

ADENO-ASSOCIATED VIRUS (AAV) PRODUCTION

SEQUENCE LISTING

[0001] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on February 7, 2022, is named 0132-0156WO1_SL.txt and is 55,665 bytes in size.

FIELD OF THE INVENTION

[0002] The present disclosure relates to methods for producing an adeno-associated virus (AAV) in an E1 complementary producer cell.

BACKGROUND

[0003] Adeno-associated virus (AAV) is a non-enveloped DNA virus. As a leading viral vector for *in vivo* gene therapy, AAV is non-pathogenic and can effectively infect a wide range of human tissues. AAV genome includes a 4.7 kb single-stranded DNA that encodes two major genes: the Rep gene encodes four isoforms (Rep78, Rep68, Rep52 and Rep40) that support AAV DNA replication, packaging, integration, and transcription; the Cap gene synthesizes three VP proteins (VP1, VP2, and VP3) for the viral protein shell or capsid construction.

[0004] Wild-type AAV cannot replicate without helper viruses, such as adenovirus (Ad) and herpes simplex virus (HSV). Adenoviral helper genes have been identified as necessary and sufficient for AAV replication, including E1A, E1B, E2A, E4, and VA. The adenovirus type 5 (Ad5) 5' 4.3 kb genomic DNA has been integrated into chromosome 19 of human embryonic kidney 293 (HEK293) cells. This Ad5 genomic fragment expresses all E1A (e.g., 289R, 243R, 171R) and E1B (e.g., 19K and 55K) isoforms. Further cell lines having E1A and E1B integration have been developed, including, e.g., PER.C6 cells and CAP® cells. See, e.g., Fallaux et al., *Human Gene Ther* 9(13):1909-1917 (1998) and Schiedner et al., *Human Gene Ther* 11(15):2105-2116 (2000). Accordingly, these cells already express E1A and E1B and therefore can support recombinant AAV (rAAV) production by the "triple plasmid transfection" approach, i.e., transfecting helper plasmid (pHelper) that encodes only E2A, E4orf6, and VA, together with plasmids that encode the Rep and Cap genes (pRepCap) and the gene of interest (pAAV-GOI).

Such cells, which have genome-integrated E1A and E1B genes and natively express E1A and E1B proteins, are known as “E1 complementary producer cells.”

[0005] Currently, over 70% of the AAV-based gene therapy pipelines in the world rely on the manufacturing platform via transient transfection in HEK293 cells with the standard triple plasmid transfection method. Productivity of the HEK293 and other E1 complementary producer cell manufacturing platforms may not be able to meet the growing demand for AAVs, e.g., for clinical development and commercialization. However, current approaches for increasing AAV productivity in E1 complementary producer cells such as HEK293 cells, e.g., high producer cell clone screening and isolation, transfection protocol optimization, and manufacturing process development, are costly and time-consuming.

SUMMARY OF THE INVENTION

[0006] In some embodiments, the disclosure provides a method of producing an adeno-associated virus (AAV) in an E1 complementary producer cell, comprising: (a) transfecting the E1 complementary producer cell with one or more vectors comprising: (i) an E1A adenovirus helper gene; (ii) an adenovirus helper gene selected from E2A, E4, or both; (iii) a viral-associated, non-coding RNA (VA RNA); and (iv) an AAV gene selected from Rep, Cap, or both; (b) culturing the transfected E1 complementary producer cell under conditions suitable for producing the AAV; and (c) purifying the AAV from the cultured E1 complementary producer cell, thereby obtaining the AAV.

[0007] In additional embodiments, the disclosure provides a method of producing an adeno-associated virus (AAV) in an E1 complementary producer cell, comprising: (a) transfecting the E1 complementary producer cell with: (i) a first vector comprising E1A operably linked to a first promoter; (ii) a second vector comprising E2A, E4, and a viral-associated, non-coding RNA (VA RNA), all operably linked to a second promoter; and (iii) a third vector comprising Rep and Cap, both operably linked to a third promoter, wherein a transfection ratio of (i) to each of (ii) and (iii) is about 1:1 to about 3:1; (b) culturing the transfected E1 complementary producer cell under conditions suitable for producing the AAV; and (c) purifying the AAV from the cultured E1 complementary producer cell, thereby obtaining the AAV. Suitably, one or more of the first, second, or third vectors further comprises a helper gene selected from E1B, NP1, NS2, or a combination thereof. Suitably, the E1B is E1B-19k or E1B-55k. Suitably, the helper gene

comprises a combination of NP1 and NS2. In embodiments, the method further comprises transfecting the E1 complementary producer cell with a vector comprising a GOI.

[0008] In further embodiments, the disclosure provides a method of producing an adeno-associated virus (AAV) in an E1 complementary producer cell, comprising: (a) transfecting the E1 complementary producer cell with: (i) a first vector comprising E1A operably linked to a first promoter and an enhancer; (ii) a second vector comprising E2A, E4, and a viral-associated, non-coding RNA (VA RNA), all operably linked to a second promoter; and (iii) a third vector comprising Rep and Cap, each operably linked to a third promoter; (b) culturing the transfected E1 complementary producer cell under conditions suitable for producing the AAV, wherein E1A is expressed at a ratio of about 1:1 to about 3:1 to each of E2A, E4, VA RNA, Rep, and Cap; and (c) purifying the AAV from the cultured E1 complementary producer cell, thereby obtaining the AAV. Suitably, one or more of the first, second, or third vectors further comprises a helper gene selected from E1B, NP1, NS2, or a combination thereof. Suitably, the E1B is E1B-19k or E1B-55k. Suitably, the helper gene comprises a combination of NP1 and NS2. In embodiments, the method further comprises transfecting the E1 complementary producer cell with a vector comprising a GOI.

[0009] In still further embodiments, the disclosure provides a method of producing an adeno-associated virus (AAV) in an E1 complementary producer cell, comprising: (a) transfecting the E1 complementary producer with: (i) a first vector comprising E1A, E2A, E4 and VA RNA, all operably linked to a first promoter; and (ii) a second vector comprising Rep and Cap, operably linked to a second promoter, wherein a copy number ratio of E1A to each of E2A, E4, VA RNA, Rep, and Cap is about 1:1 to about 3:1; (b) culturing the transfected E1 complementary producer cell under conditions suitable for producing the AAV; and (c) purifying the AAV from the cultured E1 complementary producer cell, thereby obtaining the AAV. Suitably, one or both of the first and second vectors further comprises a helper gene selected from E1B, NP1, NS2, or a combination thereof. Suitably, the E1B is E1B-19k or E1B-55k. Suitably, the helper gene comprises a combination of NP1 and NS2. In embodiments, the method further comprises transfecting the E1 complementary producer cell with a vector comprising a GOI.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] The following drawings form part of the present specification and are included to further demonstrate exemplary embodiments of certain aspects of the present invention.

[0011] Figure 1 shows the standard triple transfection plasmids for AAV production in E1 complementary producer cells, as described in embodiments herein: a pHelper plasmid containing E2A, E4, and VA RNA; a pRep-Cap plasmid containing Rep and Cap; and a pAAV-GOI plasmid containing inverted terminal repeat (ITR) sequences and a gene of interest (GOI).

[0012] Figure 2 shows the viral titer results of a transfection experiment described in embodiments herein. HEK293 cells were transfected with the standard triple transfection plasmids (“3x Tnfx”) and one or more additional helper genes (empty vector (control), E1A alone, E1B-19k alone, E1B-55k alone, NP1-NS2, E1A + E1B-19k, E1A + 19B-55k, E1B-19k + E1B-55k, and E1A + E1B-19k + E1B-55k). The crude virus titer was analyzed by droplet digital PCR (ddPCR).

