embodiments, the lysed cells are treated with a nuclease, or a combination of nucleases, to digest any contaminating high molecular weight nucleic acid present in the production culture. The examples herein illustrate a DNAse, e.g., Benzonase® digestion performed under standard conditions known in the art. For example, a final concentration of 1 unit/mL to 2.5 units/mL of Benzonase® is used at a temperature ranging from ambient temperature to 37° C. for a period of 30 minutes to several hours, or about 2 hours. In another example, a turbonuclease is used. However, one of skill in the art may utilize other suitable nuclease, or a mixture of nucleases.

[0364] In some embodiments, the lysed cells are purified using one or more of the following purification steps: tangential flow filtration (TFF) for concentrating the rAAV particles, heat inactivation of helper virus, rAAV capture by hydrophobic interaction chromatography, buffer exchange by size exclusion chromatography (SEC), and/or nanofiltration. These steps may be used alone, in various combinations, or in different orders.

[0365] In some embodiments, a Benzonase®-treated mixture is concentrated via tangential flow filtration ("TFF"). Large scale concentration of viruses using TFF ultrafiltration has been described by R. Paul et al., HUMAN GENE THERAPY, 4:609-615 (1993). TFF concentration of the feedstream enables a technically manageable volume of feedstream to be subjected to the chromatography steps used for purification and allows for more reasonable sizing of columns without the need for lengthy recirculation times. In some embodiments, the rAAV feedstream is concentrated between at least two-fold and at least ten-fold. In some

embodiments, the feedstream is concentrated between at least ten-fold and at least twenty-fold. In some embodiments, the feedstream is concentrated between at least twenty-fold and at least fifty-fold. One of ordinary skill in the art will also recognize that TFF can also be used at any step in the purification process where it is desirable to exchange buffers before performing the next step in the purification process.

[0366] In some embodiments, the rAAV particles comprise less than 10% of replication-competent adeno-associated virus particles (rcAAV). In some embodiments, the rAAV particles comprise less than 8% of replication-competent adeno-associated virus particles (rcAAV).

[0367] In some embodiments, rAAV particles comprise less than 5% of replication-competent adeno-associated virus particles (rcAAV). In some embodiments, the rAAV particles comprise less than 3% of replication-competent adeno-associated virus particles (rcAAV). In some embodiments, the rAAV particles comprise less than 1% of replication-competent adeno-associated virus particles (rcAAV).

[0368] The polynucleotide can be transformed into the packaging cell, either transiently or stably.

[0369] In certain embodiments of this invention, the AAV vector and complementary packaging gene(s), if any, are provided in the form of bacterial plasmids, AAV particles, linear polynucleotide, or any combination thereof.

[0370] In other embodiments, either the AAV vector sequence, the packaging gene(s), or both, are provided in the form of genetically altered (preferably inheritably altered) eukaryotic cells. The development of host cells inheritably altered to express the AAV vector sequence, AAV packaging genes, or both, provides an established source of the material that is expressed at a reliable level.

[0371] A variety of different genetically altered cells can thus be used in the context of this invention. By way of illustration, a mammalian host cell may be used with at least one intact copy of a stably integrated rAAV vector. An AAV packaging polynucleotide comprising at least an AAV rep gene operably linked to a promoter can be used to supply replication functions (as described in U.S. Pat. No. 5,658,776). Alternatively, a stable mammalian cell line with an AAV rep gene operably linked to a promoter can be used to supply replication functions (see, e.g., WO 95/13392; WO 98/23018; and U.S. Patent No. 5,656,785). The AAV

cap gene, providing the encapsidation proteins as described above, can be provided together with an AAV rep gene or separately (see, e.g., the above-referenced patent documents as well as WO 98/27204.

[0372] Thus, the rAAV of the disclosure can be assembled by, for example, expression of its components in a packaging host cell. The components of a virus particle (e.g., rep sequences, cap sequences, inverted terminal repeat (ITR) sequences) can be introduced into a packaging host cell using one or more viral vectors.

[0373] Once assembled, rAAV particles can be purified, if desired, using routine methods. As used herein, "purified" virus particles refer to virus particles that are removed from components in the mixture in which they were made such as, but not limited to, viral components (e.g., rep sequences, cap sequences), packaging host cells, and partially- or incompletely- assembled virus particles.

[0374] In some embodiments, the host cells for producing an AAV vector are cultured in suspension. In certain embodiment, the cells are cultured in animal component-free conditions. The animal component-free medium can be any animal component-free medium (e.g., serum-free medium) compatible with a given cell line, for example, HEK293 cells or viral production cells (VPCs), which is a clonal HEK293 cell line. Any cell line known in the art to be capable of propagating an AAV vector can be used for rAAV production using methods described herein. Exemplary cell lines that can be used to generate an AAV vector include, without limitation, HEK293, CHO, Cos-7, VPC 2.0, and NSO.

[0375] In some embodiments, a host cell for producing an AAV vector stably expresses any of the components required for AAV vector production, e.g., Rep, Cap, helper, VP1, etc. In certain embodiments, a cell line for producing an AAV vector transiently expresses any of the components required for AAV vector production.

[0376] The host cells can be modified and cultured to produce rAAV using any of the methods known in the art. For example, the methods of producing rAAV using a non-plasmid DNA have been disclosed in PCT/EP2022/061630 (WO2022/229460), US16/988,025, US17/578,004, PCT/GB2010/000165 (WO2010/086626), PCT/GB2017/052413 (WO2018033730), PCT/EP2020/075486 (WO2021048366A1), PCT/GB2020/050947 (WO2020208379) and PCT/US2022/017901 (WO2022182986) which are incorporated by reference in their entireties herein.

6.4.1. Ratio of Polynucleotide

[0377] In rAAV production methods disclosed herein, two or more polynucleotides are introduced into host cells to provide proteins required for production of rAAVs. Relative amounts of the polynucleotide introduced into the host cells can be adjusted to improve the rAAV production yields. In some embodiments, the optimal ratios between different polynucleotide are used. In the case, two or more polynucleotides are introduced into the host cells at a specific ratio. In some embodiments, the ratio among polynucleotide is a mass ratio. In some embodiments, the ratio among polynucleotide is a molar ratio.

[0378] In some embodiments, the first polynucleotide containing helper virus genes and a rep gene, the third polynucleotide containing an AAV capsid protein coding sequence, and the third polynucleotide containing an expression cassette are introduced into the host cells at a specific ratio.

[0379] In some embodiments, a molar ratio of the first polynucleotide, the second polynucleotide, and the third polynucleotide transfected in a cell is selected from: 5:1:0.2; 4:1:0.2; 3:1:0.2; 2:1:0.2; and 1:1:0.2. In some embodiments, a molar ratio of the first polynucleotide, the second polynucleotide, and the third polynucleotide transfected in a cell is selected from: 5:1:0.2; 4:1:0.2; 3:1:0.2; 2:1:0.2; 1:1:0.2; and 3:0.4:0.1. In some embodiments, a molar ratio of the first polynucleotide, the second polynucleotide, and the third polynucleotide transfected in a cell is selected from: 1:1:1 and 3:1:0.2. In some embodiments, a molar ratio of the first polynucleotide, the second polynucleotide, and the third polynucleotide transfected in a cell is selected from: 3:0.2:0.1, 3:0.2:0.5; 3:0.4:0.1; 3:0.4:0.5; 3:0.6:0.1; 3:0.4:0.5; 3:0.6:0.1; 3:0.6:0.5; 3:0.8:0.1; 3:0.8:0.5; 3:1:0.1; 3:1:0.5; 5:0.2:0.1; 5:0.2:0.5: 5:0.4:0.5; 5:0.6:0.5; 5:0.8:0.5; 5:1:0.2; 5:0.8:0.1; and 3:1:0.2.

[0380] In some embodiments, the system comprises a molar ratio of 3:6:1 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 15:72.5:12.5 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 25:70:5 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 1:1:16 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 42:42:16 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 55:35:10 of the

second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 31:59:10 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 17:66:17 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 54:41:5 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 41:54:5 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 42:30:28 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 3:3:4 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 3:5:2 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 1:6:3 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 23.8:71.4:5.8 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 2:5:3 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 55:20:25 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 52:44:4 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 54:34:12 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 6:3:1 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 35:53:12 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 4:3:3 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 45:39:16 of the second polynucleotide:first polynucleotide:third polynucleotide.

[0381] In some embodiments, the system comprises a molar ratio of 5:1:4 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 1:2:7 of the second polynucleotide:first polynucleotide:third polynucleotide. In some embodiments, the system comprises a molar ratio of 1:2:7 of the second polynucleotide:first polynucleotide:third polynucleotide.

[0382] In some embodiments, the first polynucleotide comprises a molar amount ranging from about 5% to 95% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined. In some embodiments, the first polynucleotide comprises a molar amount ranging from about 20% to 95% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined. In some embodiments, the first polynucleotide comprises a molar amount ranging from about 30% to 95% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined. In some embodiments, the first polynucleotide comprises a molar amount ranging from about 40% to 95% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined. In some embodiments, the first polynucleotide comprises a molar amount ranging from about 50% to 70% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined. In some embodiments, the first polynucleotide comprises a molar amount ranging from about 60% to 80% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined. In some embodiments, the first polynucleotide comprises a molar amount ranging from about 70% to 95% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined.

[0383] In some embodiments, the second polynucleotide comprises a molar amount ranging from about 5% to 65% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined. In some embodiments, the second polynucleotide comprises a molar amount ranging from about 5% to 60% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined. In some embodiments, the second polynucleotide comprises a molar amount ranging from about 15% to 50% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined. In some embodiments, the second polynucleotide comprises a molar amount ranging from about 25% to 40% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined.

[0384] In some embodiments, the third polynucleotide comprises a molar amount ranging from about 1% to 80% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined. In some embodiments, the third

polynucleotide comprises a molar amount ranging from about 10% to 70% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined. In some embodiments, the third polynucleotide comprises a molar amount ranging from about 20% to 70% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined. In some embodiments, the third polynucleotide comprises a molar amount ranging from about 30% to 70% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined. In some embodiments, the third polynucleotide comprises a molar amount ranging from about 40% to 70% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined. In some embodiments, the third polynucleotide comprises a molar amount ranging from about 50% to 60% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined. In some embodiments, the third polynucleotide comprises a molar amount ranging from about 5% to 35% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined. In some embodiments, the third polynucleotide comprises a molar amount ranging from about 10% to 90% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined.

[0385] In some embodiments, the second polynucleotide has a molar amount ranging from about 5% to about 80% of the total molar amount of the first, second, and third polynucleotides combined. In some embodiments, the second polynucleotide has a molar amount ranging from about 8% to about 60% of the total molar amount of the first, second, and third polynucleotides combined. In some embodiments, the second polynucleotide has a molar amount of at least 8%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, or about 60% of the total molar amount of the first, second, and third polynucleotides combined.

[0386] In some embodiments, the first polynucleotide has a molar amount ranging from about 1% to about 90% of the total molar amount of the first, second, and third polynucleotides combined. In some embodiments, the first polynucleotide has a molar amount ranging from about 38% to about 90% of the total molar amount of the first, second, and third polynucleotides combined. In some embodiments, the first polynucleotide has a molar amount of at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least

60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, or about 90% of the total molar amount of the first, second, and third polynucleotides combined.

[0387] In some embodiments, the third polynucleotide has a molar amount ranging from about 3% to about 55% of the total molar amount of the first, second, and third polynucleotides combined. In some embodiments, the third polynucleotide has a molar amount of at least 3%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, or about 55% of the total molar amount of the first, second, and third polynucleotides combined.

[0388] In some embodiments, the system comprises a molar ratio of the second polynucleotide:first polynucleotide:third polynucleotide highlighted in FIG. 17.

[0389] In some embodiments, a molar amount of the first polynucleotide introduced into the cell is at least twice of a molar amount of the second polynucleotide introduced into the cell. In some embodiments, a molar amount of the first polynucleotide introduced into the cell is two to four times of a molar amount of the second polynucleotide introduced into the cell. In some embodiments, a molar amount of the first polynucleotide introduced into the cell is at least three times of a molar amount of the second polynucleotide introduced into the cell. In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least four times of a molar amount of the third polynucleotide introduced into the cell. In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least five or six times of a molar amount of the third polynucleotide introduced into the cell. In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least five times of a molar amount of the third polynucleotide introduced into the cell. In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least ten times of a molar amount of the third polynucleotide introduced into the cell. In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least fifteen times of a molar amount of the third polynucleotide introduced into the cell. In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least twenty times of a molar amount of the third polynucleotide introduced into the cell. In some embodiments, a molar amount of the second polynucleotide introduced into the cell is at least twenty-five times of a molar amount of the third polynucleotide introduced into the cell.

6.4.2. Produced rAAVs

[0390] rAAVs produced by the method disclosed herein can be analyzed by various methods known in the art. In preferred embodiments, the method of the present disclosure provides rAAVs of a high purity at higher yields compared to conventional AAV production methods known in the art (e.g., standard three plasmid (3P) system).

[0391] In some embodiments, the method provides a higher % of full capsids. In some embodiments, the method provides a lower % of empty capsids or partially filled capsids.

[0392] In some embodiments, the rAAVs produced by the method include a lower amount of contaminant such as a recombinant plasmid contaminant or host cell DNA (hcDNA). In some embodiments, the rAAVs produced by the method of the present disclosure include less than 50% of the contaminants compared to the rAAVs produced by the conventional method. In some embodiments, the rAAVs produced by the method of the present disclosure include less than 30% of the contaminants compared to the rAAVs produced by the conventional method. In some embodiments, the rAAVs produced by the method of the present disclosure include less than 10% of the contaminants compared to the rAAVs produced by the conventional method. In some embodiments, the rAAVs produced by the method of the present disclosure include less than 5% of the contaminants compared to the rAAVs produced by the conventional method. In some embodiments, the rAAVs produced by the method of the present disclosure include less than 1% of the contaminants compared to the rAAVs produced by the conventional method. In some embodiments, the rAAVs produced by the method of the present disclosure include no recombinant plasmid contaminant.

[0393] In some embodiments, the method provides a lower % of replication-competent rAAV (rcAAV). In some embodiments, less than 10% of the rAAVs produced by the method are rcAAVs. In some embodiments, less than 5% of the rAAVs produced by the method are rcAAVs. In some embodiments, less than 3% of the rAAVs produced by the method are rcAAVs. In some embodiments, less than 1% of the rAAVs produced by the method are rcAAVs. In some embodiments, less than 0.5% of the rAAVs produced by the method are rcAAVs.

6.5. Method of Use

[0394] An rAAV produced by the methods described herein can be used in research and/or therapeutic applications. In some embodiments, the rAAV is for genetically modifying a cell

in vitro or in vivo. In some embodiments, the rAAV is used for gene therapy or for vaccination in a human or animal. More specifically, the rAAV can be used for gene addition, gene augmentation, genetic delivery of a polypeptide therapeutic, genetic vaccination, gene silencing, genome editing, gene therapy, RNAi delivery, cDNA delivery, mRNA delivery, miRNA delivery, miRNA sponging, genetic immunization, optogenetic gene therapy, transgenesis, DNA vaccination, or DNA immunization of liver cells or non-liver cells.

[0395] In some embodiments, the rAAV of the present disclosure is used for treating, ameliorating or preventing a disease or condition in a subject. In some embodiments, the disease is a disease of the central nervous system (CNS).

[0396] The rAAV of the present disclosure can be administered to a subject in a suitable pharmaceutical carrier.

[0397] The rAAV of the disclosure are typically administered in sufficient amounts to transduce or infect the desired cells and to provide sufficient levels of gene transfer and expression to provide a therapeutic benefit to subjects suffering from a disease. In particular embodiments, the rAAV is administered in sufficient amounts to provide a therapeutic benefit to subjects suffering from a disease of the central nervous system (CNS).

[0398] Conventional and pharmaceutically acceptable routes of administration include, but are not limited to, direct delivery to an organ such as, for example, the brain, intra cisterna magna (ICM), inter-digitally, intravenously, orally, intranasally, intratracheally, intrathecally, intramuscularly, intraocularly, subcutaneously, intradermally, or by other routes of administration. Routes of administration can be combined, if desired.

[0399] Transduction and/or expression of the transgene can be monitored at various time points following administration by DNA, RNA, or protein assays.

[0400] Accordingly, the present disclosure provides a method of treating and/or preventing a disease by administering the rAAV described herein.

7. EXAMPLES

7.1. Example 1: Comparison of standard three-plasmid system (3P), two plasmid 2P (2P) and new three-plasmid system (S3P)

[0401] Production of recombinant rAAV was tested using a new three-plasmid system (used interchangeably herein as "S3P") and compared to the standard three-plasmid system

("standard 3P system" or "3P") and two-plasmid system ("two plasmid 2P system" or "2P"). The tested AAV production systems are illustrated in FIG. 1.

[0402] The new 3-plasmid system (S3P) as shown in FIG. 1 contained a first plasmid with a rep gene encoding rep proteins; a second plasmid with a helper virus gene; and a third plasmid containing a capsid gene, a p41 promoter, a non-coding sequence nucleotide sequence positioned between the capsid gene and the promoter, a transgene flanked by two ITR sequences, and another non-coding sequence positioned between one ITR and the promoter (e.g., p41 promoter as illustrated in FIG. 1B). FIG. 1B shows the positioning of the non-coding sequences in the exemplary third plasmid.

[0403] The standard 3-plasmid system as shown in FIG. 1 included the following plasmids: a first plasmid containing a rep gene encoding a rep protein, a capsid gene encoding a capsid protein, and a p40 promoter positioned between the rep gene and the capsid gene; a second plasmid containing helper virus gene; and a third plasmid with a gene of interest (GOI) flanked by two ITRs.

[0404] rAAV was produced using the 3P, 2P or S3P following the process outlined below.

7.1.1. Preparation of Transfection/Cell densities and flask size

[0405] VPC 2.0 cells in F17 media were used for transfection in combination with FectoVir as a transfection reagent.