[0013] Figure 3A shows the viral titer results of a transfection experiment described in embodiments herein. HEK293 cells were transfected with the standard triple transfection plasmids (“3x Tnfx”) and one or more additional helper genes (empty vector (control), E1A alone, E1B-19k alone, E1B-55k alone, NP1-NS2, and E1A + NP1-NS2). The crude virus titer was analyzed by droplet digital PCR (ddPCR).

[0014] Figure 3B shows the protein expression results from a transfection experiment described in embodiments herein. Western blot analysis was performed to evaluate the protein expression levels of E1A (289R, 243R, and 171R isoforms), Rep (Rep78 and Rep52 isoforms), and Cap (VP1, VP2, and VP3) in HEK293 cells indicated in Figure 3A and in the Table. The levels of β -actin were measured as a control.

[0015] Figure 4A shows the protein expression results from a transfection experiment described in embodiments herein. The plasmid containing E1A was transfected at a 1:1, 2:1, or 3:1 molar ratio to each of the standard triple transfection plasmids (“3x Tnfx”), indicated as 1xE1A, 2xE1A, or 3xE1A, respectively. Western blot analysis was performed to evaluate the protein expression levels of E1A (289R, 243R, and 171R isoforms), Rep (Rep78 and Rep52 isoforms), and Cap (VP1, VP2, and VP3) in HEK293 cells transfected with the plasmids indicated in the Table.

[0016] Figure 4B shows the viral titer results of a transfection experiment described in embodiments herein. HEK293 cells were transfected with plasmid containing E1A at a 1:1, 2:1, or 3:1 molar ratio to each of the standard triple transfection plasmids (“3x Tnfx”), indicated as 1xE1A, 2xE1A, or 3xE1A, respectively. The crude virus titer was analyzed by droplet digital PCR (ddPCR).

[0017] Figure 5A shows the novel and enhanced triple transfection plasmids for AAV production in E1 complementary producer cells, as described in embodiments herein: a pLHI_Helper plasmid containing E1A, E2A, E4, and VA RNA; a pRep-Cap plasmid containing Rep and Cap; and a pAAV-GOI plasmid containing inverted terminal repeat (ITR) sequences and a gene of interest (GOI).

[0018] Figure 5B shows the viral titer results of a transfection experiment as described in embodiments herein. HEK293 cells were transfected with the standard triple transfection plasmids (as shown in Figure 1) containing pHelper, or the novel and enhanced triple transfection plasmids (as shown in Figure 5) containing pLHI_Helper for AAV2 and AAV9 production. The crude virus titer was analyzed by droplet digital PCR (ddPCR).

DETAILED DESCRIPTION OF THE INVENTION

[0019] Unless otherwise defined herein, scientific and technical terms used in the present disclosure shall have the meanings that are commonly understood by one of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The articles “a” and “an” are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

[0020] The use of the term “or” in the claims is used to mean “and/or,” unless explicitly indicated to refer only to alternatives or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.”

[0021] As used herein, the terms “comprising” (and any variant or form of comprising, such as “comprise” and “comprises”), “having” (and any variant or form of having, such as “have” and “has”), “including” (and any variant or form of including, such as “includes” and “include”) or

“containing” (and any variant or form of containing, such as “contains” and “contain”) are inclusive or open-ended and do not exclude additional, unrecited, elements or method steps.

[0022] The use of the term “for example” and its corresponding abbreviation “e.g.” (whether italicized or not) means that the specific terms recited are representative examples and embodiments of the disclosure that are not intended to be limited to the specific examples referenced or cited unless explicitly stated otherwise.

[0023] As used herein, “between” is a range inclusive of the ends of the range. For example, a number between x and y explicitly includes the numbers x and y , and any numbers that fall within x and y .

[0024] Throughout this application, the term “about” is used to indicate that a value includes the inherent variation of error for the method/device being employed to determine the value. Typically the term is meant to encompass approximately or less than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19% or 20% variability depending on the situation.

[0025] Adeno-associated virus (AAV) has emerged as the vector of choice for gene therapy in over 120 clinical trials worldwide. Improvement of the current standard for AAV production in E1 complementary producer cells, such as HEK293, PER.C6, or CAP® cells, is required to meet the fast-growing demand of recombinant AAV. As described herein, the standard AAV production method comprises the transfection of three plasmids into E1 complementary producer cells: pHelper containing E2A, E4, and VA; pRepCap containing Rep and Cap; and pAAV containing the gene of interest (GOI), termed the “standard triple transfection” and shown in Figure 1. The E1 complementary producer cells natively express the remaining helper genes E1A and E1B, and thus, E1A and E1B are not included in pHelper.

[0026] It was therefore surprisingly discovered that transfection of E1A into E1 complementary producer cells, e.g., HEK293 cells, therefore providing additional E1A above that found natively, significantly improved AAV titer. The increase in titer was generally dose-dependent on the amount of E1A introduced into the E1 complementary producer cells. Thus, provided herein are methods for AAV production in E1 complementary producer cells with higher yields as compared to the current standard triple transfection method.

[0027] In embodiments, the disclosure provides a method of producing an adeno-associated virus (AAV) in an E1 complementary producer cell, comprising: (a) transfecting the E1 complementary producer cell with one or more vectors comprising: (i) an E1A adenovirus helper gene; (ii) an adenovirus helper gene selected from E2A, E4, or both; (iii) a viral-associated, non-coding RNA (VA RNA); and (iv) an AAV gene selected from Rep, Cap, or both; (b) culturing the transfected E1 complementary producer cell under conditions suitable for producing the AAV; and (c) purifying the AAV from the cultured E1 complementary producer cell, thereby obtaining the AAV.

[0028] As used herein, a “vector” or “expression vector” refers to a nucleic acid replicon, such as a plasmid, phage, virus, or cosmid, to which another nucleic acid segment may be attached to bring about the replication and/or expression of the attached nucleic acid segment in a host cell. The term “vector” includes episomal (e.g., plasmids) and non episomal vectors. The term “vector” may also include synthetic vectors. Non-limiting examples of vectors include baculovirus vectors, bacteriophage vectors, plasmids, phagemids, cosmids, fosmids, bacterial artificial chromosomes, viral vectors (e.g. viral vectors based on vaccinia virus, poliovirus, adenovirus, adeno-associated virus, SV40, herpes simplex virus, and the like), P1-based artificial chromosomes, yeast plasmids, yeast artificial chromosomes, and any other vectors specific for specific hosts of interest (e.g., mammalian cells such as the E1 complementary producer cells described herein, including but not limited to HEK293 cells, PER.C6 cells, CAP® cells, and derivatives thereof). Vectors may be introduced into the desired host cells, e.g., HEK293 cells, PER.C6 cells, CAP® cells, or derivatives thereof, by well-known methods, including, but not limited to, transfection, transduction, cell fusion, and lipofection. Vectors can comprise various regulatory elements including, e.g., constitutive and inducible promoters, transcription enhancers, transcription terminators, and the like.

[0029] As used herein, “transfection” means the introduction of an exogenous nucleic acid molecule, e.g., a vector, into a cell. A “transfected” cell comprises an exogenous nucleic acid molecule inside the cell, and a “transformed” cell is one in which the exogenous nucleic acid molecule within the cell induces a phenotypic change in the cell. The transfected nucleic acid molecule can be integrated into the host cell’s genomic DNA and/or can be maintained by the cell, temporarily or for a prolonged period of time, extra-chromosomally. Host cells or organisms that express exogenous nucleic acid molecules or fragments are referred to as “recombinant,”

“transformed,” or “transgenic” organisms. A number of transfection techniques are generally known in the art and include various chemical and physical methods, for example, electroporation, cell injection, calcium phosphate exposure, liposome or polymer-based carrier systems, and the like. *See, e.g.,* Graham et al., *Virology* 52:456 (1973); Sambrook et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, New York (1989); Davis et al., *Basic Methods in Molecular Biology*, Elsevier (1986); and Chu et al., *Gene* 13:197 (1981). Such techniques can be used to introduce one or more exogenous nucleic acid molecules, such as the one or more vectors for producing AAVs described herein, into suitable host cells, such as HEK293 cells.

[0030] A “nucleic acid,” “nucleic acid molecule,” “polynucleotide,” or “oligonucleotide” means a polymeric compound comprising covalently linked nucleotides. The term “nucleic acid” includes RNA and DNA, both of which may be single- or double-stranded. DNA includes, but is not limited to, complementary DNA (cDNA), genomic DNA, plasmid or vector DNA, and synthetic DNA. RNA includes, but is not limited to, mRNA, tRNA, rRNA, snRNA, microRNA, or miRNA.

[0031] In embodiments, the nucleic acids herein have at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or about 100% sequence identity with a reference nucleic acid (or a fragment of the reference nucleic acid). In embodiments, the polypeptides herein have at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or about 100% sequence identity with a reference polypeptide (or a fragment of the reference polypeptide). For example, when referring to the E1A gene, one of ordinary skill in the art would understand that the E1A gene encompasses a nucleic acid having at least 70%, at least 75%, %, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or about 100% sequence identity with a wild-type adenovirus E1A gene, provided that the function of the E1A gene with respect to AAV production is not affected. As used herein, the terms “sequence identity” and “percent identity” in the context of nucleic acids, refer to the percentage of nucleotides that are the same when the nucleic acid sequences are aligned over a specified comparison window. Methods of alignment for determination of sequence identity are well-known can be performed using publicly available databases such as BLAST.

[0032] A “gene” refers to a nucleic acid that encode a polypeptide and includes cDNA and genomic DNA nucleic acid molecules. In some embodiments, “gene” also refers to a non-coding nucleic acid fragment that can act as a regulatory sequence preceding (i.e., 5’) and following (i.e., 3’) the coding sequence.

[0033] As used herein, the term “adeno-associated virus (AAV)” refers to a small sized, replicative-defective nonenveloped virus containing a single stranded DNA of the family Parvoviridae and the genus Dependoparvovirus. Over ten adeno-associated virus serotypes have been identified so far, with serotype AAV2 being the best characterized. Other non-limiting examples of AAV serotypes are ANC80, AAV1, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, and AAV11. In addition to these serotypes, AAV pseudotypes have been developed. An AAV pseudotype contains the capsid of a first serotype and the genome of a second serotype (e.g. the pseudotype AAV2/5 would correspond to an AAV with the genome of serotype AAV2 and the capsid of AAV5).

[0034] As used herein, the term “adenovirus” refers to a nonenveloped virus with an icosahedral nucleocapsid containing a double stranded DNA of the family Adenoviridae. Over 50 adenoviral subtypes have been isolated from humans and many additional subtypes have been isolated from other mammals and birds. These subtypes belong to the family Adenoviridae, which is currently divided into two genera, namely Mastadenovirus and Aviadenovirus. All adenoviruses are morphologically and structurally similar. In humans, however, adenoviruses show diverging immunological properties and are, therefore, divided into serotypes. Two human serotypes of adenovirus, namely Ad2 and Ad5, have been studied intensively and have provided the majority of general information about adenoviruses.

[0035] As used herein, the term “E1 complementary producer cell” refers to a cell line, typically a human cell line, that has the adenovirus E1A and E1B genes integrated or inserted into its genome. E1 complementary producer cells are capable of expressing E1A and E1B natively and are therefore suitable for AAV production without requiring exogenous E1A and/or E1B genes. The E1A and E1B genes may be suitably integrated in the E1 complementary producer cell as part of a longer sequence, e.g., containing additional adenovirus sequences, or the E1A and E1B genes may be suitably integrated in the E1 complementary producer cell without any additional adenovirus sequences, i.e., only the minimum required coding sequences

for expressing E1A and E1B are integrated. Integration of adenovirus genes into mammalian cells, e.g., human cells, is known in the art. E1 complementary producer cells may be derived from any suitable cell line. Exemplary E1 complementary producer cells include, but are not limited to, HEK293 cells, 911 cells, pTG6559 cells, PER.C6 cells, GH329 cells, N52.E6 cells (also known as “CAP®” cells), HeLa-E1 cells, UR cells, VLI-293 cells, Ac51 cells, Ac139 cells, and variants and derivatives thereof. E1 complementary producer cells are further described in, e.g., Kovesdi et al., *Viruses* 2(8):1681-1703 (2010) and Farson et al., *Mol Ther* 14(2):305-311 (2006). In some embodiments, the E1 complementary producer cell of the present disclosure is a HEK293 cell, a PER.C6 cell, or a CAP® cell.

[0036] As used herein, the term “human embryonic kidney 293 (HEK293) cell” refers to a cell line originally derived from human embryonic kidney cells and containing approximately 4.5 kb of Ad5 genome, including the E1A and E1B genes. HEK293 cells are an exemplary E1 complementary producer cell as described herein. Variants of HEK293 cells have been developed and include, e.g., HEK293S, HEK293T, HEK293F, HEK293FT, HEK293FTM, HEK293SG, HEK293SGGD, HEK293H, HEK293E, HEK293MSR, and HEK293A, each of which has distinct characteristics, and all of which contain the E1A and E1B genes. The term “HEK293 cell” as used herein encompasses all HEK293 cell variants and derivatives, including but not limited to the variants described herein. For example, the HEK293T cell line expresses a temperature-sensitive allele of the SV40 T antigen, which enables the amplification of vectors containing the SV40 origin. See, e.g., Yuan et al., “The Scattered Twelve Tribes of HEK293,” *Biomed Pharmacol J* 2018; 11(2).

[0037] As used herein, the term “PER.C6 cell” refers to a cell line derived from human embryonic retinal cells transformed with the E1 region of Ad5. PER.C6 cells natively express E1A and E1B and are an exemplary E1 complementary producer cell as described herein. The term “PER.C6 cell” as used herein encompasses all PER.C6 cell variants and derivatives. PER.C6 cells are further described in, e.g., Fallaux et al., *Human Gene Ther* 9(13):1909-1917 (1998).

[0038] As used herein, the term “CAP® cell” (also referred to herein as “CEVEC’s Amniocyte Production cell” or “N52.E6 cell”) refers to a cell line derived from primary human amniocytes transformed with the E1 region of Ad5 (accessible under RRID:CVCL_IQ88). CAP® cells

natively express E1A and E1B and are an exemplary E1 complementary producer cell as described herein. CAP® cell variants include, but are not limited to, CAP-T (accession ID: CVCL_WI61), which expresses the large T antigen of SV40. See, e.g., Wölfel et al., *BMC Proceedings* 5(Suppl 8):P133 (2011). The term “CAP® cell” as used herein encompasses all CAP® cell variants and derivatives.

[0039] As used herein, the term “E1A” refers to the adenovirus early region 1A (E1A) gene, which is expressed during adenovirus replication in the early phase of the viral life span. The E1A gene produces multiple protein isoforms, including but not limited to 289R, 243R, 217R, 171R, and 55R, with 289R, 243R, and 171R being the major isoforms of the Ad5 E1A gene. As used herein, the term “E1B” refers to the adenovirus early region 1B (E1B) gene, which expresses two proteins: a 55 kDa protein and a 19 kDa protein, termed “E1B-19k” and “E1B-55k,” respectively. As described herein, E1A, E1B-19k, and E1B-55k are adenovirus helper genes expressed by E1 complementary producer cells. As E1A, E1B-19k, and E1B-55k are produced by E1 complementary producer cells in sufficient amounts for AAV production, traditionally E1A and E1B genes are not exogenously introduced into E1 complementary producer cells when producing AAVs.