7.1.2. Transfection Mix

[0406] For each transfection, Basal Freestyle F17 medium or Opti-MEM was added to a transfection bottle. All the plasmids were added to the transfection mix. Polyplus FectoVir-AAV was then added to the transfection mix.

[0407] The bottle was then inverted gently 3-5 times to mix. The bottle was then incubated inside of the biological safety cabinet (BSC) at room temperature for 30 minutes.
Transfection

[0408] After the 30-minute wait period, 10X volume of the transfection mix was added to each transfection flask with the cultured VPC 2.0 cells in 30 mL or 1.4L volume. For example, for the 1.4L volume productions, 140 mL volume of transfection mix was added to each flask being transfected.

[0409] Transfection flasks were then transferred to a shaker in a 37°C incubator for 72 hours.

7.1.3. Harvesting Transfected Cells

[0410] For production, HEK293 cells are transfected with plasmids. After 48-72h the cells are lysed to release vector. The lysed material is then clarified and vector is purified by affinity chromatography.

7.1.4. Depth filtration Process

[0411] A depth filtration step was used to clarify the crude harvest. Briefly, an inlet line of a filtration system was placed into the product container containing the sample. An outlet line was placed into the product collection container. A pump attached to the tubing is set at a rate of 300 LMH, and the product is filtered through the filtration system until all the product is filtered. Once all material was pumped into the system, the lines were chased with at least 1 filter train holdup volume of chromatography equilibration buffer. Air was then pumped into the system until only foam was seen exiting in the filter train.

7.1.5. Purification of rAAV particles using a Tangential Flow Filtration (TFF) System

[0412] After crude harvesting, the samples are clarified in a feed by a concentration and buffer exchange step. A Tangential Flow Filtration (TFF) system was used for filtration for purification of the concentrated rAAV product. The TFF System includes a digital peristaltic pump, graphical LCD display, digital pressure monitor, KR2i Pump head, automatic backpressure valve, filter module stand, and KF Comm real-time data collection software. The TFF was used to concentrate the vector product (rAAV) and exchange the buffer that the product is in. A sample solution flows through the feed channel and along and tangent to the surface of the membrane as well as through the membrane. The crossflow prevents build up of molecules at the surface that can cause fouling.

[0413] For pre-chromatography TFF (TFF1), the product was concentrated to a volume such that affinity load was completed in a reasonable timeframe. Buffer exchanging into affinity equilibration buffer improved binding interactions with the affinity resin.

[0414] In some cases where $AAV9^{mut1deco1}$ capsid was used as the capsid for post-chromatography TFF (TFF2), the product was buffer exchanged into a cesium chloride buffer to allow for density gradient formation in the ultracentrifuge.

[0415] TFF filters were used such that large particles circulated unobstructed past the filter while buffers and particles smaller than the pore size permeate through the filter. The feed

line was set up at the bottom of the feed container and the retentate line was partway up the feed container. A post-concentration volume of the product was determined using the following guidelines: for less than 2L harvest, target a volume of >= 50 mL; and for 2 – 15L harvest, target a volume of >= 500 mL. The post-concentration volume concentration factor is below 30.

[0416] The working volume (wv) in the feed container was calculated as follows:

[Post-concentration Volume] – [System Holdup] = [Working Volume]

[0417] Filter pore sizes that were used included 100 and 300 kDa, resulting in the vector product becoming concentrated in the retentate. The main pump speed was set to approximately 0.11 – 0.16 mL/min per cm^2 of filter surface area.

[0418] Note: For a 5100 cm^2 surface area filter, this is approximately 550 – 820 mL/min

[0419] To perform TFF, the system was started, and the pressure was monitored for the first few minutes of operation. The valve pressure was slowly increased to 6 - 8 psi.

7.1.6. Diafiltration

[0420] Once the product container was almost completely empty, 4 working volumes of equilibration buffer was added to the product container. Once the product container was completely empty, the system was stopped.

7.1.7. Product Recovery

[0421] The retentate and feed lines were moved to a clean empty container large enough to hold the twice the working volume. The automated backpressure valve was then opened. The pump then ran until only air bubbles were coming from the retentate line. The direction of the pump was then reversed, and the pump was run again until only air bubbles were seen coming from the feed line. The pump direction was then reversed for a second time. The remaining contents of feed container was emptied into the concentrated product container.

[0422] 1.5 – 2.5 system holdup volumes of equilibration buffer were then added to the feed container, and the feed and retentate lines were returned to the feed container. The pump was then turned on, and the buffer was recirculated to rinse the system for 2 – 5 min. These steps were repeated to recover the system wash into the concentrated product container.

7.2. Example 2: rAAV production from 3P and S3P system in shaken flasks

[0423] Production of rAAV was tested in a series of experiments using a S3P system compared to 3P or 2P systems, using the experimental procedures described in Example 1.

7.2.1. Experiment 1: Production of rAAV using 3P vs S3P system with varying capsids

[0424] In this study, production of recombinant rAAV was tested using the S3P system compared to a standard 3P or 2P system in 250 ml volume flasks. The molar ratio amount of each of the polynucleotide molecules of the S3P system (helper: rep: capsid-transgene) was 1:1:0.2. For the S3P system, a rep plasmid (SEQ ID NO: 6) and a helper plasmid (SEQ ID NO: 4) was used.

[0425] Vector titres (vg/ml) for each flask were tested to determine yields. The samples were analyzed by ddPCR and were measured via analytical ultracentrifugation for % full, partial, and empty capsids. Each plasmid used in the experiments included the capsid and transgene as specified in Table 1.

[0426] Result:

[0427] The S3P system resulted in higher % full capsids (36.4%) than the 2P system (29.2%) but had lower yield (7.3x 10^{11} mg/ml) compared the 2P system (9.2x 10^{11} mg/ml), when AAV9 capsid was tested. (Table 1).

[0428] The S3P system resulted in both higher % full capsids and yields (23.3% 8.2 x 10^{11} mg/ml, respectively) as compared to the 2P system, when a variant of AAV9 capsid (AAV9 (Capsid-2_MUT1-38181)) was tested.

Table 1:

Flask	Plasmid system	Capsid	GOI	% full by SEC-MALS	Harvest Titer
1	2P	AAV9	ARSA	29.2	9.2e11
2	S3P	AAV9	ARSA	36.4	7.3e11
3	2P	AAV9 (Capsid-2_MUT1-38181)	ARSA	13.5	3.7e11

4	S3P	AAV9 (Capsid-2_MUT1-38181)	ARSA	23.3	8.2e11

7.2.2. Experiment 2: Production of rAAV using 2P vs S3P system with varying of Rep, Helper, Capsids, and Transgenes

[0429] In this study, production of recombinant rAAV was tested using the S3P system compared to a standard 3P system and 2P systems in 250 ml or 30 ml volume flasks. Different helper, rep, capsids, and transgenes were tested.

[0430] The molar ratio amount of each of the polynucleotide molecules of the S3P system (helper: rep: capsid-transgene) was 1:1:0.1.

[0431] For the S3P system, a rep plasmid (SEQ ID NO: 6) and a helper plasmid (SEQ ID NO:4) were used, except for Flask 13, where a different rep plasmid was used. The S3P system included a rep plasmid, a helper plasmid, and a capsid-transgene plasmid including a capsid, a p41 promoter, a non-coding sequence positioned between the capsid and p41 promoter, a transgene flanked by two ITR sequences, and a second non-coding sequence positioned between the p41 promoter and an ITR.

[0432] The traditional 3P system included a rep-cap plasmid, a helper plasmid, and a transgene plasmid. For the traditional 3P system, a helper plasmid (SEQ ID NO: 4) was used.

[0433] The 2P system included a rep-helper plasmid (SEQ ID NO: 1) and a capsid-transgene plasmid.

[0434] The plasmids used in this study are summarized in Table 2.

[0435] Parameters tested: Vector titres (vg/ml) for each flask were tested to determine yield. The samples were analyzed by ddPCR and were measured via analytical ultracentrifugation for % full, partial, and empty capsids.

[0436] Result:

[0437] As shown in Table 2, regardless of the type of capsid tested, the S3P system resulted in higher % full capsids than the 2P system, and comparable vector titre (vg/ml) as the 2P system.

Table 2:

Flask	Plasmid	Capsid	GOI	% full by SEC-MALS	Harvest Titer
1	S3P	AAV9	ARSA	31.4	5.93E+11
2	3P	AAV9	ARSA	20.7	2.57E+11
3	S3P	AAV9 (Capsid-2_MUT1-38181)	ARSA	17.4	2.57E+11
4	3P	AAV9 (Capsid-2_MUT1-38181)	ARSA	13.7	1.02E+11
5	S3P	Anc80L65 with peptide (from AAV9 variant #3)	UBC-ALS Tool		2.27E+11
6	3P	Anc80L65 with peptide (from AAV9 variant #3)	UBC-ALS Tool	6.9	2.51E+11
7	2P	AAV9	ARSA	23.8	6.92E+11
8	2P	AAV9 (Capsid-2_MUT1-38181)	ARSA	13.2	9.03E+11
9	2P	Anc80	UBC-ALS Tool		2.45E+11
13	Lib (S3P)	AAV9 Library	AAV9 capsid as transgene	67.9	3.57E+11
14	Lib (S3P)	AAV9 (Capsid-2_MUT1-38181) Library	AAV9 (Capsid-2_MUT1-38181) capsid as transgene	62.7	2.07E+11
15	S3P	Anc80	ARSA		1.93E+11

Flask	Plasmid	Capsid	GOI	% full by SEC-MALS	Harvest Titer
16	3P	Anc80	ARSA	8.8	1.69E+11
17	2P	Anc80	ARSA		1.69E+11
10	S3P	AAV9 variant #2	UBC-ALS Tool		6.11E+11
11	3P	AAV9 variant #2	UBC-ALS Tool		2.6E+11
12	2P	AAV9 variant #2	UBC-ALS Tool		7.22E+11

7.2.3. Experiment 3: Production of rAAV with varying capsids in 2P vs S3P system

[0438] In this study, production of recombinant rAAV was tested using the S3P system compared to a standard 2P system and 3P systems in 250 ml volume flasks. Different capsids (AAV9 or AAV9 (Capsid-2_MUT1-38181)) were tested.

[0439] The molar ratio amount of each of the polynucleotide molecules of the S3P system (helper: rep: capsid-transgene) was 1:1:0.1.

[0440] For the S3P system, a rep plasmid (SEQ ID NO: 6) and a helper plasmid (SEQ ID NO:4) were used, except Flask 3, where a different rep plasmid SEQ ID NO: 7 was used instead of the rep plasmid (SEQ ID NO: 6). The rep plasmid SEQ ID NO: 7 (also provided as AAV2 Rep S-2 of FIG. 16) does not comprise an intron/exon from an AAV2 rep. The S3P system included a rep plasmid, a helper plasmid, and a capsid-transgene plasmid including a capsid, a p41 promoter, a non-coding sequence positioned between the capsid and p41 promoter, a transgene flanked by two ITR sequences, and a second non-coding sequence positioned between the p41 promoter and an ITR.

[0441] The traditional 3P system included a rep-cap plasmid, a helper plasmid, and a transgene plasmid. For the traditional 3P system, a helper plasmid (SEQ ID NO: 4) was used.

[0442] The 2P system included a rep-helper plasmid, and a capsid-transgene plasmid. For the 2P system, a rep-helper (SEQ ID NO: 1) was used.

[0443] The plasmids used in this study are shown in Table 3:

[0444] Parameters tested: Vector titres (vg/ml) for each flask were tested to determine yields. The samples were analyzed by ddPCR and were measured via analytical ultracentrifugation for % full, partial, and empty capsids.

[0445] Result:

[0446] The crude harvest titre (vg/ml) and % full capsids for each flask are shown in Table 3 below.

Table 3:

Flask	Plasmid system	Capsid	Transgene	% full by SEC-MALS	Harvest Titer
1	S3P	AAV9 (Capsid-2_MUT1-38181)	ARSA	18.4	1.02e12
2	3P	AAV9 (Capsid-2_MUT1-38181)	ARSA	32.4	9.53e10
3	S3P	AAV9 lib	AAV9	62.7	6.64e11
4	S3P	AAV9 (Capsid-2_MUT1-38181)-lib	AAV9 (Capsid-2_MUT1-38181)	59.4	4.99e11
5	2P	AAV9 (Capsid-2_MUT1-38181)	ARSA	13.3	8.18e11
6	S3P	AAV9	ARSA	36.4	9.75e10
7	3P	AAV9	ARSA	32.5	4.65e10
8	2P	AAV9	ARSA	33.6	2.89e11

[0447] S3P systems that included a AAV9 (Capsid-2_MUT1-38181) capsid resulted in higher yield (vg/ml) compared to 3P and 2P systems, and higher % full capsids compared to 2P systems.

[0448] S3P systems that included an AAV9 capsid resulted in higher yield (vg/ml) compared to the 3P system, and slightly higher % full capsids compared to 3P and 2P systems.

7.2.4. Experiment 4: Production of rAAV using 2P vs S3P system with varying capsids

[0449] In this study, production of recombinant rAAV was tested using the S3P system compared to a standard 2P system and 3P systems in 250 ml volume flasks. 3 different capsids for each of the systems were tested. The transgenes amongst all flasks were fixed.

[0450] For the S3P system, the helper plasmid (SEQ ID NO: 4) and rep plasmid (SEQ ID NO: 6) amongst flasks were fixed. The S3P system included a rep plasmid, a helper plasmid, and a capsid-transgene plasmid including a capsid, a p41 promoter, a non-coding sequence positioned between the capsid and p41 promoter, a transgene flanked by two ITR sequences, and a second non-coding sequence positioned between the p41 promoter and an ITR. The molar ratio amount of each of the polynucleotide molecules of the S3P system (helper: rep: capsid-transgene) was 1:1:0.1.

[0451] The traditional 3P system included a rep-cap plasmid, a helper plasmid, and a transgene plasmid. For the traditional 3P system, a helper plasmid (SEQ ID NO: 4) was used.

[0452] The 2P system included a rep-helper plasmid, and a capsid-transgene plasmid. For the 2P system, a rep-helper (SEQ ID NO: 1) was used.

[0453] The plasmids used in this study are shown in Table 4:

[0454] Parameters tested:

[0455] Vector titres (vg/ml) for each flask were tested to determine yields. The samples were analyzed by ddPCR and were measured via analytical ultracentrifugation for % full, partial, and empty capsids.

[0456] Result:

[0457] The crude harvest titre (vg/ml) and % full capsids for each flask are shown in Table 4 below.

Table 4:

Flask	Plasmid system	Capsid	Transgene	% full by SEC-MALS	Harvest Titer
1	S3P	AAV9 variant #2	UBC-ALS Tool	26.5	1.17e10
2	3P	AAV9 variant #2	UBC-ALS Tool	6.8	1.21e10
3	S3P	AAV9 variant #1	UBC-ALS Tool	30.5	2.81e10
4	3P	AAV9 variant #2	UBC-ALS Tool	11.4	1.09e11
5	S3P	AAV9 variant #3	UBC-ALS Tool	27.8	6.19e11
6	3P	AAV9 variant #3	UBC-ALS Tool	12.6	1.11e11
7	2P	AAV9 variant #2	UBC-ALS Tool	1.7	1.7e11
8	2P	AAV9 variant #1	UBC-ALS Tool	18.4	1.36e11
9	2P	AAV9 variant #3	UBC-ALS Tool	17.9	412e11

[0458] Regardless of capsid tested, % full capsid amounts were higher in S3P systems compared to 3P and 2P systems.

7.2.5. Experiment 5: Production of rAAV using 2P vs S3P system with varying transgenes

[0459] In this study, production of recombinant rAAV was tested using the S3P system compared to a standard 2P system and 3P systems in 250 ml volume flasks. Different transgenes in each of the systems were tested.

[0460] For the S3P system, the helper plasmid and rep plasmid amongst flasks were fixed. The S3P system included a rep plasmid, a helper plasmid, and a capsid-transgene plasmid including a capsid, a p41 promoter, a non-coding sequence positioned between the capsid and p41 promoter, a transgene flanked by two ITR sequences, and a second non-coding sequence positioned between the p41 promoter and an ITR. The molar ratio amount of each of the polynucleotide molecules of the S3P system (helper: rep: capsid-transgene) was:1:3:0.2. The plasmids used in this study are shown in Table 5:

[0461] The traditional 3P system included a rep-cap plasmid, a helper plasmid, and a transgene plasmid. For the traditional 3P system, a rep- plasmid, and a helper plasmid (SEQ ID NO: 4) was used.

[0462] The plasmids used in this study are shown in Table 5.

[0463] Parameters tested:

[0464] Vector titres (vg/ml) for each flask were tested to determine yields. The samples were analyzed by ddPCR and were measured via analytical ultracentrifugation for % full, partial, and empty capsids.

[0465] Result:

[0466] The crude harvest titre (vg/ml) and % full capsids for each flask are shown in Table 5 below.

Table 5:

Flask	Plasmid system	GOI	% full by SEC-MALS	Harvest Titer
1	2P	BAG3	9.3	5.45e11
2	2P	BAG3	19.5	1.32e12
3	S3P	BAG3	24.4	5.61e11
4	3P	BAG3	2.7	1e11
5	S3P	BAG3	14.2	4.82e11

[0467] Vector yield (vg/ml) was higher in the S3P system with the plasmid containing BAG3 compared to 3P systems, and had similar vector yields to 2P systems. The amount of % full capsid amounts in S3P systems with plasmids containing BAG3 were higher compared to 3P and 2P systems.

7.2.6. Conclusions of Example 2:

[0468] The studies performed in this example show that the S3P system increased % full capsid particles and vector yield across multiple capsids, helper, and rep in comparison to traditional 3P and 2P systems.

7.3. Example 3: Optimization of conditions (plasmid ratio) for rAAV production using S3P system in shaken flasks

[0469] Next, the S3P system of Example 2 were tested using different molar ratios (rep: helper: capsid-transgene) and compared to 2P systems in 250 ml flasks. Production of recombinant rAAV was tested in a series of experiments using a S3P system with different molar ratio amounts of helper:rep:capsid-transgene compared to 2P systems, as described in Example 1 and FIG. 1.