[0040] As used herein, the term “E2A” refers to the adenovirus E2A helper gene, which encodes a 72 kDa DNA-binding protein that regulates viral replication. As used herein, the term “E4” refers to the adenovirus E4 helper gene, which encodes proteins that modulate viral replication and protein expression, including E4 ORF3 and E4 ORF6. In embodiments, E4 comprises one or both of E4orf3 and E4orf6. As used herein, the term “viral-associated, non-coding RNA (VA RNA)” refers to an adenovirus non-coding RNA that plays a role in regulating translation and includes VAI (or VA_I or VA RNA_I) and VAII (or VA_{II} or VA RNA_{II}). In embodiments, VA RNA comprises one or both of VAI and VAII. In the context of AAV production, the adenovirus genes E2A, E4, and VA RNA mediate AAV replication. As described herein, current methods for producing AAVs in E1 complementary producer cells include transfecting the E1 complementary producer cell with a pHelper plasmid that includes the E2A, E4, and VA RNA genes.

[0041] As used herein, the term “Rep” refers to an AAV coding region or functional homolog thereof that encodes the replication proteins of the virus, which are collectively required for

replicating the viral genome. Functional homologs of the AAV Rep include, e.g., the Rep coding region from the human herpesvirus 6 (HHV-6), which is also known to mediate AAV DNA replication. The Rep coding region encodes at least the genes encoding for AAV Rep78, Rep68, Rep52, and Rep40, or functional homologs thereof. The Rep coding region may be derived from any AAV serotype, e.g., as described herein. The Rep coding region is not required to include all wild-type genes but may be altered (e.g., by insertion, deletion, or mutation of one or more nucleotides) so long as the Rep genes present provide for sufficient replication functions when expressed in the E1 complementary producer cell.

[0042] As used herein, the term “Cap” refers to an AAV coding region or functional homolog thereof that encodes the capsid proteins of the virus. Non-limiting examples of capsid proteins are the AAV capsid proteins VP1, VP2, and VP3. The Cap genes described herein can be derived from any AAV serotype or a combination of AAV serotypes.

[0043] In some embodiments, the E1 complementary producer cell is transfected with one or more vectors comprising one or more of (i) E1A, (ii) one or both of E2A and E4, (iii) VA RNA, and (iv) one or both of Rep and Cap. In certain embodiments, (i), (ii), (iii), and (iv) are on a single vector. In further embodiments, (i), (ii), (iii), and (iv) are on more than one vector. Suitably, (i), (ii), and (iii) can be on a first vector, and (iv) can be on a second vector. In embodiments, (ii) comprises both E2A and E4. In further embodiments, (iv) comprises both Rep and Cap. For example, a first vector can comprise E1A, E2A, E4, and VA RNA, and a second vector can comprise Rep and Cap. Suitably, (i) can be on a first vector, (ii) and (iii) can be on a second vector, and (iv) is on a third vector. For example, a first vector can comprise E1A, a second vector can comprise E2A, E4, and VA RNA, and a third vector can comprise Rep and Cap. Suitably, any one of (i), (ii), (iii), and (iv) can be on a separate vector, e.g., each of (i), (ii), (iii), and (iv) can be on a separate vector. For example, a first vector can comprise E1A, a second vector can comprise E2A and E4, a third vector can comprise VA RNA, and a fourth vector can comprise Rep and Cap. Suitably, the E1 complementary producer cell is a HEK293 cell, a PER.C6 cell, or a CAP® cell.

[0044] In some embodiments, E1A is transfected into the E1 complementary producer cell in a higher amount than any one of E2A, E4, VA RNA, Rep, and Cap. As discussed herein, it was unexpectedly discovered that AAV titers were dose-dependent on amount of E1A expressed in

the E1 complementary producer cells. Thus, in embodiments, the E1A is introduced into the E1 complementary producer cell at a 1.1-fold, 1.2-fold, 1.3-fold, 1.4-fold, 1.5-fold, 1.6-fold, 1.7-fold, 1.8-fold, 1.9-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, or more than 10-fold higher amount than any of E2A, E4, VA RNA, Rep, and Cap. Methods of increasing expression of a particular gene can include, e.g., increasing the amount of the gene introduced into the cell and increasing the transcription level of the gene in the cell. Suitably, the E1 complementary producer cell is a HEK293 cell, a PER.C6 cell, or a CAP® cell.

[0045] Suitably, the vector comprising E1A can be transfected into the E1 complementary producer cell at a ratio of about 1:1 to about 10:1 to each of the vectors comprising E2A, E4, VA RNA, Rep, and/or Cap, or about 1:1 to about 9:1, or about 1:1 to about 8:1, or about 1:1 to about 7:1, or about 1:1 to about 6:1, or about 1:1 to about 5:1, or about 1:1 to about 4:1, or about 1:1 to about 3:1, or about 1:1 to about 2:1, or about 1:1 to about 1.5:1, to each of the vectors comprising E2A, E4, VA RNA, Rep, and/or Cap. In some embodiments, the ratio of the vector comprising E1A to each of the vectors comprising E2A, E4, VA RNA, Rep, and/or Cap transfected into the E1 complementary producer cell is 1:1. In further embodiments, the ratio of the vector comprising E1A to each of the vectors comprising E2A, E4, VA RNA, Rep, and/or Cap transfected into the E1 complementary producer cell is 2:1. In yet further embodiments, the ratio of the vector comprising E1A to each of the vectors comprising E2A, E4, VA RNA, Rep, and/or Cap transfected into the E1 complementary producer cell is 3:1. Suitably, the E1 complementary producer cell is a HEK293 cell, a PER.C6 cell, or a CAP® cell.

[0046] In some embodiments, the expression level of E1A in the E1 complementary producer cell (e.g., HEK293 cell, a PER.C6 cell, or a CAP® cell) is higher as compared to each of E2A, E4, VA RNA, Rep, and/or Cap. Suitably, each of E1A, E2A, E4, VA RNA, Rep, and/or Cap can be operably linked to one or more promoters. In some embodiments, each of E1A, E2A, E4, VA RNA, Rep, and/or Cap is operably linked to a separate promoter. As used herein, the terms “operably linked,” “in operable combination,” and “in operable order” mean that a polynucleotide of interest (e.g., E1A) is linked to a regulatory element (e.g., a promoter) in a manner that allows for expression of the polynucleotide of interest. As used herein, the terms “promoter,” “promoter sequence,” and “promoter region” refer to a polynucleotide capable of binding RNA polymerase and initiating transcription of a downstream coding or non-coding gene sequence. Promoters include constitutive promoters, which allow for continual transcription

of its associated gene; and inducible promoters, which can be switched from an off state to an on state upon addition of an inducer molecule. Promoters can be classified as “strong” or “weak” based their affinity for RNA polymerase and/or sigma factor. A strong promoter has a high rate of transcription initiation as compared to a weak promoter. Non-limiting examples of promoters include spleen focus-forming virus (SFFV) promoter, the human polypeptide chain elongation factor (EF1 α) promoter, the phosphoglycerate kinase (PGK) promoter, the ubiquitin C (UBC) promoter, the cytomegalovirus (CMV) promoter, the chicken beta-actin (CBA) promoter, the tetracycline-controlled transactivator system (also known as the “Tet-On” system or tTA-dependent system, or the reverse tetracycline-controlled transactivator system (also known as the “Tet-Off” system or rtTA-dependent system), the Rous sarcoma virus long terminal repeat (RSV) promoter, the mouse mammary tumor virus (MMTV) promoter, the actin promoter, the cytokeratin 14 promoter, or the cytokeratin 18 promoter. In some embodiments, the promoter operably linked to E1A is a stronger promoter as compared to the promoter(s) operably linked to any of E2A, E4, VA RNA, Rep, and Cap.

[0047] Suitably, the promoter operably linked to E1A can provide 1.1-fold, 1.2-fold, 1.3-fold, 1.4-fold, 1.5-fold, 1.6-fold, 1.7-fold, 1.8-fold, 1.9-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, or more than 10-fold higher expression as compared to the promoter(s) operably linked to any of E2A, E4, VA RNA, Rep, and Cap. In further embodiments, the promoter operably linked to E1A provides substantially a same expression level as compared to the promoter(s) operably linked to any of E2A, E4, VA RNA, Rep, and Cap. As used herein, “substantially similar” or “substantially the same” expression level means that the expression level is within 20%, within 15%, within 10%, within 5%, or within 1% of a reference expression level.

[0048] In some embodiments, E1A is operably linked to a promoter and further operably linked to an enhancer, and each of E2A, E4, VA RNA, Rep, and/or Cap is suitably not linked to an enhancer. As used herein, the term “enhancer” refers to a regulatory element that activate transcription to a higher level than would be the case in their absence, e.g., by recruiting transcription factors. Enhancers can activate promoter transcription over large distances (e.g., up to 1 Mbp away from the transcription start site) and independently of orientation. Non-limiting examples of enhancers include the CMV enhancer, α -fetoprotein MERII enhancer, MCK enhancer, or SV40 polyA enhancer. Suitably, the E1A, which is operably linked to a promoter

and an enhancer, can provide higher expression levels as compared to E2A, E4, VA RNA, Rep, and/or Cap, which are not linked to enhancers.

[0049] Suitably, the one or more vectors can include an origin of replication to increase the number of plasmids produced by the host cell, which leads to increased levels of the proteins encoded by the genes on the one or more vectors. Exemplary origins of replication include, but are not limited to, the SV40 origin of replication, the Epstein-Barr (EBV) origin of replication, and others known to one of ordinary skill in the art.

[0050] In some embodiments, the number of copies of E1A, referred to herein as “copy number,” present on the one or more vectors is higher than the number of copies of each of E2A, E4, VA RNA, Rep, and/or Cap on the one or more vectors. In an example, a single vector can contain 2 or more copies of E1A and a single copy of each of E2A, E4, VA RNA, Rep, and Cap. In another example, for each copy of E2A, E4, VA RNA, Rep, and Cap present on the one or more vectors, at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 copies of E1A are present on the one or more vectors. Suitably, the copy number ratio of E1A to each of E2A, E4, VA RNA, Rep, and/or Cap on the one or more vectors can be about 1:1 to about 10:1, or about 1:1 to about 9:1, or about 1:1 to about 8:1, or about 1:1 to about 7:1, or about 1:1 to about 6:1, or about 1:1 to about 5:1, or about 1:1 to about 4:1, or about 1:1 to about 3:1, or about 1:1 to about 2:1, or about 1:1 to about 1.5:1. In some embodiments, the copy number ratio of E1A to each of E2A, E4, VA RNA, Rep, and/or Cap on the one or more vectors is about 1:1. In further embodiments, the copy number ratio of E1A to each of E2A, E4, VA RNA, Rep, and/or Cap on the one or more vectors is about 2:1. In yet further embodiments, the copy number ratio of E1A to each of E2A, E4, VA RNA, Rep, and/or Cap on the one or more vectors is about 3:1.

[0051] In some embodiments, the method comprises transfecting an additional helper gene into the E1 complementary producer cell. Thus, in embodiments, the one or more vectors further comprises (i) E1A, (ii) one or both of E2A and E4, (iii) VA RNA, (iv) one or both of Rep and Cap; and (v) a helper gene selected from E1B, NP1, NS2, or a combination thereof. Suitably, the E1 complementary producer cell is a HEK293 cell, a PER.C6 cell, or a CAP® cell.

[0052] As discussed herein, E1B is an adenovirus helper gene and encodes the E1B-19k and E1B-55k helper proteins for AAV production. As used herein the terms “NP1” and “NS2” refer

to human bocavirus (HBoV1) genes that encode the NP1 and NS2 nonstructural proteins, respectively. NP1 and NS2 have been discovered as helper genes for AAV replication and were shown to enhance AAV titers when co-expressed with the conventional adenovirus helper genes for E1 complementary producer cells, i.e., E2A, E4, and VA RNA as described herein. See, e.g., Wang et al., *Mol Ther Methods Clin Dev* 11:40-51 (2018). In some embodiments, transfecting one or more of E1B, NP1, and NS2, in combination with (i), (ii), (iii), and (iv) as described herein, further increases AAV titers in E1 complementary producer cells. In certain embodiments, (v) comprises E1B. In further embodiments, (v) comprises NP1 and NS2. In yet further embodiments, (v) comprises E1B, NP1, and NS2. Suitably, the E1 complementary producer cell is a HEK293 cell, a PER.C6 cell, or a CAP® cell.

[0053] In some embodiments, (i), (ii), (iii), (iv), and (v) are on a single vector. In further embodiments, (i), (ii), (iii), (iv), and (v) are on more than one vector. For example, (i), (ii), and (iii) can be on a first vector, (iv) can be on a second vector, and (v) can be on a third vector. In another example, (i), (ii), (iii), and (v) can be on a first vector, and (iv) can be on a second vector. In a further example, (i), (ii), and (iii) can be on a first vector, and (iv) and (v) can be on one or more additional vectors. In a yet further example, (i) and (v) can be on a first vector, and (ii), (iii), and (iv) can be on one or more additional vectors. Suitably, any one of (i), (ii), (iii), (iv), and (v) can be on a separate vector, e.g., each of (i), (ii), (iii), (iv), and (v) can be on a separate vector. In embodiments, a first vector comprises E1A, E2A, E4, and VA RNA, and a second vector comprises Rep and Cap. In additional embodiments, a first vector comprises E1A, E2A, E4, and VA RNA, a second vector comprises Rep and Cap, and a third vector comprises E1B, NP1, NS2, or a combination thereof.

[0054] The E1 complementary producer cells (e.g., HEK293 cells, PER.C6 cells, or CAP® cells) can be cultured, following transfection of (i) E1A, (ii) one or both of E2A and E4, (iii) VA RNA, (iv) one or both of Rep and Cap; (v) a helper gene selected from E1B, NP1, NS2, or a combination thereof; and/or the GOI, using any suitable device, facility, and method described herein. Suitably, expression of (i), (ii), (iii), (iv), and (v) can be induced during the culturing. In certain embodiments, the promoter for any of (i), (ii), (iii), (iv), and/or (v) is a constitutive promoter. In further embodiments, the promoter for any of (i), (ii), (iii), (iv), and/or (v) is an inducible promoter, requiring an inducer molecule to activate transcription as described herein. In one aspect, the expression level of a gene under the control of an inducible promoter can be

modulated by regulating the amount of the inducer molecule. In another aspect, the same inducible promoter can be regulated using more than one inducer molecule to effect different expression levels. In a further aspect, the promoter operably linked to (i) is a stronger promoter than the promoter(s) operably linked to (ii), (iii), (iv), and/or (v). For example, (i) can be under control of a relatively strong promoter such as the CMV or SV40 promoter, and (ii), (iii), (iv), and/or (v) can be under control of a relatively weaker promoter such as the UBC or PGK promoter. In a further aspect, the promoter operably linked to (i) is induced by a different inducer molecule than the promoter operably linked to (ii), (iii), (iv), and/or (v). For example, (i) can be under control of a Tet-On system that is inducible by tetracycline (Tet) or an analog such as doxycycline (Dox); (ii), (iii), (iv), and/or (v) can be under control of a constitutive promoter such as CMV, SV40, UBC, or PGK; and the level of E1A expression can be regulated by the amount of inducer molecule (e.g., Tet or Dox) added to the cell culture. Thus, the amount of inducer molecule added to the cell culture can determine the relative amounts of (i), (ii), (iii), (iv), and/or (v) expressed in the cell culture.