[0470] rAAV was produced using the 2P or S3P system following the process (e.g., Transfection, harvesting, and purification) outlined in Example 1.

7.3.1. Experiment 1: Production of rAAV using 2P vs S3P system with varying the molar amount of helper plasmid

[0471] In this study, production of recombinant rAAV was tested using the S3P system in 250 ml volume flasks. The molar amount of a helper plasmid was varied.

[0472] The molar ratio amount of each of the polynucleotide molecules of the S3P system (helper: rep: capsid-transgene) was varied. The transgene used was also varied. The plasmids used in this study included a rep plasmid (SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16), a helper plasmid (SEQ ID NO: 4), and a capsid-transgene plasmid that includes a AAV9 (Capsid-2_MUT1-38181) capsid, a p41 promoter, a non-coding sequence between the 41 promoter and capsid, a transgene flanked by two ITR sequences, and a second non-coding sequence positioned between the ITR and p41 promoter. The plasmids used in this study are shown in Table 6:

Table 6:

Flask	Capsid	Ratio (helper:rep:cap-transgene)	% full by SEC-MALS	Harvest Titer (vg/ml)
1	AAV9 (Capsid-2_MUT1-38181)	1:3:0.2	ND	ND
2	AAV9 (Capsid-2_MUT1-38181)	1.36:3:0.2	ND	$1.33x10^{11}$
3	AAV9 (Capsid-2_MUT1-38181)	0.91:3:0.2	ND	$5.96 x10^{10}$

Flask	Capsid	Ratio (helper:rep:cap-transgene)	% full by SEC-MALS	Harvest Titer (vg/ml)
4	AAV9 (Capsid-2_MUT1-38181)	0.82:3:0.2	49%	9.25×10^{10}
5	AAV9 (Capsid-2_MUT1-38181)	0.5:3:0.2	ND	6.35×10^{10}

[0473] Parameters tested:

[0474] Vector titres (vg/ml) for each flask were tested to determine yield. The samples were analyzed by ddPCR and were measured via analytical ultracentrifugation for % full, partial, and empty capsids.

[0475] Result:

[0476] Results showed that the S3P system had acceptable levels of % full capsids and yield (vgs/ml).

7.3.2. Experiment 2: Production of rAAV using 2P vs S3P system with varying the molar amount of rep plasmid

[0477] In this study, production of recombinant rAAV was tested using the S3P system in 250 ml volume flasks compared to a 2P system. The molar amount of a helper plasmid was varied. The plasmids used in this study included a rep plasmid (SEQ ID NO: 6), a helper plasmid (SEQ ID NO: 4), a capsid-transgene plasmid capsid, a promoter, a non-coding sequence between the promoter and capsid, a transgene (BAG3) flanked by two ITR sequences, and a second non-coding sequence between the p41 promoter and an ITR.

[0478] The 2P system included a rep-helper plasmid (SEQ ID NO: 1), and a capsid-transgene plasmid. The capsid-transgene plasmid included a capsid, and a transgene (BAG3) flanked by two ITR sequences. The molar ratio amount of rep-helper:capsid-transgene is 4:1.

[0479] The plasmid systems used in this study are shown in Table 7 below:

[0480] Parameters tested:

[0481] Vector titres (vg/ml) for each flask were tested to determine yield. The samples were analyzed by ddPCR and were measured via analytical ultracentrifugation for % full, partial, and empty capsids.

[0482] Results

[0483] The crude harvest titre (vg/ml) and % full capsids for each flask are shown in Table 7 below.

Table 7:

Flask	Plasmid system	Molar Ratio H:R:Cap-Transgene	% full by SEC-MALS	Harvest Titer
1	S3P	1 : 1 : 0.1	22.7	2.16e11
2	S3P	2 : 1 : 0.1	19.2	2.08e11
3	S3P	1 : 2 : 0.1	31	8.59e10
4	S3P	3 : 1 : 0.1	17.2	1.41e11
5	S3P	1 : 3 : 0.1	26.7	1.65e11
6	S3P	1 : 1 : 0.2	16.2	2.7e11
7	S3P	2 : 1 : 0.2	12.1	1.71e11
8	S3P	1 : 2 : 0.2	18	2.67e11
9	S3P	3 : 1 : 0.2	12.2	1.82e11
10	S3P	1 : 3 : 0.2	33.1	1.59e11
11	2P	4 : 1	1.5	1.7e11

[0484] As shown in Table 7, the molar ratio of the S3P system showing the highest amount of vector yield was 1:2:0.2 (flask 8). This molar ratio resulted in approximately higher vector yield (2.67×10^{11} vg/ml) than the 2P system (1.7×10^{11}) (flask 11), and higher % full capsids (18%) compared to the 2P system 33.1% (Flask 11).

[0485] Varying the amount of rep was an important metric to balance % full capsids and vector yield.

7.3.3. Experiment 3: Production of rAAV using 2P vs S3P system with varying the molar ratio of helper:rep:capsid-transgene plasmids

[0486] In this study, production of recombinant rAAV was tested using the S3P system in 250 ml volume flasks compared to a 2P system. The molar amount of a helper plasmid was varied. The plasmids used in this study included a rep plasmid (SEQ ID NO: 6), a helper plasmid (SEQ ID NO: 4), and a capsid-transgene plasmid capsid. The capsid-transgene plasmid included a p41 promoter, a non-coding sequence between the promoter and capsid, a transgene (BAG3) flanked by two ITR sequences, and a second non-coding sequence between the p41 promoter and an ITR.

[0487] The 2P system included a rep-helper plasmid (SEQ ID NO: 1), and a capsid-transgene plasmid. The capsid-transgene plasmid included a capsid, and a transgene (BAG3) flanked by two ITR sequences. The molar ratio amount of rep-helper:capsid-transgene is 4:1.

[0488] The plasmid systems used in this study are shown in Table 8.

[0489] Parameters tested:

[0490] Vector titres (vg/ml) for each flask were tested to determine yield. The samples were analyzed by ddPCR and were measured via analytical ultracentrifugation for % full, partial, and empty capsids.

[0491] Results

[0492] The crude harvest titre (vg/ml) and % full capsids for each flask are shown in Table 8 below.

Table 8:

Flask	Plasmid system	Molar Ratio (H:R:Cap-transgene)	% full by SEC-MALS	Harvest Titer
1	S3P	ND	ND	ND
2	S3P	0.5 : 3 : 0.2	39.8	3.96e11
3	S3P	0.5 : 1 : 0.5	13.8	5.76e11
4	S3P	0.5 : 3 : 0.5	22.4	4.56e11
5	S3P	1 : 1 : 0.2	21.2	6.2e11

Flask	Plasmid system	Molar Ratio (H:R:Cap-transgene)	% full by SEC-MALS	Harvest Titer
6	S3P	1 : 3 : 0.2	35.5	6.39e11
7	S3P	1 : 1 : 0.5	15.2	6.16e11
8	S3P	1: 3 : 0.5	24.5	6.56e11
9	S3P	0.5 : 4 : 1	16.5	3.86e11
10	S3P	1 : 4 : 1	18.9	6.11e11
11	S3P	1: 1 :1	8.3	3.99e11
12	S3P	1 : 3 : 0.2	32.5	4.26e11
13	2P	4 : 1	14.7	5.95e11
14	S3P	1 : 3 : 0.2	34.2	5.28e11

[0493] The molar ratio of the S3P system showing the highest amount of vector yield was 1:3:0.5 (6.56 x 1011) (flask 8). This molar ratio resulted in slightly higher vector yield (6.56 x 10^{11}) (flask 8) than the 2P system (5.95 x 10^{11}) (flask 13), and increased % full capsids (24.5%) compared to the 2P system 14.7% (Flask 13). Flask 2 with molar ratio of 0.5:3:0.2 had the highest % full capsids (39.8%) compared to the other S3P molar ratios and the 2P system.

[0494] Varying the amount of rep was an important metric to balance % full capsids and vector yield.

7.3.4. Experiment 4: Production of rAAV using 2P vs S3P system with varying the molar ratio of helper:rep:capsid-transgene plasmids

[0495] In this study, production of recombinant rAAV was tested using the S3P system in 250 ml volume flasks compared to a 2P system. The molar amount of a helper plasmid was varied. The S3P system used in this study included a rep plasmid (SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16) except Flask 14 used a rep plasmid (SEQ ID NO: 6), a helper plasmid (SEQ ID NO: 4), a capsid-transgene plasmid containing a capsid, a p41 promoter, a non-coding sequence between the promoter and capsid, a transgene (BAG3) flanked by two ITR

sequences, and a second non-coding sequence between the p41 promoter and an ITR. Flasks 14-15, in the cap-transgene plasmid, included libraries encoding the capsid instead of BAG3.

[0496] The 2P system included a rep-helper plasmid (SEQ ID NO: 1), and a capsid-transgene plasmid. The capsid-transgene plasmid included a capsid, and a transgene (BAG3) flanked by two ITR sequences. The molar ratio amount of rep-helper:capsid-transgene is 4:1.

[0497] The plasmid systems used in this study are shown in Table 8.

[0498] Parameters tested:

[0499] Vector titres (vg/ml) for each flask were tested to determine yield. The samples were analyzed by ddPCR and were measured via analytical ultracentrifugation for % full, partial, and empty capsids.

[0500] Results

[0501] The crude harvest titre (vg/ml) and % full capsids for each flask are shown in Table 9 below.

Table 9:

Flask	Plasmid system	Capsid	Molar ratio (H:R:Cap-transgene)	% full by SEC-MALS	Harvest Titer
1	2P	capsid /BAG3	4:1 (R-H:Cap-transgene)	7	4.96e10
2	S3P	capsid /BAG3	1 : 1 : 0.1	30.1	2.47e11
3	S3P	capsid /BAG3	1 : 3 : 0.1	48.2	1.28e11
4	S3P	capsid /BAG3	1: 3 : 0.2	29.6	1.84e11
5	S3P	capsid /BAG3	1 : 3 : 0.3	19.1	7.25e10
6	S3P	capsid /BAG3	1 : 4 : 0.2	33.9	2.43e11
7	S3P	capsid /BAG3	1 : 4 : 0.3	28.1	2.02e11
8	S3P	capsid /BAG3	1 : 5 : 0.2	37.9	
9	S3P	capsid /BAG3	1 : 5 : 0.3	26	1.56e11
10	S3P	capsid /BAG3	1 : 3 : 0.2	25.5	2.78e11
11	S3P	capsid /BAG3	1 : 3 : 0.2	28.3	3.45e11

Flask	Plasmid system	Capsid	Molar ratio (H:R:Cap-transgene)	% full by SEC-MALS	Harvest Titer
12	S3P	capsid /BAG3	1 : 3 : 0.2	28	2.56e11
13	S3P	capsid /BAG3	0.5 : 3 : 0.2	24.6	1.57e11
14	S3P	capsid library	1 : 3 : 0.2	79.4	1.69e11
15	S3P	capsid library	1 : 3 : 0.2	70.5	2.87e11

[0502] The molar ratio of the S3P system showing the highest amount of vector yield was 1:3:0.2 (3.45×10^{11}) (flask 11). This molar ratio resulted in higher vector yield 1:3:0.2 (3.45×10^{11}) (flask 11) than the 2P system (4.96 x 1010) (flask 1), and increased % full capsids (28.3%) compared to the 2P system 7% (Flask 1). Flask 3 with molar ratio of 1:3:0.1 (flask 3) had the highest % full capsids (48.2) compared to the other S3P molar ratios and the 2P system (7%) (flask 1).

7.3.5. Conclusions of Experiments 1-4

[0503] As shown in FIG. 3, the S3P system, at specific molar ratios, showed an increase in vector yield (molar ratio 1:3:0.2 of helper:rep:capsid-transgene) and % full capsid particles (molar ratio 1:5:0.2 of helper:rep:capsid-transgene). Varying the amount of rep was an important metric to balance % full capsids and vector yield.

[0504] Similar experiments were performed across various molar ratios among helper/rep/capsid-transgene (Cap GOI) plasmids and ratios providing good AAV yields and full capsid production are highlighted in FIG. 17. FIG. 17 shows the molar ratio of each plasmid out of the total amount of all three plasmids in each side of the triangle. The study shows that the system including a large amount of Rep plasmid is generally preferred.

7.4. Example 4: Helper plasmids

[0505] Production of recombinant rAAV was tested in a series of experiments in 250 ml volume flasks. Helper plasmids of varying sizes, and molar ratios of helper:rep:capsid-transgene using the S3P system were tested.

[0506] rAAV was produced using the S3P system following the process (e.g., Transfection, harvesting, and purification) outlined in Example 1.

[0507] Two different helper plasmid sizes in the S3P system were tested: H-2, which was 13,641 bps (SEQ ID NO: 4), and H-1 was 11,596 bps (SEQ ID NO: 3). The smaller helper plasmid, H-1, was generated by removing about 2000bp of sequences within the larger helper plasmid, H-2, such as fiber and hexon genes.

7.4.1. Experiment 1: Production of rAAV using S3P system with varying the size of helper plasmid, and the molar ratio

[0508] In this study, production of recombinant rAAV was tested using the S3P system. The helper plasmid sizes, and molar ratio amount of helper:rep:capsid-transgene were varied. The capsid-transgene plasmid used in the S3P system included a AAV9 (Capsid-2_MUT1-38181) capsid, a p41 promoter, a non-coding sequence between the p41 promoter and capsid, a transgene (BAG3) flanked by two ITR sequences, and a second non-coding sequence between an ITR and p41 non-coding sequence. The rep plasmid used in the S3P system was the rep plasmid (SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16).

[0509] The plasmid systems used in this study are shown in Table 10:

[0510] Parameters tested:

[0511] Vector titres (vg/ml) for each flask were tested to determine yield. The samples were analyzed by ddPCR and were measured via analytical ultracentrifugation for % full, partial, and empty capsids.

[0512] Results:

[0513] As shown in FIG. 4 and Table 10, S3P systems with a smaller helper plasmid (H-1; SEQ ID NO: 3) resulted in higher vector yield and % full capsids compared to S3P systems with a larger helper plasmid (H-2; SEQ ID NO: 4).

Table 10:

Flask	Plasmid system	Capsid/GOI	Molar Ratio (H:R:C-GOI)	% full by SEC-MALS	Harvest Titer (vg/ml)
1	larger helper (SEQ ID NO: 4)	AAV9 (Capsid-2_MUT1-38181) /BAG3	1:3:0.2	46.5	5.16e11
2	larger helper (SEQ ID NO: 4)	AAV9 (Capsid-2_MUT1-38181) /BAG3	1:4:0.2	35.6	3.68e11

Flask	Plasmid system	Capsid/GOI	Molar Ratio (H:R:C-GOI)	% full by SEC-MALS	Harvest Titer (vg/ml)
3	larger helper(SEQ ID NO: 4)	AAV9 (Capsid-2_MUT1-38181)//BAG3	1:5:0.2	49.8	2.97e11
4	smaller helper (SEQ ID NO: 3)	AAV9 (Capsid-2_MUT1-38181) /BAG3	1:3:0.2	61.3	6.24e11
5	smaller helper (SEQ ID NO: 3)	AAV9 (Capsid-2_MUT1-38181) //BAG3	1:4:0.2	70	4.75e11
6	smaller helper (SEQ ID NO: 3)	AAV9 (Capsid-2_MUT1-38181) /BAG3	1:5:0.2	65.1	3.63e11

7.4.2. Experiment 2: Production of rAAV using S3P system with varying the size of helper plasmid, the molar ratio, and the transgene

[0514] In this study, production of recombinant rAAV was tested using the S3P system described in Examples 1-3. The helper plasmid sizes, promoter of the capsid-transgene plasmid, and molar ratio amount of helper:rep:capsid-transgene were varied. The S3P system included a rep plasmid (SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16) which was fixed.

[0515] The plasmid systems used in this study are shown in Table 11:

[0516] Parameters tested:

[0517] Vector titres (vg/ml) for each flask were tested to determine yields. The samples were analyzed by ddPCR and were measured via analytical ultracentrifugation for % full, partial, and empty capsids.

[0518] Results

[0519] As shown in Table 11, regardless of transgene used in the capsid-transgene, S3P systems with a smaller helper plasmid (H-1; SEQ ID NO: 3) resulted in increased vector yield and % full capsids compared to S3P systems with a larger helper plasmid (H-2; SEQ ID NO: 4). The results showed that increasing the amount of rep in the S3P plasmid system increased % full capsid particles.

Table 11:

Flask	Plasmid system	Capsid/GOI	Molar Ratio (H:R:Cap-transgene)	% full by SEC-MALS	Harvest Titer (vg/ml)
1	larger helper (SEQ ID NO: 3)	capsid /BAG3	1:3:0.2	28	$6.37x10^{11}$
2	larger helper (SEQ ID NO: 4)	capsid /BAG3	1:4:0.2	36.7	$5.13 x10^{11}$
3	larger helper(SEQ ID NO: 4)	capsid /BAG3	1:5:0.2	51.6	$3.64 x10^{11}$
4	smaller helper (SEQ ID NO: 3)	capsid /BAG3	1:3:0.2	42.3 (38.1/3.9/ 58)	$6.6 x10^{11}$
5	smaller helper (SEQ ID NO: 3)	capsid /BAG3	1:4:0.2	74 (35/ 2.9/ 61.5)	$5.02 x10^{11}$
6	smaller helper (SEQ ID NO: 3)	capsid /BAG3	1:5:0.2	34.4	$4.17 x10^{11}$
7	larger helper (SEQ ID NO: 4)	capsid / BAG3	1:3:0.2	72.3 (50/ 0/ 48.5)	$7.32 x10^{11}$
8	smaller helper (SEQ ID NO: 3)	capsid / BAG3	1:3:0.2	53 (37/ 29/ 60.1)	$7.83 x10^{11}$
9	smaller helper (SEQ ID NO: 3)	capsid / BAG3	1:4:0.2	46.8	$6.59 x10^{11}$
10	smaller helper (SEQ ID NO: 3)	capsid / BAG3	1:5:0.2	70.4	$5.34 x10^{11}$

7.4.3. Experiment 3: Production of rAAV using S3P system with varying the molar ratio

[0520] In this study, production of recombinant rAAV was tested using the S3P system to determine optimal ratios for increased rAAV production. The molar ratio amount of helper:rep:capsid-transgene was varied, while the helper plasmid (SEQ ID NO: 3), rep plasmid (SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16), and transgene were fixed. The capsid-transgene plasmid included a capsid, a p41 promoter, a non-coding sequence between the p41 promoter and capsid, a transgene (BAG3 flanked by two ITRs, and a second non-coding sequence between p41 promoter and an ITR.