[0055] In embodiments where the one or more vectors comprise (i), (ii), (iii), and (iv), the culturing comprises regulating the expression of (i), (ii), (iii), and (iv), such that E1A is expressed at a ratio of 1:1 to about 10:1 to each of (ii), (iii), and (iv), or about 1:1 to about 9:1, or about 1:1 to about 8:1, or about 1:1 to about 7:1, or about 1:1 to about 6:1, or about 1:1 to about 5:1, or about 1:1 to about 4:1, or about 1:1 to about 3:1, or about 1:1 to about 2:1, or about 1:1 to about 1.5:1 to each of (ii), (iii), and (iv). Suitably, the ratio of the expression of E1A to the expression of each of (ii), (iii), and (iv) is about 1:1. In further embodiments, the ratio of the expression of E1A to the expression of each of (ii), (iii), and (iv) is about 2:1. In yet further embodiments, the ratio of the expression of E1A to the expression of each of (ii), (iii), and (iv) is about 3:1.

[0056] In embodiments where the one or more vectors comprises (i), (ii), (iii), (iv), and (v), the culturing comprises regulating the expression of (i), (ii), (iii), (iv), and (v), such that E1A is expressed at a ratio of 1:1 to about 10:1, or about 1:1 to about 9:1, or about 1:1 to about 8:1, or about 1:1 to about 7:1, or about 1:1 to about 6:1, or about 1:1 to about 5:1, or about 1:1 to about 4:1, or about 1:1 to about 3:1, or about 1:1 to about 2:1, or about 1:1 to about 1.5:1 to each of (ii), (iii), (iv), and (v). Suitably, the ratio of the expression of E1A to the expression of each of (ii), (iii), (iv), and (v) is about 1:1, or about 2:1, or about 3:1.

[0057] In some embodiments, the one or more vectors further comprises a gene of interest (GOI). Suitably, the GOI is on a separate vector from each of (i), (ii), (iii), (iv), and (v). Thus, in embodiments, the method further comprises transfecting the E1 complementary producer cell with a vector comprising a GOI, wherein the GOI is on a separate vector from each of (i), (ii), (iii), (iv), and (v). Suitably, the vector comprising (i) is transfected into the E1 complementary producer cell at a ratio of about 1:1 to about 10:1 to the vector comprising the GOI, or about 1:1 to about 9:1, or about 1:1 to about 8:1, or about 1:1 to about 7:1, or about 1:1 to about 6:1, or about 1:1 to about 5:1, or about 1:1 to about 4:1, or about 1:1 to about 3:1, or about 1:1 to about 2:1, or about 1:1 to about 1.5:1 to the vector comprising the GOI. Suitably, the ratio of the vector comprising (i) to the ratio of the vector comprising the GOI transfected into the E1 complementary producer cell is about 1:1, about 2:1, or about 3:1. Suitably, the E1 complementary producer cell is a HEK293 cell, a PER.C6 cell, or a CAP® cell.

[0058] The vector comprising the GOI can include two inverted terminal repeat (ITR) sequences that flank the GOI. As known in the art, these ITR sequences are single-stranded nucleotide sequences followed downstream by its reverse complement. ITR sequences represent the minimal sequence required for replication, rescue, packaging, and integration of the AAV genome.

[0059] In general, as used herein, the term “GOI” refers to a heterologous gene, i.e., a gene that is not normally joined together and/or not normally associated with a particular host cell. In some embodiments, a heterologous gene is a construct where the coding sequence itself is not found in nature (e.g., a synthetic sequence having codons different from the native gene). Suitably, the GOI can be a reporter gene, a selection gene, or a gene of therapeutic interest.

[0060] As referred to herein, a “reporter gene” is a gene that, upon expression, confers a phenotype upon a cell that can be easily identified and measured. In some embodiments, the reporter gene encodes a fluorescent protein. In further embodiments, the reporter gene comprises a selection gene.

[0061] As referred to herein, a “selection gene” is a gene that encodes a protein having an enzymatic activity, which confers to the host cell the ability to grow in medium lacking an otherwise essential nutrient. Alternatively or additionally, a selection gene may confer resistance to an antibiotic or drug upon the host cell in which the selection gene is expressed. A selection

gene may be used to confer a particular phenotype upon the host cell. When a host cell expresses a selection gene to grow in selective medium, the gene is said to be a positive selection gene. A selection gene can also be used to select against host cells containing a particular gene; a selection gene used in this manner is referred to as a negative selection gene.

[0062] As used herein, the term “gene of therapeutic interest” refers to any functionally relevant nucleotide sequence. Thus, the gene of therapeutic interest of the present disclosure can comprise any desired gene that encodes a protein that is defective or missing from a target cell genome or that encodes a non-native protein having a desired biological or therapeutic effect (e.g., an antiviral function), or the sequence can correspond to a molecule having an antisense or ribozyme function. Representative (non-limiting) examples of suitable genes of therapeutic interest include those used for the treatment of inflammatory diseases, autoimmune, chronic and infectious diseases, including such disorders as AIDS, cancer, neurological diseases, cardiovascular disease, hypercholesterolemia; various blood disorders including various anemias, thalassemia and hemophilia; genetic defects such as cystic fibrosis, Gaucher's Disease, adenosine deaminase (ADA) deficiency, emphysema, etc. Several antisense oligonucleotides (e.g., short oligonucleotides complementary to sequences around the translational initiation site (AUG codon) of an mRNA) that are useful in antisense therapy for cancer and for viral diseases have been described in the art and are also examples of suitable genes of therapeutic interest.

[0063] In additional embodiments, the disclosure provides a method of producing an adeno-associated virus (AAV) in an E1 complementary producer cell, comprising: (a) transfecting the E1 complementary producer cell with: (i) a first vector comprising E1A operably linked to a first promoter; (ii) a second vector comprising E2A, E4, and a viral-associated, non-coding RNA (VA RNA), all operably linked to a second promoter; and (iii) a third vector comprising Rep and Cap, both operably linked to a third promoter, wherein a transfection ratio of (i) to each of (ii) and (iii) is about 1:1 to about 3:1; (b) culturing the transfected E1 complementary producer cell under conditions suitable for producing the AAV; and (c) purifying the AAV from the cultured E1 complementary producer cell, thereby obtaining the AAV. Suitably, one or more of the first, second, or third vectors further comprises a helper gene selected from E1B, NP1, NS2, or a combination thereof. Suitably, the E1B is E1B-19k or E1B-55k. Suitably, the helper gene comprises a combination of NP1 and NS2. In embodiments, the method further comprises

transfecting the E1 complementary producer cell with a vector comprising a GOI. Suitably, the E1 complementary producer cell is a HEK293 cell, a PER.C6 cell, or a CAP® cell.

[0064] In further embodiments, the disclosure provides a method of producing an adeno-associated virus (AAV) in an E1 complementary producer cell, comprising: (a) transfecting the E1 complementary producer cell with: (i) a first vector comprising E1A operably linked to a first promoter and an enhancer; (ii) a second vector comprising E2A, E4, and a viral-associated, non-coding RNA (VA RNA), all operably linked to a second promoter; and (iii) a third vector comprising Rep and Cap, each operably linked to a third promoter; (b) culturing the transfected E1 complementary producer cell under conditions suitable for producing the AAV, wherein E1A is expressed at a ratio of about 1:1 to about 3:1 to each of E2A, E4, VA RNA, Rep, and Cap; and (c) purifying the AAV from the cultured E1 complementary producer cell, thereby obtaining the AAV. Suitably, one or more of the first, second, or third vectors further comprises a helper gene selected from E1B, NP1, NS2, or a combination thereof. Suitably, the E1B is E1B-19k or E1B-55k. Suitably, the helper gene comprises a combination of NP1 and NS2. In embodiments, the method further comprises transfecting the E1 complementary producer cell with a vector comprising a GOI. Suitably, the E1 complementary producer cell is a HEK293 cell, a PER.C6 cell, or a CAP® cell.

[0065] In still further embodiments, the disclosure provides a method of producing an adeno-associated virus (AAV) in an E1 complementary producer cell, comprising: (a) transfecting the E1 complementary producer cell with: (i) a first vector comprising E1A, E2A, E4 and VA RNA, all operably linked to a first promoter; and (ii) a second vector comprising Rep and Cap, operably linked to a second promoter, wherein a copy number ratio of E1A to each of E2A, E4, VA RNA, Rep, and Cap is about 1:1 to about 3:1; (b) culturing the transfected E1 complementary producer cell under conditions suitable for producing the AAV; and (c) purifying the AAV from the cultured E1 complementary producer cell, thereby obtaining the AAV. Suitably, one or both of the first and second vectors further comprises a helper gene selected from E1B, NP1, NS2, or a combination thereof. Suitably, the E1B is E1B-19k or E1B-55k. Suitably, the helper gene comprises a combination of NP1 and NS2. In embodiments, the method further comprises transfecting the E1 complementary producer cell with a vector comprising a GOI. Suitably, the E1 complementary producer cell is a HEK293 cell, a PER.C6 cell, or a CAP® cell.

[0066] In embodiments, provided herein is a method of treatment in a subject in need thereof with an AAV comprising: transfecting a E1 complementary producer cell (e.g., HEK293 cell, PER.C6 cell, or CAP® cell) with one or more vectors comprising: (i) an E1A adenovirus helper gene; (ii) an adenovirus helper gene selected from E2A, E4, or both; (iii) a viral-associated, non-coding RNA (VA RNA); and (iv) an AAV gene selected from Rep, Cap, or both; producing the AAV; harvesting the AAV; and administering the AAV to the subject. Methods of producing the AAV are further described herein. Suitably, the methods are used to treat the subject with a GOI, e.g., a gene of therapeutic interest. Administration to a human subject can include, for example, inhalation, injection, or intravenous administration, as well as other administration methods known in the art.

[0067] As discussed herein, methods of the present disclosure advantageously provide a higher titer of the AAV produced by the E1 complementary producer cells, as compared to a method that does not comprise transfecting the E1 complementary producer cell with E1A. In embodiments, the methods of the present disclosure provide at least 1.1-fold, at least 1.2-fold, at least 1.3-fold, at least 1.4-fold, at least 1.5-fold, at least 1.6-fold, at least 1.7-fold, at least 1.8-fold, at least 1.9-fold, at least 2-fold, at least 3-fold, at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold, or at least 10-fold or higher titer of the AAV as compared to a method that does not comprise transfecting the E1 complementary producer cell with E1A. Suitably, the E1 complementary producer cell is a HEK293 cell, a PER.C6 cell, or a CAP® cell.

[0068] The methods of producing the AAVs can be used in a continuous manufacturing system. Culturing conditions for E1 complementary producer cells, e.g., HEK293 cells, PER.C6 cells, or CAP® cells, are known in the field. In general, HEK293 cells, PER.C6 cells, and CAP® cells are suspension cultures, which are grown in culture flasks or large suspension vats that allow for a large surface area for gas and nutrient exchange. Suspension cell cultures often utilize a stirring or agitation mechanism to provide appropriate mixing. Media and conditions for maintaining cells in suspension are known to one of ordinary skill in the art. In exemplary embodiments, the use of a suspension cell culture allows for the production of large volumes of AAV, with high productivity and prolonged culture conditions to allow for multiple harvests of AAVs for each batch of starting cells.

[0069] Production methods can utilize any suitable reactor(s) including but not limited to stirred tank, airlift, fiber, microfiber, hollow fiber, ceramic matrix, fluidized bed, fixed bed, and/or spouted bed bioreactors. As used herein, “reactor” can include a fermenter or fermentation unit, or any other reaction vessel and the term “reactor” is used interchangeably with “fermenter.” For example, in some aspects, an example bioreactor unit can perform one or more, or all, of the following: feeding of nutrients and/or carbon sources, injection of suitable gas (e.g., oxygen), inlet and outlet flow of fermentation or cell culture medium, separation of gas and liquid phases, maintenance of temperature, maintenance of oxygen and CO₂ levels, maintenance of pH level, agitation (e.g., stirring), and/or cleaning/sterilizing. Example reactor units, such as a fermentation unit, may contain multiple reactors within the unit, for example the unit can have 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, or 100, or more bioreactors in each unit and/or a facility may contain multiple units having a single or multiple reactors within the facility. In various embodiments, the bioreactor can be suitable for batch, semi fed-batch, fed-batch, perfusion, and/or a continuous fermentation processes. Any suitable reactor diameter can be used. In embodiments, the bioreactor can have a volume between about 100 mL and about 50,000 L. Non-limiting examples include a volume of 100 mL, 250 mL, 500 mL, 750 mL, 1 liter, 2 liters, 3 liters, 4 liters, 5 liters, 6 liters, 7 liters, 8 liters, 9 liters, 10 liters, 15 liters, 20 liters, 25 liters, 30 liters, 40 liters, 50 liters, 60 liters, 70 liters, 80 liters, 90 liters, 100 liters, 150 liters, 200 liters, 250 liters, 300 liters, 350 liters, 400 liters, 450 liters, 500 liters, 550 liters, 600 liters, 650 liters, 700 liters, 750 liters, 800 liters, 850 liters, 900 liters, 950 liters, 1000 liters, 1500 liters, 2000 liters, 2500 liters, 3000 liters, 3500 liters, 4000 liters, 4500 liters, 5000 liters, 6000 liters, 7000 liters, 8000 liters, 9000 liters, 10,000 liters, 15,000 liters, 20,000 liters, and/or 50,000 liters. Additionally, suitable reactors can be multi-use, single-use, disposable, or non-disposable and can be formed of any suitable material including metal alloys such as stainless steel (e.g., 316L or any other suitable stainless steel) and Inconel, plastics, and/or glass.

[0070] In embodiments and unless stated otherwise herein, the devices, facilities, and methods described herein can also include any suitable unit operation and/or equipment not otherwise mentioned, such as operations and/or equipment for separation, purification, and isolation of such products. Any suitable facility and environment can be used, such as traditional stick-built facilities, modular, mobile and temporary facilities, or any other suitable construction, facility, and/or layout. For example, in some embodiments modular clean-rooms can be used.

Additionally and unless otherwise stated, the devices, systems, and methods described herein can be housed and/or performed in a single location or facility or alternatively be housed and/or performed at separate or multiple locations and/or facilities.

[0071] All references cited herein, including patents, patent applications, papers, textbooks and the like, and the references cited therein, to the extent that they are not already, are hereby incorporated herein by reference in their entirety.