[0521] The plasmid systems used in this study are shown in Table 12 below:

Table 12:

Flask	Molar Helper (SEQ ID NO: 3)	Molar Rep (SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16)	Molar Cap/transgene	% full by SEC-MALS	Harvest Titer (vg/ml)
1	0.2	3	0.1	26	2.49×10^{11}
2	0.2	3	0.5	17	6.40×10^{11}
3	0.4	3	0.1	84	3.75×10^{11}
4	0.4	3	0.5	*	*
5	0.6	3	0.1	*	*
6	0.6	3	0.5	27	1.04×10^{12}
7	0.8	3	0.1	74	5.60×10^{11}
8	0.8	3	0.5	46	1.11×10^{12}
9	1	3	0.1	*	*
10	1	3	0.5	*	*
11	0.2	5	0.1	*	*
12	0.2	5	0.5	*	*

Flask	Molar Helper (SEQ ID NO: 3)	Molar Rep (SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16)	Molar Cap/transgene	% full by SEC-MALS	Harvest Titer (vg/ml)
13	0.4	5	0.5	*	*
14	0.6	5	0.5	23.2	4.68×10^{11}
15	0.8	5	0.5	25.9	5.53×10^{11}
16	1	5	0.2	68.2	5.22×10^{11}
17	0.8	5	0.1	4.3	3.45×10^{11}
18	1	3	0.2	87.7	8.13×10^{11}

[0522] Parameters tested:

[0523] Vector titres (vg/ml) for each flask were tested to determine yields. The samples were analyzed by ddPCR and were measured via analytical ultracentrifugation for % full, partial, and empty capsids.

[0524] Results

[0525] Flask 18 with molar ratio of 1:3:0.2 (helper:rep:capsid-transgene) resulted in highest % full capsids (87.7%) compared to Flasks 1-17.

[0526] Flask 8 with molar ratio of 0.6:3:0.5 (helper:rep:capsid-transgene) resulted in highest yield (1.04×10^{12} vg/ml) compared to Flasks 1-7 and 9-18.

7.4.4. Experiment 4: Production of rAAV using S3P system with varying the molar ratio

[0527] This study repeats the study performed in Experiment 3 to test for reproducibility. Production of recombinant rAAV was tested using the S3P system to determine optimal ratios for increased rAAV production. The molar ratio amount of helper:rep:capsid-transgene was varied, while the helper plasmid (SEQ ID NO: 3), rep plasmid (SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16), and transgene were fixed. The capsid-transgene plasmid included a capsid, a p41 promoter, a non-coding sequence between the p41 promoter and capsid, a transgene (BAG3) flanked by two ITRs, and a second non-coding sequence between p41promoter and an ITR.

[0528] The plasmid systems used in this study are shown in Table 13 below:

Table 13:

Flask	Molar Helper (SEQ ID NO: 3)	Molar Rep (SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16)	Molar Cap/transgene	% full by SEC-MALS	Harvest Titer (vg/ml)
1	0.2	3	0.1	22.9	2.30×10^{11}
2	0.2	3	0.5	37	4.60×10^{11}
3	0.4	3	0.1	50	2.77×10^{11}
4	0.4	3	0.5	29.5	6.61×10^{11}
5	0.6	3	0.1	13.4	4.20×10^{11}
6	0.6	3	0.5	42.2	9.31×10^{11}
7	0.8	3	0.1	43.5	4.64×10^{11}
8	0.8	3	0.5		9.70×10^{11}
9	1	3	0.1	13.3	6.47×10^{11}
10	1	3	0.5	27.6	1.29×10^{12}
11	0.2	5	0.1		2.60×10^{10}
12	0.2	5	0.5	32.6	1.52×10^{11}
13	0.4	5	0.5	17.3	2.90×10^{11}
14	0.6	5	0.5	48.6	4.13×10^{11}
15	0.8	5	0.5	47.1	5.39×10^{11}
16	1	5	0.2	53	4.01×10^{11}
17	0.8	5	0.1	39.3	2.37×10^{11}
18	1	3	0.2	44.4	<LLOQ can retest if

Flask	Molar Helper (SEQ ID NO: 3)	Molar Rep (SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16)	Molar Cap/transgene	% full by SEC-MALS	Harvest Titer (vg/ml)
					needed. Almost no amplification

[0529] Results:

[0530] Flasks 4, 6, 8, 9, and 15 resulted in yields greater than 5 x 10^{11} vg/ml. Of flasks 4, 6, 8, 9, and 15, Flasks 6 and 15 resulted in % full capsids greater than 40%.

7.4.5. Experiment 5: Production of rAAV using S3P system with varying the size of helper plasmid and the molar ratio

[0531] In this study, production of recombinant rAAV was tested using the S3P system to determine optimal ratios for increased rAAV production using a larger helper plasmid compared to the helper plasmid of Experiments 6 and 7. The molar ratio amount of helper:rep:capsid-transgene was varied, while the helper plasmid (SEQ ID NO: 4), rep plasmid (SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16), and transgene were fixed. capsid-transgene. The capsid-transgene plasmid included a capsid, a p41 promoter, a non-coding sequence between the p41 promoter and capsid, and a transgene (BAG3) flanked by two ITRs.

[0532] The plasmid systems used in this study are shown in Table 14 below.

Table 14:

Flask	Molar Helper (SEQ ID NO: 4)	Molar Rep (SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16)	Molar Cap/transgene	% full by SEC-MALS	Harvest Titer (vg/ml)
1	0.2	3	0.1	1.31 x 10^{11}	38.8
2	0.2	3	0.5	5.10 x 10^{11}	22.2

Flask	Molar Helper (SEQ ID NO: 4)	Molar Rep (SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16)	Molar Cap/transgene	% full by SEC-MALS	Harvest Titer (vg/ml)
3	0.4	3	0.1	2.68×10^{11}	44
4	0.4	3	0.5	8.95×10^{11}	26.3
5	0.6	3	0.1	3.79×10^{11}	42.1
6	0.6	3	0.5	1.22×10^{12}	27.1
7	0.8	3	0.1	4.48×10^{11}	41.3
8	0.8	3	0.5	1.30×10^{12}	28.3
9	1	3	0.1	3.77×10^{11}	45.4
10	1	3	0.5	1.52×10^{12}	26.4
11	0.2	5	0.1	3.14×10^{10}	NA
12	0.2	5	0.5	2.05×10^{11}	28.1
13	0.4	5	0.5	4.36×10^{11}	23
14	0.6	5	0.5	5.91×10^{11}	34
15	0.8	5	0.5	8.32×10^{11}	30
16	1	5	0.2	4.52×10^{11}	40
17	0.8	5	0.1	1.64×10^{11}	53
18	1	3	0.2	7.71×10^{11}	34.6

[0533] Results:

[0534] As shown in FIG. 6, the design and size of the helper plasmid of Experiments 4-5 had a direct impact on the ability to achieve both high percent full capsid particles greater than 40%, while maintaining harvest crude titre over $6x10^{11}$ vg/ml.

[0535] Flasks 2, 4, 6, 8, 10, 14, 15, and 18 resulted in yields greater than 5 x 10^{11} vg/ml. None of flasks 2, 4, 6, 8, 10, 14, 15, and 18, resulted in % full capsids greater than 40%. Therefore, based on Experiments 2-4, smaller sized helper plasmids resulted in higher yield and % full capsids compared to larger sized helper plasmids shown in Experiment 5.

7.4.6. Experiment 6: Production of rAAV using S3P system with varying the transgene and molar ratios

[0536] In this study, production of recombinant rAAV was tested using the S3P system to determine optimal ratios for increased rAAV production. The molar ratio amount of helper:rep:capsid-transgene, the size of the helper plasmid, and the transgene were varied. The capsid-transgene plasmid included a capsid, a promoter, a non-coding sequence between the promoter and capsid, a transgene (BAG3) flanked by two ITR sequences, and a second non-coding sequence between the p41 promoter and an ITR. The rep plasmid (SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16) in the S3P system was fixed.

[0537] The plasmid systems used in this study are shown in Table 15 below.

Table 15:

Flask	Helper SEQ ID NO:	Capsid-transgene plasmid	Molar Ratio Helper/Rep/Capsid-transgene	% full by SEC-MALS	Harvest Titer
1	4	capsid- BAG3	1:3:0.2	32.2	3.89 x 10^{11}
2	4	capsid- BAG3	1:3:0.2	33.4	5.71 x 10^{11}
3	3	capsid- BAG3	1:3:0.2	53.9	4.56 x 10^{11}
4	3	capsid- BAG3	0.4:3:0.1	65.9	9.31 x 10^{10}
5	3	capsid- BAG3	1:4:0.2	53.1	3.18 x 10^{11}
6	3	capsid- BAG3	1:5:0.2	52.1	2.59 x 10^{11}
7	3	capsid- BAG3	1:3:0.2	53.6	5.76 x 10^{11}
8	3	capsid- BAG3	0.4:3:0.1	58.4	1.34 x 10^{11}
9	3	capsid- BAG3	1:4:0.2	54.4	4.66 x 10^{11}
10	3	capsid- BAG3	1:5:0.2	52.1	2.59 x 10^{11}

[0538] Result:

[0539] Optimization of plasmid ratios in the S3P system resulted in 3 plasmid ratios (Flasks 3, 7, and 9) with crude harvest titre over 5 x 10^{11} vg/ml and % full capsid particles over 50%.

[0540] Conclusions of Experiments 1-6

[0541] As shown in FIG. 4, the S3P system with smaller sized helper plasmids (SEQ ID NO: 3) at particular ratios (1:3:0.2; 0.4:3:0.1) resulted in higher yield and % full capsids compared to larger sized helper plasmids (SEQ ID NO: 4).

7.4.7. Experiment 7: Production of rAAV using S3P system with varying the transgene and molar ratios compared to traditional 3P system

[0542] In this study, production of recombinant rAAV was tested using the S3P system to determine optimal ratios for increased rAAV production, and compared to the traditional 3P system.

[0543] The S3P system included a rep plasmid (SEQ ID NO: 6), a helper plasmid (SEQ ID NO: 3), and a capsid-transgene. The molar ratio amount of helper:rep:capsid-transgene was varied. The capsid-transgene plasmid included a capsid, a promoter, a non-coding sequence between the promoter and capsid, a transgene (BAG3) flanked by two ITR sequences, and a second non-coding sequence between the p41 promoter and an ITR.

[0544] The traditional 3P system included a rep-cap plasmid, a helper plasmid, and a capsid-transgene plasmid including a capsid and a transgene flanked by two ITR sequences.

[0545] The plasmid systems used in this study are shown in Table 16 below.

Table 16:

Flask	**Plasmid System**	Helper SEQ ID NO:	Capsid-transgene plasmid	Molar Ratio Helper/Rep/ Capsid-transgene	Harvest Titer
1	3P	4	capsid- BAG3	1:1:1	6.52E+10

Flask	**Plasmid System**	Helper SEQ ID NO:	Capsid-transgene plasmid	Molar Ratio Helper/Rep/ Capsid-transgene	Harvest Titer
2	S3P	3	capsid- BAG3	1:3:0.2	4.62E+11
3	3P	4	capsid- BAG3	1:1:1	2.80E+11
4	S3P	3	capsid- BAG3	1:3:0.2	ND

[0546] Results: The studies performed in this example show that the S3P system increased vector yield (Flask 2: $4.62x10^{11}$ vg/ml) at a molar ratio of 1:3:0.2 (Rep:helper:cap-transgene) in comparison to traditional 3P system.

7.5. Example 5: rAAV production of 3P, 2P and S3P systems tested in 3L bioreactors

[0547] Based on the results of small scale experiments performed in 250 shaken flasks, rAAV production using 3P, 2P and S3P systems was tested in 3L bioreactors.

7.5.1. Experiment 1: Impact of plasmid ratio on S3P system at 3L bioreactor scale:

[0548] In this study, production of recombinant rAAV was tested in 3L bioreactors (BRs) using the S3P system to determine optimal ratios for increased rAAV production. The molar ratio amount of helper:rep:capsid-transgene, was varied, while the transgene, helper (SEQ ID NO: 4), and rep (SEQ ID NO: 6) were fixed. The capsid-transgene plasmid included a capsid, a p41 promoter, a non-coding sequence between the p41 promoter and capsid, a transgene (BAG3 (1725 bps)) flanked by two ITR sequences, and a second non-coding sequence between the p41 promoter and an ITR.

[0549] The plasmid systems used in this study are shown in Table 17.

Table 17:

BR	Capsid-transgene plasmid	Molar ratio Helper/Rep /Capsid-transgene	hcDNA (pg/1e13vg)	HCP (ng/mml)	Harvest crude Titer	AUC full %	% full by SEC-MALs
1	capsid-BAG3	1:1:0.1	ND	<LLOQ	ND	31.6	29.2
2	capsid-BAG3	1:1:0.1	270.5	<LLOQ	5.16×10^{11}	40.3	30.4
3	capsid-BAG3	1:3:0.2	ND	<LLOQ	ND	47.3	42.1
4	capsid-BAG3 (1725 bps)	1:3:0.2	116.9	<LLOQ	8.18×10^{11}	50.1	43.7

[0550] Results:

[0551] Similar to the experiments performed in 250 ml shaken flasks, the results of the 3L Bioreactor rAAV production shows that plasmid ratios impact vector quality and yield. As shown in FIG. 5, the S3P system with an increased molar amount of the rep plasmid (a molar ratio of 1:3:0.2 (helper:rep:capsid-transgene) showed increased % full capsid particles (42.1% and 43.7% of Flasks 3 and 4, respectively) compared to S3P system with a molar ratio of 1:1:0.1 (helper:rep:capsid-transgene).

[0552] The S3P system with the molar ratio of 1:1:1 (helper:rep:capsid-transgene) resulted in increased vector yield (8.18×10^{11} vg/ml) compared to S3P system with less molar amount of the rep plasmid (5.16×10^{11}) vg/ml (a molar ratio of 1:1:0.1 (helper:rep:capsid-transgene)).

7.5.2. Experiment 2: Production of rAAV using S3P system compared to traditional 3P and 2P systems at Bioreactor Scale

[0553] In this study, production of recombinant rAAV was tested in 3L bioreactors using the 3P, 2P, and S3P system to determine optimal ratios for increased rAAV production.

[0554] For bioreactors with the S3P system, the molar ratio amount of helper:rep:capsid-transgene was varied, while the transgene (BAG3 (1725 bps)), helper plasmid (SEQ ID NO: 4), and rep plasmid (SEQ ID NO: 6) were fixed. The capsid-transgene plasmid included a capsid, a p41 promoter, a non-coding sequence between the p41 promoter and capsid, a transgene (BAG3 (1725 bps)) flanked by two ITR sequences, and a second non-coding sequence between the p41 promoter and an ITR.

[0555] The plasmid systems used in this study are shown in Table 18.

Table 18:

				% Full capsids		
BR Name	Plasmid Name	Molar Ratio H:R:Cap-transgene	Harvest (vg/ml)	SEC-MALS	AUC	Refeyn
1	S3P	1 : 3 : 0.2	9.18E+11	47.7	48.5	49.6
2	S3P	1 : 3 : 0.2	2.64E+11	28.3	35.4	ND
3	3P	1 : 1.92 : 0.47 (rep-cap: Helper: transgene)	1.61E+11	9	15.3	11.5
4	2P	4 : 1	4.94E+11	19.3	25.4	25.1

[0556] Results: As shown in FIG. 7 and Table 18, similar to the experiments performed in 250 ml shaken flasks, the results of the 3L Bioreactor rAAV production shows that the S3P system outperforms 2P and traditional 3P systems in terms of % full capsid particles (48.5% and 35.4% of BR 1 and 2, respectively). Total crude titre yield was highest in S3P of BR 1 (9.18x 1011).

7.5.3. Experiment 3: Production of rAAV using S3P system compared to 2P systems at Bioreactor Scale:

[0557] In this study, production of recombinant rAAV was tested in 3L bioreactors using the S3P system.

[0558] For bioreactors with the S3P system, the molar ratio amount of helper:rep:capsid-transgene was 1:3:0.2 (helper:rep:capsid-transgene). The S3P system included a rep plasmid (SEQ ID NO: 6), a helper plasmid (SEQ ID NO: 4; H-2), and a capsid-transgene plasmid. The capsid-transgene plasmid included a capsid, a p41 promoter, a non-coding sequence between the p41 promoter and capsid, a transgene (BAG3) flanked by two ITR sequences, and a second non-coding sequence between the p41 promoter and an ITR.

[0559] The plasmid systems used in this study are shown in Table 19:

Table 19:

			Molar Ratio H:R:Cap-transgene		% Full capsids	
Capsid	Plasmid system	Media		Harvest (vg/ml)	SEC-MALS	AUC
AAV9 (Capsid-2_MUT1-38181	S3P	F17 media	1 : 3 : 0.2	2.83E+11	50.2	60.1
AAV9	S3P	F17 media		2.62E+11	38.8	52.1
X1	S3P	Affinia media		6.75E+11	45.6	63.8
AAV9	S3P	Affinia media		6.35E+11	47.2	49.2

[0560] Results: Similar to the experiments performed in 250 ml shaken flasks of Examples 1-3, the results of the 3L Bioreactor rAAV production shows that the S3P system shows vector yield and % full capsid particles similar to Examples 1-3 in 250 ml shaken flasks, and thus is able to scale up.

7.5.4. Experiment 5: Production of rAAV using S3P system at 3L Bioreactor Scale (different capsid)

[0561] In this study, production of recombinant rAAV was tested in 3L bioreactors using the S3P system.

[0562] For bioreactors with the S3P system, the molar ratio amount of helper:rep:capsid-transgene was 1:3:0.2 (helper:rep:capsid-transgene). The S3P system included a rep plasmid (SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16), a helper plasmid (SEQ ID NO: 3; H-1), and a capsid-transgene plasmid that is different from Experiment 4. The capsid-transgene plasmid included a capsid, a p41 promoter, a non-coding sequence between the p41 promoter and capsid, a transgene (UBC-ARSA) flanked by two ITR sequences, and a second non-coding sequence between the p41 promoter and an ITR.

[0563] The plasmid systems used in this study are shown in Table 20:

Table 20:

BR (3L)	Plasmid System	Harvest (vg/ml)	% full SEC-MALS
1	S3P	8.29E+11	35.9
2	S3P	9.4E+11	30.1

[0564] Results: Similar to the experiments performed in 250 ml shaken flasks of Examples 1-3, the results of the 3L Bioreactor rAAV production shows that the S3P system shows vector yield and % full capsid particles similar to Examples 1-3 in 250 ml shaken flasks, and thus is able to scale up.

7.5.5. Experiment 6: Production of rAAV using S3P system at 3L Bioreactor Scale (different capsid)

[0565] In this study, production of recombinant rAAV was tested in 3L bioreactors using the S3P system.

[0566] For bioreactors with the S3P system, the molar ratio amount of helper:rep:capsid-transgene was 1:3:0.2 (helper:rep:capsid-transgene). The S3P system included a rep plasmid (SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16), a helper plasmid (SEQ ID NO: 3; H-1), and a capsid-transgene plasmid that is different from Experiment 5. The capsid-transgene plasmid included a capsid, a p41 promoter, a non-coding sequence between the p41 promoter and capsid, a transgene (UBC-ARSA) flanked by two ITR sequences, and a second non-coding sequence between the p41 promoter and an ITR.

[0567] The plasmid systems used in this study are shown in Table 21:

Table 21:

BR (3L)	Plasmid System	Molar Ratio (H:R:Cap-transgene)	Harvest (vg/ml)	% full
1	S3P	1:3:0.2	8.65E+11	46.6%
2	S3P	1:3:0.2	5.14E+11	
3	S3P	1:3:0.2	8.4E+11	46.6%
4	S3P	1:3:0.2	1.08E+12	

[0568] Results: Similar to the experiments performed in 250 ml shaken flasks of Examples 1-3, the results of the 3L Bioreactor rAAV production shows that the S3P system shows vector

yield and % full capsid particles similar to Examples 1-3 in 250 ml shaken flasks, and thus is able to scale up.

7.5.6. Experiment 6: Production of rAAV using S3P system at 50L Bioreactor Scale:

[0569] In this study, production of recombinant rAAV was tested in 50L bioreactors using the S3P system to determine scalability of the S3P system.

[0570] For 50L bioreactors with the S3P system, the molar ratio amount of helper:rep:capsid-transgene was 1:3:0.2. The S3P system included a rep plasmid, a helper plasmid, and a capsid-transgene plasmid which included a capsid, a promoter, a non-coding sequence between the promoter and capsid, and a transgene (BAG3) flanked by two ITR sequences.

[0571] Results: S3P system tested in 50L bioreactors resulted in 53.5% full capsid particles and a crude titre of $8.8x10^{11}$ vg/ml, similar to results of the S3P system tested in 250 ml shaken flasks and 3L bioreactors of Examples 1-4.

[0572] As shown in FIG. 8, the S3P system tested in 50 L BRs were compared to 3L BR data. The S3P system tested in 50L bioreactors maintained crude harvest yield (8.8×10^{11} vg/ml) and % full capsid particles (53.5%) across all scales (250 ml shaken flask, 3L Bioreactor, and 50L bioreactors).

7.5.7. Conclusion of Example 5

[0573] The S3P system scales tested in 3L and 50L bioreactors result in similar % full capsids and crude harvest yield as that of the 250 ml shaken flasks of Examples 1-3.

7.6. Example 6: Production of rAAV using S3P system with different helper plasmids at varying molar ratios

[0574] In this study, production of recombinant rAAV was tested in flasks using the S3P system described in Examples 1-3 and as shown in FIG. 9. The type of helper plasmid and molar ratio amount of helper:rep:capsid-transgene were varied. The S3P system included a rep plasmid (SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16), a H-1 helper plasmid or a H-3 (FIG. 9), and a AAV9 capsid-transgene plasmid as illustrated in FIG. 9 and was tested at varying ratios.

[0575] The plasmid systems used in this study are shown in Table 22:

Table 22:

Flask	Ratio (H:R:Cap-GOI)	Helper	Titer	% Full	% Partial	% Empty
Flask 2	0.16: 0.03: 0.83	S3P H-1	1.40E+11	4.7	0	95.3
Flask 3	0.3:0.2: 0.5	S3P H-1	1.71E+12	26.4	N/A	73.5
Flask 4	0.6:0.3: 0.1	S3P H-1	1.94E+12	39.8	1.4	58.8
Flask 6	0.1:0.2: 0.7	S3P H-1	8.93E+11	17	0.6	82.4
Flask 7	0.1:0.6: 0.3	S3P H-1	1.62E+12	45.5	N/A	54.5
Flask 8	0.5:0.1: 0.4	S3P H-1	1.15E+12	15.6	0	84.4
Flask 9	0.3:0.5: 0.2	S3P H-1	2.60E+12	46.7	N/A	53.3
Flask 10	0.2:0.4:0.4	S3P H-1	2.51E+12	42.2	0.7	57.1
Flask 12	0.24:0.05: 0.71	S3P H-3	3.17E+11	6.5	0	93.5
Flask 13	0.16:0.03:0.83	S3P H-3	2.15E+11	5.1	0	94.9
Flask 14	0.3:0.2:0.5	S3P H-3	1.30E+12	54.9	1.3	43.8
Flask 15	0.6:0.3:0.1	S3P H-3	1.54E+12	59	3.3	37.7
Flask 16	0.7:0.1: 0.2	S3P H-3	1.12E+12	25.1	0.7	74.2
Flask 17	0.1:0.2: 0.7	S3P H-3	7.50E+11	20.5	2.1	77.4
Flask 18	0.1:0.6:0.3	S3P H-3	8.59E+11	43.5	0.9	55.6
Flask 19	0.5:0.1:0.4	S3P H-3	8.28E+11	13.9	0	86.1
Flask 20	0.3:0.5:0.2	S3P H-3	1.38E+12	58.8	0	41.2
Flask 21	0.2:0.4: 0.4	S3P H-3	1.36E+12	45.4	0	54.6
Flask 22	0.4:0.3: 0.3	S3P H-3	1.52E+12	46	0	54
Flask 45	1:1:1	3P H-1	6.19E+11	38.5	3.1	58.4
Flask 46	1:1:1	3P H-3	4.44E+11	63	0	37

[0576] Parameters tested:

[0577] Vector titres (vg/ml) for each flask were tested to determine yields. The samples were analyzed by ddPCR and were measured via analytical ultracentrifugation for % full, partial, and empty capsids.

[0578] Results:

[0579] The S3P system with the molar ratio of 0.3:0.5: 0.2 (helper:rep:capsid-transgene) with helper H-1 and the molar ratio 0.6:0.3:0.1 (helper: rep: cap-transgene) with the H-3 helper plasmid (FIG. 9) resulted in increased vector yield (2.60×10^{12} and 1.54×10^{12} vg/ml, respectively) compared to S3P system ((6.19E+11 and 4.44E+11 vg/ml, respectively) (helper:rep:capsid-transgene)). There was also an increase in % full capsid particles in the S3P system with the molar ratio of 0.3:0.5: 0.2 (helper:rep:capsid-transgene) with helper H-1

(46.7%) and the molar ratio 0.6:0.3:0.1 (helper: rep: cap-transgene) with the H-3 helper (59%) compared to S3P system (38.5% when using the H-1 helper).

7.7. Example 7: Production of rAAV using a 4-plasmid system with split rep

[0580] In this study, production of recombinant rAAV was tested in flasks using a 4-plasmid system. The rep proteins were split onto two separate plasmids (rep1 and rep2 of Table 23 below). The 4-plasmid system included 2 different rep plasmids, an H-1 helper plasmid, and an AAV9 capsid-transgene plasmid, as exemplified in FIG. 10. Results were compared to the S3P system that included 3 plasmids.

[0581] The plasmid systems used in this study are shown in Table 23:

Table 23:

Flask #	Description	Plasmid Ratio H: Rep 1 + Rep 2:Cap-Car	Titer (vg/mL)	% Full	Helper	Rep1	Rep2	CapCar
1	AAV2/2 Ctrl	0.125-0.75-0.125	2.60e11	43.7	H-1	(FIG. 16; AAV2 Rep S-1)AAV2: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter	N/A	AAV9
10	AAV2/2 R1 - High Small	0.125-0.75-0.125	3.71e11	30.7	H-1	AAV2: Rep 78 and Rep 68 + p5 promoter	AAV2: Rep 52 and Rep40 + AAV2 p19 promoter	AAV9
11	AAV2/2 R1 - 50/50	0.125-0.75-0.125	2.37e11	37.6	H-1	AAV2: Rep 78 and Rep 68 + P5 promoter	AAV2: Rep 52 and Rep40 + AAV2 p19 promoter	AAV9
12	AAV2/2 R1 - High Large	0.125-0.75-0.125	3.16e11	30	H-1	AAV2: Rep 78 and Rep 68 + P5 promoter	AAV2: Rep 52 and Rep40 + AAV2 p19 promoter	AAV9
6	No exon large	0.2-0.6-0.2	3.64e11	24.4	H-1	AAV2: Rep 78 and Rep 68 + p5 promoterwithout intron/exon native to AAV2 rep	AAV2: Rep 52 and Rep40 + AAV2 p19 promoter	AAV9
2	AAV2/2 Ctrl	0.2-0.6-0.2	6.57e11	38.5	H-1	(FIG. 16; AAV2 Rep S-1) AAV2: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter	N/A	AAV9

13	AAV2/2 R2 - High Small	0.2-0.6-0.2	2.94e11	36.5	H-1	AAV2: Rep 78 and Rep 68 + P5 promoter	AAV2: Rep 52 and Rep40 + AAV2 p19 promoter	AAV9
14	AAV2/2 R2 - 50/50	0.2-0.6-0.2	3.30e11	30.2	H-1	AAV2: Rep 78 and Rep 68 + P5 promoter	AAV2: Rep 52 and Rep40 + AAV2 p19 promoter	AAV9
15	AAV2/2 R2 - High Large	0.2-0.6-0.2	3.29e11	26.6	H-1	AAV2: Rep 78 and Rep 68 + P5 promoter	AAV2: Rep 52 and Rep40 + AAV2 p19 promoter	AAV9
3	AAV2/2 Ctrl	0.3-0.4-0.3	9.39e11	26.7	H-1	(FIG. 16; AAV2 Rep S-1) AAV2: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter	N/A	AAV9
16	AAV2/2 R3 - High Small	0.3-0.4-0.3	4.83e11	24	H-1	AAV2: Rep 78 and Rep 68 + P5 promoter	AAV2: Rep 52 and Rep40 + AAV2 p19 promoter	AAV9
17	AAV2/2 R3 - 50/50	0.3-0.4-0.3	3.22e11	21.7	H-1	AAV2: Rep 78 and Rep 68 + P5 promoter	AAV2: Rep 52 and Rep40 + AAV2 p19 promoter	AAV9
18	AAV2/2 R3 - High Large	0.3-0.4-0.3	2.32e11	13.8	H-1	AAV2: Rep 78 and Rep 68 + P5 promoter	AAV2: Rep 52 and Rep40 + AAV2 p19 promoter	AAV9
4	AAV1/2 Ctrl	0.2-0.6-0.2	7.78e11	37	H-1	AAV1: 50% of Rep78 and 50% Rep 68 + AAV2: 50% of Rep78 and 50% Rep 68 + AAV1 p19 promoter; AAV2: Rep 52 and Rep 40 + p5 promoter	N/A	AAV9
19	AAV1/2 R2 - High Small	0.2-0.6-0.2	2.27e11	25.3	H-1	AAV1: Rep 78 and Rep 68 + p5 promoter	AAV2: Rep 52 and Rep40 + AAV2 p19 promoter	AAV9
20	AAV1/2 R2 - 50/50	0.2-0.6-0.2	2.36e11	35.8	H-1	AAV1: Rep 78 and Rep 68 + p5 promoter	AAV2: Rep 52 and Rep40 + AAV2 p19 promoter	AAV9
21	AAV1/2 R2 - High Large	0.2-0.6-0.2	2.96e11	23.7	H-1	AAV1: Rep 78 and Rep 68 + p5 promoter	AAV2: Rep 52 and Rep40 + AAV2 p19 promoter	AAV9

5	AAV9/2 Ctrl	0.2-0.6-0.2	5.90e11	35.5	H-1	AAV9: 50% of Rep78 and 50% Rep 68 + AAV2: 50% of Rep78 and 50% Rep 68 + AAV9 p19 promoter; AAV2: Rep 52 and Rep 40 + p5 promoter	N/A	AAV9
22	AAV9/2 R2 - High Small	0.2-0.6-0.2	2.11e11	23.7	H-1	AAV9: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter	AAV2: Rep 52 and Rep40 + AAV2 p19 promoter	AAV9
23	AAV9/2 R2 - 50/50	0.2-0.6-0.2	2.16e11	28	H-1	AAV9: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter	AAV2: Rep 52 and Rep40 + AAV2 p19 promoter	AAV9
24	AAV9/2 R2 - High Large	0.2-0.6-0.2	1.75e11	16	H-1	AAV9: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter	AAV2: Rep 52 and Rep40 + AAV2 p19 promoter	AAV9

[0582] Parameters tested:

[0583] Vector titres (vg/ml) for each flask were tested to determine yields. The samples were analyzed by ddPCR and were measured via analytical ultracentrifugation for % full, partial, and empty capsids.

[0584] Results:

[0585] The S3P system consistently resulted in higher vector yield (vg/ml) compared to the 4-plasmid system. However, splitting the rep proteins into 2 different plasmids in the 4-plasmid system provided increased packaging efficiency (% full capsid particles) with more small rep (Rep 52 and Rep 40) at lower amounts of total rep proteins.

7.8. Example 8: Production of rAAV using S3P system with split rep on the cap-transgene (e.g., cap-car)

[0586] In this study, production of recombinant rAAV was tested in flasks using a S3P system, to understand the effects of moving one portion of the rep proteins to the capsid-transgene plasmid in order to optimize the rep protein ratios without requiring a fourth plasmid or to increase rep protein expression for a single rep protein. The S3P systems tested included rep variations on rep plasmid or on the rep plasmid and the cap-transgene construct; and a helper plasmid (H-1), as exemplified in FIG. 11.

[0587] Parameters tested:

[0588] Vector titres (vg/ml) for each flask were tested to determine yields. The samples were analyzed by ddPCR and were measured via analytical ultracentrifugation for % full, partial, and empty capsids.

[0589] The plasmid systems used in this study are shown in Table 24:

Table 24:

Flask Info			Results		Ratio		
Flask #	**pRep**	**CapCar Rep**	**Titer (vg/mL)**	**% Full**	**Helper**	**Rep**	**CapCar**
2	AAV2 Full: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter (FIG. 16; AAV2 Rep S-1)	AAV9-transgene	2.43E+12	48.8	2	7.5	0.5
1 SP3 CTRL			2.56E+12	35.7	2	6	2
3 SP3 CTRL			2.44E+12	28.4	2	4.5	3.5
5	AAV2: Rep 78 and Rep 68 and Rep 52 and Rep 40 + p5 promoter (FIG. 16; AAV2 Rep S-1)	AAV2 Small: Rep 52 and Rep 40; AAV9-transgene	7.5E+11	51.7	2	7.5	0.5
4			1.81E+12	61.9	2	6	2
6			2.02E+12	52.4	2	4.5	3.5
8	AAV2 Large: Rep 78 and Rep 68+ p5 promoter		7.98E+11	85.5	2	7.5	0.5
7			1.5E+12	72.1	2	6	2
9			1.55E+12	66.2	2	4.5	3.5
11	AAV2: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter (FIG. 16; AAV2 Rep S-1)	AAV2 Large: Rep 78 and Rep 68; AAV9-transgene	3.35E+11	87.2	2	7.5	0.5
10			5.61E+11	69.3	2	6	2
12			5.86E+11	49.8	2	4.5	3.5
14	AAV2: Rep 52 and Rep 40 + p19 promoter		4.27E+11	86.3	2	7.5	0.5
13			8.64E+11	73.6	2	6	2
15			8.29E+11	48.8	2	4.5	3.5
17	AAV2: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter	AAV9 Large: Rep 78 and Rep 68;	1.26E+11	71.1	2	7.5	0.5
16			3.17E+11	36.8	2	6	2
18			2.67E+11	21	2	4.5	3.5

	(FIG. 16; AAV2 Rep S-1)	AAV9-transgene					
20	AAV2 small: Rep 52 and Rep 40 + p19 promoter		1.61E+11	78.6	2	7.5	0.5
19			4.11E+11	43.4	2	6	2
21			4.29E+11	25.9	2	4.5	3.5
22	AAV2 Rep 78 and Rep 68+ p5 promoter	AAV9-transgene	9.31E+11	12.5	2	6	2
23	AAV2 Rep 52 and Rep 40 + p19 promoter		[LLOQ]	1.6	2	6	2

[0590] Results: The S3P system with large rep proteins (Rep 78 and Rep 68) separated from small rep proteins (Rep 52 and Rep 40) onto different plasmids resulted in greater packaging efficiency (see e.g., Flasks 7-9 with 72.1%, 85.5%, a 66.2% full capsid particles). The greater titer (vg/ml) is shown in flask 6 (2.02x 10^{12} vg/ml). The packaging from Flask 6 is 1.8x the tier of Flask 3. The titer (vg/ml) is also 0.8 x the titer (vg/ml) of S3P control system having AAV2 Rep 78, Rep 68, Rep 52, and Rep 40 on a single rep plasmid (Flask 3).

7.9. Example 9: Production of rAAV using S3P system with varied wild-type Rep proteins

[0591] In this study, production of recombinant rAAV was tested in flasks using a S3P system, to understand the effects on different AAV rep serotypes on vector yield and % full capsid proteins. The S3P systems tested included rep proteins from different wild-type AAV serotypes the rep plasmid, a cap-transgene construct as shown in FIG. 9; and a helper plasmid (H-1) as shown in FIG. 9.

[0592] Additionally, the present inventors previously found that adding UL12 to the rep plasmid improved both yield (vg/ml) and packaging (% full) using a 1:3:0.2 (H-R-Cap-GOI) ratio. Thus, the S3P systems in this study also tested AAV2 rep plasmids that have added helper virus genes such as UL12 or NS2.

[0593] Parameters tested:

[0594] Vector titres (vg/ml) for each flask were tested to determine yields. The samples were analyzed by ddPCR and were measured via analytical ultracentrifugation for % full, partial, and empty capsids.

[0595] The plasmid systems used in this study are shown in Table 25 below:

Table 25:

Flask Information		Plasmid Descriptions					Plasmid Ratios		
Category	**Flask #**	**Helper**	**Rep**	**CapCar**	**Titer**	**% Full**	**Helper**	**Rep**	**CapCar**
WT Reps Rep 78, Rep 68, Rep 52, Rep 40	1	H-1 helper	AAV1	AAV-transgene as shown in FIG. 9	1.0E+12	6.2	2	6	2
	2		AAV2		1.4E+12	10.5	2	6	2
	3				ND	ND	ND	ND	ND
	4		AAV4		1.5E+12	9.7	2	6	2
	10		AAV10		1.6E+12	11.6	2	6	2
	11		AAV11		1.8E+12	9.8	2	6	2
	12		AAV12		9.5E+11	5.8	2	6	2
P7 AAV5 Reps	14		P7 AAV5		low	20	2	6	2
Rep w/ helper virus genes UL12 and/or NS2	15		AAV2		7.4E+11	13.6	1	3	0.2
	16		AAV2 + NS2		1.0E+12	11.2	2	6	2
	17		AAV2 + NS2		5.3E+11	18.8	1	3	0.2
	18		AAV2 + UL12		1.5E+12	17.1	2	6	2
	19		AAV2 + UL12		1.1E+12	20.3	1	3	0.2
	20		AAV2 + NS2+UL12		8.3E+11	10.4	2	6	2
	21		AAV2 + NS2+UL12		5.4E+11	12.6	1	3	0.2
Ctrl	22		Rep Classic (FIG. 2)		8.7E+11	12.6	2	6	2
Rep-Cap-Car Repeats	23		AAV2 Rep + UL12 (FV)	Large Rep- (Rep 78 and Rep 68 as shown in FIG. 11; AAV9-transgene	1.4E+11	42.8	1	3	0.2
	24		AAV2 Rep (FV) AAV2: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter (FIG. 16; AAV2 Rep S-1)		2.3E+11	50.1	1	3	0.2
	25		AAV2 Rep + UL12 (Mirus)		1.2E+11	45.6	1	3	0.2
	26		AAV2 Rep (Mirus) AAV2: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter (FIG. 16; AAV2 Rep S-1)		1.5E+11	41	1	3	0.2

[0596] Results:

[0597] As shown in Table 25, the results showed that:

[0598] rep proteins from AAV serotypes AAV2, AAV4, AAV10, and AAV11 had high vector titer (vg/ml) and % full capsid particles;

[0599] Rep proteins with added helper virus gene UL12 provides increased % full capsid particles and thus provides benefits to packaging; and

[0600] Capsid-transgene plasmids with rep proteins, at specific ratios, resulted in high (e.g., 5x higher) % full capsid particles.

7.10. Example 10: Production of rAAV using S3P system with varied capsid, promoter, and Rep AAV serotype

[0601] The present inventors previously showed that packaging efficiency was improved when the S3P system included an AAV5 capsid with a p40 promoter on the capsid-transgene plasmid (data not shown). The present inventors previously showed that AAV1 rep protein showed good performance with AAV9-based capsids.

[0602] In this study, a S3P system was tested varying the capsid in the capsid-transgene plasmid and the promoter (p40 or p41), and varying the rep protein to rep proteins of different AAV serotypes (AAV2, AAV5). The aim of this study was to evaluate the potential for using the standard S3P construct (FIG. 2) with WT rep constructs in a S3P system.

[0603] Different transfection reagents were also used during transfection (Mirus, PEIPro, and fectovir). The plasmid systems used in this study are provided in Table 26 below, with the helper plasmid H-1 being fixed.

Table 26:

Flask #	Cap-GOI Promoter	Cap-GOI	Ratio	Rep	Helper	TFX Reagent	Titer	% Full
1	P41	AAV5-transgene (FIG. 2)	R2 (0.31-0.59-0.1)	(SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16): AAV2 Rep 78, Rep 68, Rep 52, Rep 40	H-1	FV	7.7E+11	20.2
2	P41	AAV5-transgene (FIG. 2)	R2 (0.31-0.59-0.1)	(SEQ ID NO: 7; AAV2 Rep S-2 of FIG.	H-1	FV	6.2E+11	15.5

				16) : AAV2 Rep 78, Rep 68, Rep 52, Rep 40				
3	P41	AAV5-transgene (FIG. 2)	R2 (0.31-0.59-0.1)	AAV1: Rep 78 and Rep 68 + p5 promoter	H-1	FV	2.9E+11	12.7
4	P41	AAV5-transgene (FIG. 2)	R2 (0.31-0.59-0.1)	AAV2: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter (FIG. 16; AAV2 Rep S-1)	H-1	FV	4.7E+11	11.9
5	P41	AAV5-transgene (FIG. 2)	R2 (0.31-0.59-0.1)	AAV5: Rep 22+p5 promoter	H-1	FV	LLOQ	2.2
6	P41	AAV5-transgene (FIG. 2)	R2 (0.31-0.59-0.1)	P7-AAV5: Rep 22+p7 promoter	H-1	FV	LLOQ	10.9
7	P41	AAV5-transgene (FIG. 2)	R2 (0.31-0.59-0.1)	(SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16) : AAV2 Rep 78, Rep 68, Rep 52, Rep 40	H-1	PEI	5.8E+11	30.5
8	P41	AAV5-transgene (FIG. 2)	R2 (0.31-0.59-0.1)	(SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16) : AAV2 Rep 78, Rep 68, Rep 52, Rep 40	H-1	Mirus	5.3E+11	31.1

9	P40	AAV5-transgene (FIG. 12)	R2 (0.31-0.59-0.1)	AAV1 Large rep: Rep 78 and Rep 68 + p5 promoter	H-1	FV	4.9E+11	19.7
10	P40	AAV5-transgene (FIG. 12)	R2 (0.31-0.59-0.1)	AAV2: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter (FIG. 16; AAV2 Rep S-1)	H-1	FV	9.4E+11	27.4
11	P40	AAV5-transgene (FIG. 12)	R2 (0.31-0.59-0.1)	AAV5: Rep 22 "Rep40-like protein" + p5	H-1	FV	LLOQ	17.4
12	P40	AAV5-transgene (FIG. 12)	R2 (0.31-0.59-0.1)	P7-AAV5: Rep 22+p7 promoter	H-1	FV	LLOQ	22.1
13	P40	AAV5-transgene (FIG. 12)	R2 (0.31-0.59-0.1)	(SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16) : AAV2 Rep 78, Rep 68, Rep 52, Rep 40	H-1	PEI	4.8E+11	52.2
14	P40	AAV5-transgene (FIG. 12)	R2 (0.31-0.59-0.1)	(SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16) : AAV2 Rep 78, Rep 68, Rep 52, Rep 40	H-1	Mirus	4.6E+11	49
15	P40	AAV5-transgene	R1 (0.24-0.71-0.05)	(SEQ ID NO:	H-1	FV	3.2E+11	34.2

		(FIG. 12)		7; AAV2 Rep S-2 of FIG. 16) : AAV2 Rep 78, Rep 68, Rep 52, Rep 40				
16	P40	AAV5-transgene (FIG. 12)	R2 (0.31-0.59-0.1)	(SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16) : AAV2 Rep 78, Rep 68, Rep 52, Rep 40	H-1	FV	6.1E+11	37.9
17	P40	AAV5-transgene (FIG. 12)	R3 (0.42-0.3-0.28)	(SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16) : AAV2 Rep 78, Rep 68, Rep 52, Rep 40	H-1	FV	8.5E+11	34.8
18	P40	AAV5-transgene (FIG. 12)	R4 (0.42-0.42-0.16)	(SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16) : AAV2 Rep 78, Rep 68, Rep 52, Rep 40	H-1	FV	9.3E+11	38.7
19	P40	AAV5-transgene (FIG. 12)	R5 (0.28-0.5-0.22)	FIG. 2 (SEQ ID NO: 7) : AAV2 Rep 78, Rep 68, Rep 52, Rep 40	H-1	FV	9.1E+11	39
20	P40	AAV5-transgene (FIG. 12)	R6 (0.17-0.66-0.17)	FIG. 2 (SEQ ID NO: 7) :	H-1	FV	5.4E+11	44.9

				AAV2 Rep 78, Rep 68, Rep 52, Rep 40				
21	P40	AAV5-transgene (FIG. 12)	R7 (0.1-0.8-0.1)	(SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16) : AAV2 Rep 78, Rep 68, Rep 52, Rep 40	H-1	FV	2.0E+11	45.5
22	P40	AAV5-transgene (FIG. 12)	R8 (0.42-0.53-0.05)	(SEQ ID NO: 7; AAV2 Rep S-2 of FIG. 16) : AAV2 Rep 78, Rep 68, Rep 52, Rep 40	H-1	FV	4.3E+11	38.7
23	P40	ITR-GOI-ITR	3P (1-1-1)	AAV2 Rep + AAV5 Cap	H-1	FV	2.8E+11	32.5
24	P40	ITR-GOI-ITR	3P (1-1-1)	AAV2 Rep + AAV5 Cap	H-1	PEI	1.6E+11	39.4
25	P40	ITR-GOI-ITR	3P (1-1-1)	AAV2 Rep + AAV5 Cap	H-1	Mirus	1.7E+11	13.3

[0604] Results:

[0605] Capsid-transgene constructs with a p40 or p41 promoter resulted in similar yield (vg/ml). However, the % full capsid particles were 2x higher when the capsid-transgene included a p40 promoter instead of a p41 promoter. Additionally, certain ratios had higher vector yield without loss of % full capsid particles when a p41 promoter was used in the capsid-transgene.

7.11. Example 11: Production of rAAV using S3P system with varying ratios and varying rep AAV serotypes

[0606] In this study, AAV2 Rep plasmids or non-AAV2 rep plasmids were tested in the S3P system at different molar ratios compared to a standard 3P system at a molar ratio of 1:1:1 (H:R:Cap-GOI). Table 27 shows the S3P plasmid systems tested in this study:

Table 27:

Flask	Rep	Ratio (H:R:Cap-GOI)	Helper	Cap-GOI
1	AAV2 (AAV2 Rep S-2 of FIG. 16; SEQ ID NO: 7)	59-30.8-10.3	H-1	AAV2-transgene + p41 promoter
2	AAV2 (AAV2 Rep S-2 of FIG. 16; SEQ ID NO: 7) + p5 promoter	45-30-25	H-1	AAV2-transgene + p41 promoter
3	AAV2 (AAV2 Rep S-2 of FIG. 16; SEQ ID NO: 7) + p5 promoter	45-45-10	H-1	AAV2-transgene + p41 promoter
4	AAV2 (AAV2 Rep S-2 of FIG. 16; SEQ ID NO: 7) + p5 promoter	30-30-40	H-1	AAV2-transgene + p41 promoter
5	AAV2 (AAV2 Rep S-2 of FIG. 16; SEQ ID NO: 7) + p5 promoter	35-42.5-22.5	H-1	AAV2-transgene + p41 promoter
6	AAV2 (AAV2 Rep S-2 of FIG. 16; SEQ ID NO: 7) + p5 promoter	30-60-10	H-1	AAV2-transgene + p41 promoter
7	AAV2 (AAV2 Rep S-2 of FIG. 16; SEQ ID NO: 7) + p5 promoter	22.5-42.5-35	H-1	AAV2-transgene + p41 promoter
8	AAV2 (AAV2 Rep S-2 of FIG. 16; SEQ ID NO: 7) + p5 promoter	22.5-55-22.5	H-1	AAV2-transgene + p41 promoter
9	AAV2 (AAV2 Rep S-2 of FIG. 16; SEQ ID NO: 7) + p5 promoter	15-72.5-12.5	H-1	AAV2-transgene + p41 promoter
10	AAV2 (AAV2 Rep S-2 of FIG. 16; SEQ ID NO: 7) + p5 promoter	10-47.5-42.5	H-1	AAV2-transgene + p41 promoter
11	AAV2 (AAV2 Rep S-2 of FIG.	10-62.5-27.5	H-1	AAV2-transgene + p41 promoter

	16; SEQ ID NO: 7) + p5 promoter			
12 (3P)	3P Ctrl (AAV2 Rep-Cap) + p5 promoter	33-33-33	H-1	ITR-GOI-ITR
13	AAV1 Rep: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter	22.5-55-22.5	H-1	AAV2-transgene + p41 promoter
14	AAV2 Rep: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter (FIG. 16; AAV2 Rep S-1)	22.5-55-22.5	H-1	AAV2-transgene + p41 promoter
15	AAV4 Rep: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter	22.5-55-22.5	H-1	AAV2-transgene + p41 promoter
16	AAV7 Rep: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter	22.5-55-22.5	H-1	AAV2-transgene + p41 promoter
17	AAV10 Rep: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter	22.5-55-22.5	H-1	AAV2-transgene + p41 promoter
18	AAV2 Rep (FIG. 14) + + p5 promoter + NS2 gene + CMV promoter	22.5-55-22.5	H-1	AAV2-transgene + p41 promoter
19	AAV2 Rep (FIG. 13) + p5 promoter + UL12 + Ef1a promoter	22.5-55-22.5	H-1	AAV2-transgene + p41 promoter
20	AAV2 Rep NS2 + CMV promoter + UL12 + Ef1a promoter (FIG. 15)	22.5-55-22.5	H-1	AAV2-transgene + p41 promoter

[0607] Results: Flasks 19 and 20 showed an increase in vector crude titer (vg/ml) compared to 3P at the ratio tested. The best condition was Flask 6, with a molar ratio of 30:60:10 (H:R:Cap-GOI) which resulted in a vector crude titer of 1E+11 and 14.3% full capsid particles compared to 3P control (1.64×10^{10} vg/ml and 4.4% full capsid particles). This ratio tested resulted in a 6x increase in yield and a 3x packaging increase compared to the traditional 3P system.

7.12. Example 12: Production of rAAV using S3P system with varied capsids and varied rep AAV serotypes

[0608] In this study, AAV2, AAV4, AAV10, AAV11 Rep plasmids were tested in the S3P system compared to a standard 3P system. Table 28 shows the S3P plasmid systems tested in this study:

Table 28:

Sample #	Capsid	Rep	Helper	Cap-GOI	Titer	% Full
1	AAV9	AAV1 Large Rep (Rep 78 and Rep 68) without intron/exon native to AAV1 Rep + p5	H-1	AAV9-transgene + p41 promoter (FIG. 2)	4.99E+11	0
2		AAV4 Rep (Rep 78, Rep 68 Rep 52, Rep 40) + p5 promoter	H-1	AAV9-transgene + p41 promoter (FIG. 2)	3.10E+12	20.5
3		AAV10 Rep (Rep 78, Rep 68 Rep 52, Rep 40) + p5 promoter	H-1	AAV9-transgene + p41 promoter (FIG. 2)	3.47E+12	20.8
4		AAV11 Rep (Rep 78, Rep 68 Rep 52, Rep 40) + p5 promoter	H-1	AAV9-transgene + p41 promoter (FIG. 2)	3.36E+12	20
5		AAV2 Rep (Rep 78, Rep 68 Rep 52, Rep 40) + p5 promoter, with Rep intron/Exon (FIG. 16; AAV2 Rep S-1)	H-1	AAV9-transgene + p41 promoter (FIG. 2)	2.51E+12	23.4
6		AAV2 Rep (FIG. 16 S-2, includes extraneous noncoding sequences: part of E4 ORF6 from helper, part of fiber gene, part of ad5 genome)	H-1	AAV9-transgene + p41 promoter (FIG. 2)	1.44E+12	21.6
7		AAV2 Rep (FIG. 16, S-3) ; includes extraneous noncoding sequences: part of E4 ORF6 from	H-1	AAV9-transgene + p41 promoter (FIG. 2)	1.46E+12	21.4

		helper, part of ad5 genome				
8		AAV2 Rep (FIG. 16, S-4); same as FIG. 16, S-2 but with 2 point mutations	H-1	AAV9-transgene + p41 promoter (FIG. 2)	2.53E+12	20.5
9		AAV2 Rep (with Codon Optimized p40). FIG. 16 S-1 but P40 is functionally inactivated in AAV2 Rep.	H-1	AAV9-transgene + p41 promoter (FIG. 2)	3.03E+12	20.1
10		AAV2 Rep (FIG. 16 S-2 but (Codon Optimized). P40 is functionally inactivated in AAV2 Rep	H-1	AAV9-transgene + p41 promoter (FIG. 2)	1.78E+12	19.1
11		AAV2 Rep (FIG. 16 S-3 but Codon Optimized P40 is functionally inactivated in AAV2 Rep	H-1	AAV9-transgene + p41 promoter (FIG. 2)	1.17E+12	17.6
12		AAV2 Rep (FIG. 16 S-4 but Codon Optimized P40 is functionally inactivated in AAV2 Rep	H-1	AAV9-transgene + p41 promoter (FIG. 2)	1.95E+12	18
13	AAV5	AAV1 Large Rep (Rep 78 and Rep 68 but missing intron/exon native to AAV1 rep)	H-1	AAV5-transgene + p40 promoter (FIG. 2)	6.18E+11	8.2
14		AAV4 Rep (Rep 78, Rep 68 Rep 52, Rep 40) + p5 promoter	H-1	AAV5-transgene + p40 promoter (FIG. 2)	1.05E+12	13.2
15		AAV10 Rep (Rep 78, Rep 68 Rep 52, Rep 40) + p5 promoter	H-1	AAV5-transgene + p40 promoter (FIG. 2)	1.27E+12	16
16		AAV11 Rep (Rep 78, Rep 68 Rep 52, Rep 40) + p5 promoter	H-1	AAV5-transgene + p40 promoter (FIG. 2)	1.18E+12	13.3
17		AAV2 Rep (Rep 78, Rep 68 Rep 52, Rep 40)	H-1	AAV5-transgene +	9.42E+11	13.6

		+ p5 promoter, with Rep intron/Exon (FIG. 16; AAV2 Rep S-1)		p40 promoter (FIG. 2)		
18		AAV2 Rep (FIG. 16 S-2), includes extraneous noncoding sequences: part of E4 ORF6 from helper, part of fiber gene, part of ad5 genome	H-1	AAV5-transgene + p40 promoter (FIG. 2)	9.70E+11	15.6
19		AAV2 Rep (FIG. 16, S-3) ; includes extraneous noncoding sequences: part of E4 ORF6 from helper, part of ad5 genome	H-1	AAV5-transgene + p40 promoter (FIG. 2)	1.51E+12	15.5
20		AAV2 Rep (FIG. 16, S-4); same as FIG. 16, S-2 but with 2 point mutations	H-1	AAV5-transgene + p40 promoter (FIG. 2)	9.98E+11	16.7
21		AAV2 Rep (with Codon Optimized p40). FIG. 16 S-1 but P40 is functionally inactivated in AAV2 Rep.	H-1	AAV5-transgene + p40 promoter (FIG. 2)	1.00E+12	12.6
22		AAV2 Rep (FIG. 16 S-2 but (Codon Optimized). P40 is functionally inactivated in AAV2 Rep	H-1	AAV5-transgene + p40 promoter (FIG. 2)	1.00E+12	17.9
23		AAV2 Rep (FIG. 16 S-3 but Codon Optimized P40 is functionally inactivated in AAV2 Rep	H-1	AAV5-transgene + p40 promoter (FIG. 2)	8.33E+11	18.5
24 (high CV)		AAV2 Rep (FIG. 16 S-4 but Codon Optimized P40 is functionally inactivated in AAV2 Rep	H-1	AAV5-transgene + p40 promoter (FIG. 2)	7.36E+11	15

[0609] An aim of this study was to determine whether codon optimization of p40 in the rep protein changed the yield and packaging efficiency.

[0610] Results:

[0611] S3P system with an AAV5 capsid-transgene plasmid performed significantly better than when the capsid is driven by a p40 promoter.

[0612] Using a P41 promoter instead of a P40 promoter in the capsid-transgene construct significantly decreased rcAAV by removing homologous sequences between rep and cap.

[0613] As shown in Table 29 below, AAV4, AAV10, and AAV11 rep proteins showed an improvement on crude vector yield (vg/ml).

Table 29:

Titer vs whether rep sequence is codon optimized				
Description	**WT**	**CO (codon optimized)**	**WT**	**CO**
	AAV9 Capsid-GOI		**AAV5 Capsid-GOI**	
AAV1 Large Rep	4.99E+11		6.18E+11	
AAV4 Rep	3.10E+12		1.05E+12	
AAV10 Rep	3.47E+12		1.27E+12	
AAV11 Rep	3.36E+12		1.18E+12	
AAV2 Rep (FIG. 16 S-1)	2.51E+12	3.03E+12	9.42E+11	1.00E+12
AAV2 Rep (FIG. 16 S-2)	1.44E+12	1.78E+12	9.70E+11	1.00E+12
AAV2 Rep (FIG. 16 S-3)	1.46E+12	1.17E+12	1.51E+12	8.33E+11
AAV2 Rep (FIG. 16 S-4)	2.53E+12	1.95E+12	9.98E+11	7.36E+11

7.13. Example 13: Production of rAAV using S3P system with varied capsids and varied rep AAV serotypes

[0614] In this study, AAV2, AAV9, and AAV10 Rep plasmids were tested in the S3P system, and AAV9. The helper (H-1) was fixed. Table 30 shows the S3P plasmid systems tested in this study:

Table 30:

Description	**Titer**	**% Full**	**Rep**	**Cap-Car**
	Avg	Avg		

AAV2, AAV2Rep	2.6E+11	10.2	AAV2 Rep: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter (FIG. 16; AAV2 Rep S-1)	AAV2 + p41 promoter
AAV2, AAV10Rep	4.9E+11	12.4	AAV1- Rep: Rep 78, Rep 68, Rep 52, Rep 40	AAV2 + p41 promoter
AAV2 3P	1.4E+11	5.4	AAV2 Rep/Cap	
AAV9, AAV2Rep	4.7E+11	7.7	AAV2 Rep: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter (FIG. 16; AAV2 Rep S-1)	AAV9 + 765 bp spacer + p41 promoter
AAV9, AAV10Rep	6.8E+11	18.0	AAV10 Rep: Rep 78, Rep 68, Rep 52, Rep40 + p5 promoter	AAV9 with a 765 bp spacer + P41
AAV9 3P	1.7E+11	35.7		
AAV9 MLC2 AAV2 pA 1172	2.8E+12	38.8	AAV2 Rep: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter (FIG. 16; AAV2 Rep S-1)	AAV9 + p41 promoter
AAV9 MLC2 SV40 pA	2.8E+12	39.7	AAV2 Rep: Rep 78, Rep 68, Rep 52, Rep 40 + p5 promoter (FIG. 16; AAV2 Rep S-1)	AAV9 + SV40 pA

[0615] Result: A S3P plasmid system with rep proteins of an AAV10 serotype resulted in increased crude vector titer (vg/ml) and % full capsid particles compared to 3P control.

7.14. Example 14: Production of rAAV using S3P system with varied promoters driving Cap-transgene and Rep plasmids

[0616] The present inventors previously found that crude titer (vg/ml) and/or % full capsid particle packaging efficiency was improved when using a p40 promoter instead of a p41 promoter in the cap-transgene construct. In this study, production of recombinant rAAV was tested in 30L bioreactors using the S3P system to evaluate UL12 protein on a rep plasmid and the effect of different promoter sequences.

[0617] Table 31 provides a list of S3P plasmid systems tested in this study:

Table 31:

Results					Rep				Capcar	
Flask #	Titer (vg/mL)	% Full	Purpose	Ratio	Promoter 1	Protein 1	Promoter 2	Protein 2	Promoter	Cap/GOI
1	2.8E+12	56	Evaluate Inducible Promoter	3:5:2	Ef1a	tTA before Rep	P5	AAV2Rep (no P40)	TRE	AAV9 [MLC2 BAG3]

2	1.6E+12	65		3:5:2	P5	tTa after AAV2 Rep (Rep 78, Rep 68, Rep 52 and Rep 40)	N/A	tTA		
3	1.5E+12	69		3:5:2	P5	tTa after AAV2 Rep (noP40) (Rep 78, Rep 68, Rep 52 and Rep 40)	Ef1a	tTA		
4	<1e8	34.6		High Rep	P5					
5	1.3E+12	61.8		High Capcar	P5					
6	4.4E+11	67.9		3:5:2	P5	TRE without tTa AAV2 Rep (Rep 78, Rep 68, Rep 52 and Rep 40)	N/A			
7	2.5E+12	39.6		3:5:2	P5	tTa without TRE AAV2Rep (noP40) (Rep 78, Rep 68, Rep 52 and Rep 40)	Ef1a	tTA	P41	
8	2.7E+12	36	AAV10 Rep Ctrl	3:5:2	P5	AAV10 Rep (Rep 78, Rep 68, Rep 52 and Rep 40)	N/A		P41	
9 (no cloning sites in capcar)	2.5E+12	45.2		High Rep						
10 (no cloning sites in capcar)	2.4E+12	36	AAV2 Rep Ctrl	3:5:2	P5	AAV2 Rep (Rep 78, Rep 68, Rep 52 and Rep 40)	N/A			
11	2.6E+12	34.6	No Cloning Sites	3:5:2	P5	AAV2 Rep (Rep 78, Rep 68, Rep 52 and Rep 40)	N/A			
12	2.0E+12	53.1	P40 Capcar	3:5:2	P5	AAV2 Rep (Rep 78, Rep 68, Rep	N/A		P40	

						52 and Rep 40)			
13	1.4E+12	53.4		High Rep	P5	AAV2 Rep (Rep 78, Rep 68, Rep 52 and Rep 40)	N/A		
14	2.1E+12	40.2		High Capcar	P5	AAV2Rep (Rep 78, Rep 68, Rep 52 and Rep 40)	N/A		
15	1.9E+12	55.5	Ef1a Capcar	3:5:2	P5	AAV2 Rep	N/A		EF1a
16	9.7E+10	43		3:5:2	P5	AAV2 Rep (Rep 78, Rep 68, Rep 52 and Rep 40)	N/A		
17	2.1E+12	38.6		3:5:2	P5	AAV2 Rep (Rep 78, Rep 68, Rep 52 and Rep 40)	N/A		
18	1.1E+11	42	SV40 Capcar	3:5:2	P5	AAV2 Rep (Rep 78, Rep 68, Rep 52 and Rep 40)	N/A		SV40
19	2.4E+12	42.2	UL12	3:5:2	P5	AAV10 Rep (Rep 78, Rep 68, Rep 52 and Rep 40)	Ef1a	UL12	P41
20	3.0E+12	41.1		3:5:2	Ef1a	UL12	P5	AAV10Rep	
21	2.4E+12	40.4		High Rep					
22	1.6E+12	44.4	Split Rep	3:5:2	P5	AAV10 Large Rep (Rep 78 and Rep 68)	P19	AAV2 Small Rep	
23	7.8E+11	68		High Rep					
24	4.0E+11	23.7		High Capcar					

[0618] Results:

[0619] As shown in Flask 20, adding the UL12 into the rep plasmid upstream of the Rep protein sequence produced a higher vector crude titer (vg/ml) compared to flask 19, where the UL12 sequence is downstream of the AAV10 rep sequence.

[0620] The inducible promoter as shown in Flasks 1-7 increased vector crude titer and significantly increased % full capsid particles.

[0621] S3P systems that included rep proteins from an AAV10 serotype outperformed rep proteins from an AAV2 serotype (see e.g., Flask 8 and Flask 10).

8. EQUIVALENTS AND INCORPORATION BY REFERENCE

[0622] While the invention has been particularly shown and described with reference to a preferred embodiment and various alternate embodiments, it will be understood by persons skilled in the relevant art that various changes in form and details can be made therein without departing from the spirit and scope of the invention.

[0623] All references, issued patents and patent applications cited within the body of the instant specification are hereby incorporated by reference in their entirety, for all purposes. US provisional application 63/565,528 filed on March 14, 2024 has been incorporated by reference in its entirety.

What is claimed is:

1. A system for production of recombinant adeno-associated virus (rAAV) comprising:

a first polynucleotide comprising a rep gene encoding a Rep protein, and a first promoter operably linked to the rep gene;

a second polynucleotide comprising a helper virus gene,

a third polynucleotide comprising (i) a capsid gene encoding a Capsid protein, a second promoter operably linked to the capsid gene; and (ii) an expression cassette comprising a transgene flanked by two inverted terminal repeats (ITRs).

2. The system of claim 1, wherein the first polynucleotide comprises a sequence encoding a Rep 78 protein, a Rep 68 protein, a Rep 52 protein, or a Rep 40 protein.

3. The system of claim 1, wherein the first polynucleotide comprises two, three or four sequences, wherein each of the two, three, or four sequences encodes a Rep 78 protein, a Rep 68 protein, a Rep 52 protein, or a Rep 40 protein.

4. The system of any one of claims 1-3, wherein the first polynucleotide comprises sequences encoding a Rep 78 protein, a Rep 68 protein, a Rep 52 protein, and a Rep 40 protein.

5. The system of claim 1, wherein:

the first polynucleotide comprises sequences encoding AAV2 Rep 78, AAV2 Rep 68, AAV2 Rep 52, and AAV2 Rep 40 proteins.

6. The system of claim 1, wherein the first polynucleotide comprises sequences encoding AAV4 Rep 78, AAV4 Rep 68, AAV4 Rep 52, and AAV4 Rep 40 proteins.

7. The system of claim 1, wherein the rep gene of the first polynucleotide comprises a sequence encoding a rep protein from an AAV serotype selected from: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, and AAV13.

8. The system of claim 7, wherein the first polynucleotide comprises sequences encoding AAV10 Rep 78, AAV10 Rep 68, AAV10 Rep 52, and AAV10 Rep 40 proteins.

9. The system of claim 1, wherein the first polynucleotide comprises sequences encoding:

30-50% of AAV2 and AAV10 Rep 78 proteins;

30-50% of AAV2 and AAV10 Rep 68 proteins;

an AAV2 Rep 52 protein; and

an AAV4 Rep 40 protein.

10. The system of any one of claims 1-9, wherein the first promoter is from an AAV serotype that is different from the origin of the rep gene.

11. The system of any one of claims 1-9, wherein the first promoter is from an AAV serotype that is the same as the origin of the rep gene.

12. The system of any one of claims 1-10, wherein the first promoter is a p5 promoter.

13. The system of claim 12, wherein the p5 promoter is an AAV2 p5, AAV4 p5, or AAV10 p5 promoter.

14. The system of any one of claims 1-13, wherein the first polynucleotide further comprises a p19 promoter.

15. The system of claim 14, wherein the p19 promoter is a AAV2 p19, AAV4 p19, or AAV10 p19 promoter.

16. The system of any one of claims 1-15, wherein the first promoter comprises a P7 promoter.

17. The system of any one of claims 1-15, wherein the first promoter comprises a p40 promoter.

18. The system of claim 17, wherein the p40 promoter is an AAV2 p40, AAV4 p40, or AAV10 p40 promoter.

19. The system of any one of claims 1-16, wherein the first polynucleotide is devoid of a p40 promoter.

20. The system of any one of claims 1-13, wherein the first polynucleotide is devoid of: a p19 promoter or a p40 promoter, or both a p19 promoter and a p40 promoter.

21. The system of any one of claims 1-12, wherein the first polynucleotide further comprises one or more additional promoters.

22. The system of claim 21, wherein the one or more additional promoters is selected from: p19, CMV, SV40, Ef1a, UBC, p5, and a PGK promoter.

23. The system of claim 1, wherein:

the first polynucleotide comprises sequences encoding AAV2 Rep 78, AAV2 Rep 68, AAV2 Rep 52, and AAV2 Rep 40 proteins, wherein the first promoter is a AAV2 p5 promoter, and wherein the first polynucleotide further comprises a p19 promoter;

the second polynucleotide comprises sequences encoding Adeno E2A, Adeno E4, and Adeno VA; and

the third polynucleotide further comprises:

a non-coding nucleotide sequence positioned between one of the two ITR sequences and the second promoter; and

an AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and an AAV2 3' UTR sequence at the 3' end of the capsid coding sequence.

24. The system of claim 1 wherein:

the first polynucleotide comprises sequences encoding AAV4 Rep 78, AAV4 Rep 68, AAV4 Rep 52, and AAV4 Rep 40 proteins, wherein the first promoter is a AAV2 p5 promoter, and wherein the first polynucleotide further comprises a p19 promoter;

the second polynucleotide comprises sequences encoding Adeno E2A, Adeno E4, and Adeno VA;

the second promoter of the third polynucleotide is a p41 promoter, and

the third polynucleotide further comprises:

a non-coding sequence positioned between one of the two ITR sequences and the second promoter; and

a AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and AAV2 3' UTR sequence at the 3' end of the capsid coding sequence.

25. The system of claim 1, wherein:

the first polynucleotide comprises sequences encoding AAV10 Rep 78, AAV10 Rep 68, AAV10 Rep 52, and AAV10 Rep 40 proteins, wherein the first promoter is a AAV2 p5 promoter, and wherein the first polynucleotide further comprises a p19 promoter;

the second polynucleotide comprises sequences encoding Adeno E2A, Adeno E4, and Adeno VA;

the second promoter of the third polynucleotide is a p41 promoter, and

the third polynucleotide further comprises:

a non-coding sequence positioned between one of the two ITR sequences and the second promoter and a AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and AAV2 3' UTR sequence at the 3' end of the capsid coding sequence.

26. The system of claim 1, wherein:

the first polynucleotide comprises sequences encoding:

AAV2 and AAV10 Rep 78 proteins;

AAV2 and AAV10 Rep 68 proteins;

an AAV2 Rep 52 protein; and

an AAV4 Rep 40 protein,

wherein the first promoter is an AAV2 p5 promoter, and wherein the first polynucleotide further comprises a p19 promoter;

the second polynucleotide comprises coding sequences of Adeno E2A, Adeno E4, and Adeno VA;

the second promoter of the third polynucleotide is a p41 promoter, and

the third polynucleotide further comprises: a non-coding sequence positioned between one of the two ITR sequences and the second promoter and an AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and AAV2 3' UTR sequence at the 3' end of the capsid coding sequence.

27. The system of any one of claims 1-26, wherein the first polynucleotide is devoid of a helper virus gene.

28. The system of claim 27, wherein the first polynucleotide is devoid of adenovirus helper genes.

29. The system of any one of claims 1-28, wherein the first polynucleotide is devoid of a capsid gene.

30. The system of any one of claims 1-29, wherein the first polynucleotide further comprises a nucleotide sequence encoding an additional protein.

31. The system of claim 30, wherein the additional protein is from a Herpes simplex virus type 1 (HSV-1) or a bocavirus.

32. The system of claim 31, wherein the additional protein is a helper virus gene selected from: NS2 and UL12.

33. The system of claim 1, wherein the first polynucleotide comprises sequences encoding:

an AAV2 Rep 78 protein and an AAV2 Rep 68 protein; or

a non-AAV2 Rep 78 protein and a non-AAV2 Rep 68 protein.

34. The system of claim 33, wherein the first promoter of the first polynucleotide is a p5 promoter.

35. The system of any one of claims 33-34, wherein the first polynucleotide is devoid of a p19 promoter.

36. The system of any one of claims 33-35, wherein the third polynucleotide comprises a promoter selected from: p19 promoter, CMV, SV40, Ef1a, UBC, p5, and a PGK promoter.

37. The system of any one of claims 33-36, wherein the third polynucleotide further comprises a Rep 52 protein and a Rep 40 protein positioned between:

two promoter sequences and downstream the 3'ITR sequence; or

between the second promoter and the capsid protein.

38. The system of claim 37, wherein the Rep 52 protein is a non-AAV2 or an AAV2 Rep 52 protein; and wherein the Rep 40 protein is a non-AAV2 or an AAV2 Rep 40 protein.

39. The system of claim 37, wherein the two promoters are a p19 promoter and a p40 promoter.

40. The system of claim 1, wherein the first polynucleotide comprises sequences encoding a Rep 52 protein and a Rep 40 protein.

41. The system of claim 40, wherein the first polynucleotide or third polynucleotide is devoid of a p19 promoter.

42. The system of any one of claims 40-41, wherein the third polynucleotide further comprises coding sequences of a Rep 78 protein and a Rep 68 protein positioned between:

2 promoters and downstream the 3' ITR sequence; or

between the second promoter and the capsid protein.

43. The system of claim 42, wherein the Rep 78 protein is a non-AAV2 or an AAV2 Rep 78 protein; and wherein the Rep 68 protein is a non-AAV2 or an AAV2 Rep 68 protein.

44. The system of claim 1, wherein the first polynucleotide comprises sequences encoding a Rep 78 protein and a Rep 68 protein, optionally wherein the first polynucleotide comprises mutated coding sequences of Rep 52 and Rep 40 proteins with a mutated start codon.

45. The system of claim 44, wherein the first polynucleotide comprises sequences encoding an AAV2 Rep 78 protein and an AAV2 Rep 68 protein.

46. The system of claim 44, wherein the first promoter of the first polynucleotide is a p5 promoter.

47. The system of any one of claims 44-46, wherein the first polynucleotide is devoid of a p19 promoter.

48. The system of any one of claims 44-47, wherein the system further comprises a fourth polynucleotide comprising sequences encoding Rep 52 and Rep 40 proteins.

49. The system of claim 48, wherein the fourth polynucleotide comprises a p19 promoter.

50. The system of any one of claims 48-49, wherein the fourth polynucleotide is smaller in size compared to the first polynucleotide.

51. The system of claim 50, wherein the fourth polynucleotide comprises a p19 promoter and a p40 promoter.

52. The system of any one of claims 1-51, wherein the second polynucleotide comprises two helper virus genes from Adenovirus 5 or Adenovirus 2.

53. The system of any one of claims 1-51, wherein the second polynucleotide comprises two helper virus genes selected from Adeno E2A, Adeno E2B, Adeno E4, Adeno VA, Adeno L3 and Adeno L4.

54. The system of any one of claims 1-51, wherein the second polynucleotide comprises three helper virus genes selected from Adeno E2A, Adeno E2B, Adeno E4, Adeno VA, Adeno L3 and Adeno L4.

55. The system of any one of claims 1-51, wherein the second polynucleotide comprises Adeno E2A, Adeno E4, and Adeno VA.

56. The system of any one of claims 1-51, wherein the second polynucleotide comprises Adeno E2B, Adeno VA, Adeno L3, Adeno E2A, Adeno L4, and Adeno E4.

57. The system of any one of claims 1-51, wherein the second polynucleotide comprises E1 Open Reading Frame (ORF1), E4 Open Reading Frame (ORF2), ORF3, ORF4 and ORF 6/7.

58. The system of claim 55, wherein the second polynucleotide further comprises a helper virus gene selected from: NS2 and UL12.

59. The system of claim 55, wherein the second polynucleotide further comprises helper virus genes NS2 and UL12.

60. The system of any one of claims 1-51, wherein the second polynucleotide lacks Adeno E2B, Adeno VA, Adeno L3, or Adeno L4.

61. The system of any one of claims 1-51, wherein the second polynucleotide lacks two or more helper virus genes selected from Adeno E2B, Adeno VA, Adeno L3, and Adeno L4.

62. The system of any one of claims 1-51, wherein the second polynucleotide lacks Adeno E2B, Adeno VA, Adeno L3, and Adeno L4.

63. The system of any one of claims 1-51, wherein the second polynucleotide has a length less than 14,000 nucleotides.

64. The system of claim 63, wherein the second polynucleotide has a length less than 12,000 nucleotides.

65. The system of claim 63, wherein the second polynucleotide has a length ranging from 9,000 to 13,000 nucleotides.

66. The system of claim 63, wherein the second polynucleotide has a length ranging from 11,000 to 12,000 nucleotides.

67. The system of any one of claims 1-22 and 27-66, wherein the third polynucleotide further comprises a 5' UTR nucleotide sequence between the second promoter and the capsid gene.

68. The system of claim 67, wherein the 5' UTR sequence is a non-coding nucleotide sequence.

69. The system of any one of claims 67-68, wherein the second promoter of the third polynucleotide is a p41 promoter, p40 promoter, CMV promoter, SV40 promoter, Ef1a promoter, TRE, UBC, or a PGK promoter.

70. The system of any one of claims 67-69, wherein the 5'UTR sequence of the third polynucleotide has a length ranging from 200-400 nucleotides.

71. The system of any one of claims 67-70, wherein the capsid gene and the second promoter in the third polynucleotide are positioned outside of the two ITR sequences.

72. The system of any one of claims 67-71, wherein the two ITR sequences are from an AAV serotype selected from: AAV1, AAV2, AAV3, AAV4, AA5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, and AAV13.

73. The system of any one of claims 67-72, wherein the capsid gene and the second promoter in the third polynucleotide are positioned downstream of the 3'-ITR sequence.

74. The system of any one of claims 67-72, wherein the capsid gene and the second promoter in the third polynucleotide are positioned upstream of the 5'-ITR sequence.

75. The system of any one of claims 67-74, wherein the third polynucleotide further comprises a non-coding nucleotide sequence positioned between one of the two ITR sequences and the second promoter.

76. The system of claim 75, wherein the non-coding nucleotide sequence of the third polynucleotide has a length ranging from 165-1300 nucleotides.

77. The system of any one of claims 67-76, wherein the third polynucleotide comprises the AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and a AAV2 3' UTR sequence positioned at the 3' end of the capsid coding sequence.

78. The system of any one of claims 67-76, wherein the third polynucleotide comprises the AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and a transcription termination sequence (polyA sequence) positioned at the 3' end of the capsid coding sequence.

79. The system of any one of claims 67-76, wherein the third polynucleotide further comprises a AAV2 3' UTR sequence positioned at the 3' end of the capsid coding sequence.

80. The system of any one of claims 67-76, wherein the AAV2 5' UTR sequence is positioned between the second promoter and the 5' end of the capsid coding sequence.

81. The system of any one of claims 67-76, wherein the AAV2 3' UTR sequence is positioned at the 3' end of the capsid coding sequence.

82. The system of any one of claims 67-76, wherein the third polynucleotide comprises the AAV2 5' UTR sequence positioned between the second promoter and the 5' end of the capsid coding sequence and a transcription termination sequence (e.g., polyA sequence) at the 3' end of the capsid coding sequence.

83. The system of claim 82, wherein the polyA sequence is a SV40 polyA sequence.

84. The system of any one of claims 67-83, wherein the third polynucleotide further comprises a rep gene encoding a Rep protein.

85. The system of claim 84, wherein the third polynucleotide comprises sequences encoding one or more of: Rep 78 protein, Rep 68 protein, Rep 52 protein, and Rep 40 protein.

86. The system of claim 85, wherein the third polynucleotide comprises sequences encoding a Rep 52 protein and Rep 40 protein.

87. The system of claim 85, wherein the third polynucleotide comprises sequences encoding Rep 78, Rep 68, Rep 52, and Rep 40 proteins.

88. The system of any one of claims 1-87, wherein the Capsid protein has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 100% amino acid sequence identity to the sequence of a AAV capsid protein of an AAV selected from the group consisting of: AAV9; Anc80L65; Anc80-55, Anc80-129, Anc80-156, Anc80-751, Anc80-1029, Anc80-1712, AAV2; AAV1; AAV6; AAV3; AAV LK03; AAV7; AAV8; AAV hu.37; AAV rh.10; AAV hu.68; AAV10; AAV5; AAV3-3; AAV4-4; AAV1-A; hu.46-A; hu.48-A; hu.44-A; hu.43-A; AAV6-A; hu.34-B; hu.47-B; hu.29-B; rh.63-B; hu.56-B; hu.45-B; rh.57-B; rh.35-B; rh.58-B; rh.28-B; rh.51-B; rh.19-B; rh.49-B; rh.52-B; rh.13-B; AAV2-B; rh.20-B; rh.24-B; rh.64-B; hu.27-B; hu.21-B; hu.22-B; hu.23-B; hu.7-C; hu.61-C; rh.56-C; hu. 9-C; hu.54-C; hu.53-C; hu.60-C; hu.55-C; hu.2-C; hu.1-C; hu.18-C; hu.3-C; hu.25-C; hu.15-C; hu.16-C; hu.11-C; hu.10-C; hu.4-C; rh.54-D; rh.48-D; rh.55-D; rh.62-D; AAV7-D; rh.52-E; rh.51-E; hu.39-E; rh.53-E; hu.37-E; rh.43-E; rh.50-E; rh.49-E; rh.61-E; hu.41-E; rh.64-E; rh74; hu.42-E; rh.57-E; rh.40-E; hu.67-E; hu.17-E; hu.6-E; hu.66-E; rh.38-E; hu.32-F; AAV9/hu; hu.31-F; Anc80; Anc81; Anc82; Anc83; Anc84; Anc94; Anc113; Anc126; Anc127; Anc80L27; Anc80L59; Anc80L60;

Anc80L62; Anc80L33; Anc80L36; Anc80L44; Anc80L1; Anc110; Anc80DI; CAPSID-1_AAV9_1000, Capsid-3_38181, and AAV9 (Capsid-2_MUT1-38181).

89. The system of claim 88, wherein the capsid protein is an AAV9 capsid variant protein, wherein the AAV9 capsid variant protein comprises a 6 to 12-mer peptide inserted into an AAV9 capsid protein.

90. The system of claim 2, wherein:

the first polynucleotide comprises sequences encoding Rep 78, Rep 68, Rep 52, and Rep 40 proteins, wherein the first promoter is a AAV2 p5 promoter, and wherein the first polynucleotide further comprises a p19 promoter;

the second polynucleotide comprises sequences encoding Adeno E2A, Adeno E4, and Adeno VA;

the second promoter of the third polynucleotide is a p41 promoter, and

the third polynucleotide further comprises: a second non-coding sequence positioned between one of the two ITR sequences and the second promoter and a AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and AAV2 3' UTR at the 3' end of the capsid coding sequence.

91. The system of claim 90, wherein: the two ITR sequences are AAV2 ITR sequences.

92. The system of any one of claims 90-91, wherein the first polynucleotide comprises sequences encoding a AAV2 Rep 78 protein, a AAV2 Rep 68 protein, a AAV2 Rep 52 protein, and a AAV2 Rep 40 protein.

93. The system of claim 1, wherein:

the first polynucleotide comprises sequences encoding Rep 78, Rep 68, Rep 52, and Rep 40 proteins, wherein the first promoter is an AAV2 p5 promoter;

the second polynucleotide comprises sequences encoding Adeno E2A, Adeno E4, and Adeno VA;

the second promoter of the third polynucleotide is selected from: a p41, p40, CMV, SV40, Ef1a, Ef1a core, PGK, CAG, UBC, CBh, or a TRE promoter; and

the third polynucleotide further comprises: a non-coding sequence positioned between one of the two ITR sequences and the second promoter and a AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and AAV2 3' UTR at the 3' end of the capsid coding sequence.

94. The system of claim 1, wherein:

the first polynucleotide comprises sequences encoding Rep 78, Rep 68, Rep 52, and Rep 40 proteins, wherein the first promoter is a AAV2 p5 promoter;

the second polynucleotide comprises sequences encoding Adeno E2A, Adeno E4, and Adeno VA; .

the second promoter of the third polynucleotide is a p41 promoter, and

the third polynucleotide further comprises: a non-coding sequence positioned between one of the two ITR sequences and the second promoter and a AAV2 5' UTR sequence positioned at the 5' end of the capsid coding sequence and a transcription termination sequence (polyA sequence) at the 3' end of the capsid coding sequence.

95. The system of claim 1, wherein the first polynucleotide comprises sequences encoding: AAV2 Rep 78, AAV2 Rep 68, AAV2 Rep 52, and AAV2 Rep 40 proteins, and a UL12 or transthyretin (ttR) protein, and wherein the first promoter is an AAV2 p5 promoter.

96. The system of claim 95, wherein the second polynucleotide comprises sequences encoding Adeno E2A, Adeno E4, and Adeno VA.

97. The system of claim 95, wherein the second promoter of the third polynucleotide is selected from: a p41, p40, CMV, SV40, Ef1a, Ef1a core, PGK, CAG, UBC, CBh, or a TRE promoter.

98. The system of any one of claims 95-97, wherein the first polynucleotide further comprises a coding sequence of UL12 or transthyretin(ttR).

99. The system of any one of claims 95-98, wherein the first polynucleotide further comprises a coding sequence of transthyretin(ttR).

100. The system of any one of claims 1-99, wherein the first polynucleotide is in a plasmid.

101. The system of any one of claims 1-100, wherein the second polynucleotide is in a plasmid.

102. The system of any one of claims 1-101, wherein the third polynucleotide is in a plasmid.

103. The system of any one of claims 1-102, wherein the first polynucleotide is in a first plasmid, the second polynucleotide is in a second plasmid, and the third polynucleotide construct is in a third plasmid.

104. A packaging cell comprising the system of any one of claims 1-103.

105. A method of producing recombinant AAV, comprising:

providing the system of any one of claims 1-103;

introducing the first polynucleotide, the second polynucleotide and the third polynucleotide of the system into a cell; and

culturing the cell under conditions suitable for recombinant AAV (rAAV) production.

106. The method of claim 105, wherein in step (b), a molar amount of the first polynucleotide introduced into the cell is at least twice of a molar amount of the second polynucleotide introduced into the cell.

107. The method of claim 106, wherein a molar amount of the first polynucleotide introduced into the cell is two to four times of a molar amount of the second polynucleotide introduced into the cell.

108. The method of claim 107, wherein a molar amount of the first polynucleotide introduced into the cell is at least three times of a molar amount of the second polynucleotide introduced into the cell.

109. The method of claim 108, wherein a molar amount of the second polynucleotide introduced into the cell is at least four times of a molar amount of the third polynucleotide introduced into the cell.

110. The method of claim 109, wherein a molar amount of the second polynucleotide introduced into the cell is at least five or six times of a molar amount of the third polynucleotide introduced into the cell.

111. The method of claim 110, wherein a molar amount of the second polynucleotide introduced into the cell is at least five times of a molar amount of the third polynucleotide introduced into the cell.

112. The method of claim 111, wherein a molar amount of the second polynucleotide introduced into the cell is at least ten times of a molar amount of the third polynucleotide introduced into the cell.

113. The method of claim 112, wherein a molar amount of the second polynucleotide introduced into the cell is at least fifteen times of a molar amount of the third polynucleotide introduced into the cell.

114. The method of claim 113, wherein a molar amount of the second polynucleotide introduced into the cell is at least twenty times of a molar amount of the third polynucleotide introduced into the cell.

115. The method of claim 114, wherein a molar amount of the second polynucleotide introduced into the cell is at least twenty-five times of a molar amount of the third polynucleotide introduced into the cell.

116. The method of any one of claims 105-115, wherein the first polynucleotide comprises a molar amount ranging from about 5% to 95% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined.

117. The method of claim 105-115, wherein the first polynucleotide comprises a molar amount ranging from about 5% to about 35% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined.

118. method of claim 105-115, wherein the first polynucleotide comprises a molar amount ranging from about 10% to about 90% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined.

119. The method of claim 105-115, wherein the second polynucleotide comprises a molar amount ranging from about 5% to 80% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined.

120. The method of claim 116, wherein the second polynucleotide comprises a molar amount of about 60% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined.

121. The method of claim 105, wherein the third polynucleotide comprises a molar amount ranging from about 1% to 90% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined.

122. The method of claim 116, wherein the third polynucleotide comprises a molar amount of about 70% of the total molar amount of the first polynucleotide, the second polynucleotide, and the third polynucleotide combined.

123. The method of claim 105, wherein the system comprises a molar ratio of 6:3:1 of the second polynucleotide:first polynucleotide:third polynucleotide.

124. The method of claim 105, wherein the system comprises a molar ratio of 5:1:4 of the second polynucleotide:first polynucleotide:third polynucleotide.

125. The method of claim 105, wherein the system comprises a molar ratio of 1:2:7 of the second polynucleotide:first polynucleotide:third polynucleotide.

126. The method of any one of claims 105-125, wherein culturing the cell under conditions suitable for recombinant AAV production comprises expanding the cell.

127. The method of any one of claims 105-125, wherein the method comprises collecting the secreted recombinant AAV production expressed by the cell.

128. The method of any one of claims 105-125, further comprising lysing the cell.

129. The method of claim 125, further comprising purifying the lysed cell to collect rAAV particles.

130. The method of claim 129, wherein the collected rAAV particles comprise less than 5% of replication-competent adeno-associated virus particles (rcAAV).

131. The method of claim 130, wherein the collected rAAV particles comprise less than 1% of replication-competent adeno-associated virus particles (rcAAV).

132. A recombinant adeno-associated virus (rAAV) produced by the method of any one of claims 105-131, using the system of any one of claims 1-103, or from the packaging cell of claim 104.

133. A pharmaceutical composition comprising the recombinant adeno-associated virus (rAAV) of claim 132.