Additional Exemplary Embodiments

[0072] Embodiment 1 is a method of producing an adeno-associated virus (AAV) in an E1 complementary producer cell, comprising: (a) transfecting the E1 complementary producer cell with one or more vectors comprising: (i) an E1A adenovirus helper gene; (ii) an adenovirus helper gene selected from E2A, E4, or both; (iii) a viral-associated, non-coding RNA (VA RNA); and (iv) an AAV gene selected from Rep, Cap, or both; (b) culturing the transfected E1 complementary producer cell under conditions suitable for producing the AAV; and (c) purifying the AAV from the cultured E1 complementary producer cell, thereby obtaining the AAV.

[0073] Embodiment 2 includes the method of embodiment 1, wherein (i), (ii), (iii), and (iv) are on a single vector.

[0074] Embodiment 3 includes the method of embodiment 1, wherein (i), (ii), (iii), and (iv) are on more than one vector.

[0075] Embodiment 4 includes the method of embodiment 3, wherein (i), (ii), and (iii) are on a first vector, and (iv) is a second vector.

[0076] Embodiment 5 includes the method of embodiment 3, wherein (i) is on a first vector, (ii) and (iii) are on a second vector, and (iv) is on a third vector.

[0077] Embodiment 6 includes the method of embodiment 3, wherein each of (i), (ii), (iii), and (iv) is on a separate vector.

[0078] Embodiment 7 includes the method of embodiment 5 or 6, wherein the vector comprising (i) is transfected into the E1 complementary producer cell at a ratio of about 1:1 to about 5:1 to each of the vectors comprising (ii), (iii), and (iv).

[0079] Embodiment 8 includes the method of embodiment 7, wherein the ratio of the vector comprising (i) to each of the vectors comprising (ii), (iii), and (iv) transfected into the E1 complementary producer cell is 1:1.

[0080] Embodiment 9 includes the method of embodiment 7, wherein the ratio of the vector comprising (i) to each of the vectors comprising (ii), (iii), and (iv) transfected into the E1 complementary producer cell is 2:1.

[0081] Embodiment 10 includes the method of embodiment 7, wherein the ratio of the vector comprising (i) to each of the vectors comprising (ii), (iii), and (iv) transfected into the E1 complementary producer cell is 3:1.

[0082] Embodiment 11 includes the method of any one of embodiments 1 to 10, wherein (i), (ii), (iii), and (iv) are operably linked to one or more promoters.

[0083] Embodiment 12 includes the method of embodiment 11, wherein each of (i), (ii), (iii), and (iv) is operably linked to a separate promoter.

[0084] Embodiment 13 includes the method of embodiment 12, wherein the promoter operably linked to (i) provides substantially a same expression level as compared to the promoters operably linked to (ii), (iii), and (iv).

[0085] Embodiment 14 includes the method of embodiment 12, wherein the promoter operably linked to (i) provides at least 2-fold higher expression as compared to the promoters operably linked to (ii), (iii), and (iv).

[0086] Embodiment 15 includes the method of embodiment 12, wherein the promoter operably linked to (i) provides at least 3-fold higher expression as compared to the promoters operably linked to (ii), (iii), and (iv).

[0087] Embodiment 16 includes the method of any one of embodiments 11 to 15, wherein (i) is operably linked to a promoter and further operably linked to an enhancer, and wherein (ii), (iii), and (iv) are not operably linked to an enhancer.

[0088] Embodiment 17 includes the method of any one of embodiments 1 to 16, wherein a copy number ratio of (i) to each of (ii), (iii), and (iv) on the one or more vectors is about 1:1 to about 5:1.

[0089] Embodiment 18 includes the method of embodiment 17, wherein the copy number ratio of (i) to each of (ii), (iii), and (iv) on the one or more vectors is 1:1.

[0090] Embodiment 19 includes the method of embodiment 17, wherein the copy number ratio of (i) to each of (ii), (iii), and (iv) on the one or more vectors is 2:1.

[0091] Embodiment 20 includes the method of embodiment 17, wherein the copy number ratio of (i) to each of (ii), (iii), and (iv) on the one or more vectors is 3:1.

[0092] Embodiment 21 includes the method of any one of embodiments 1 to 20, wherein (ii) comprises E2A and E4.

[0093] Embodiment 22 includes the method of any one of embodiments 1 to 21, wherein (iv) comprises Rep and Cap.

[0094] Embodiment 23 includes the method of any one of embodiments 1 to 22, wherein the culturing comprises expressing E1A at a ratio of about 1:1 to about 5:1 to each of (ii), (iii), and (iv).

[0095] Embodiment 24 includes the method of embodiment 23, wherein the ratio of the expression of E1A to the expression of each of (ii), (iii), and (iv) is 1:1, 2:1, or 3:1.

[0096] Embodiment 25 includes the method of any one of embodiments 1 to 24, wherein the one or more vectors further comprises (v) a helper gene selected from E1B, NP1, NS2, or a combination thereof.

[0097] Embodiment 26 includes the method of embodiment 25, wherein the E1B is E1B-19k or E1B-55k.

[0098] Embodiment 27 includes the method of embodiment 25, wherein (v) comprises NP1 and NS2.

[0099] Embodiment 28 includes the method of any one of embodiments 25 to 27, wherein (i) and (v) are on a single vector.

[00100] Embodiment 29 includes the method of any one of embodiments 25 to 27, wherein (v) is on a separate vector from (i), (ii), (iii), and (iv).

[00101] Embodiment 30 includes the method of any one of embodiments 1 to 29, wherein the one or more vectors further comprises a gene of interest.

[00102] Embodiment 31 includes the method of any one of embodiments 1 to 29, further comprising transfecting the E1 complementary producer cell with a vector comprising a gene of interest, wherein the gene or interest is on a separate vector from (i), (ii), (iii), and (iv).

[00103] Embodiment 32 includes the method of any one of embodiments 1 to 31, wherein the method produces at least 1.5-fold or higher titer of the AAV as compared to a method that does not comprise transfecting an E1 complementary producer cell with an E1A adenovirus helper gene.

[00104] Embodiment 33 is a method of producing an adeno-associated virus (AAV) in an E1 complementary producer cell, comprising: (a) transfecting the E1 complementary producer cell with: (i) a first vector comprising E1A operably linked to a first promoter; (ii) a second vector comprising E2A, E4, and a viral-associated, non-coding RNA (VA RNA), all operably linked to a second promoter; and (iii) a third vector comprising Rep and Cap, both operably linked to a third promoter, wherein a transfection ratio of (i) to each of (ii) and (iii) is about 1:1 to about 3:1; (b) culturing the transfected E1 complementary producer cell under conditions suitable for producing the AAV; and (c) purifying the AAV from the cultured E1 complementary producer cell, thereby obtaining the AAV.

[00105] Embodiment 34 is a method of producing an adeno-associated virus (AAV) in an E1 complementary producer cell, comprising: (a) transfecting the E1 complementary producer cell with: (i) a first vector comprising E1A operably linked to a first promoter and an enhancer; (ii) a second vector comprising E2A, E4, and a viral-associated, non-coding RNA (VA RNA), all operably linked to a second promoter; and (iii) a third vector comprising Rep and Cap, each operably linked to a third promoter; culturing the transfected E1 complementary producer cell under conditions suitable for producing the AAV, wherein E1A is expressed at a ratio of about 1:1 to about 3:1 to each of E2A, E4, VA RNA, Rep, and Cap; and purifying the AAV from the cultured E1 complementary producer cell, thereby obtaining the AAV.

[00106] Embodiment 35 is a method of producing an adeno-associated virus (AAV) in an E1 complementary producer cell, comprising: (a) transfecting the E1 complementary producer cell with: (i) a first vector comprising E1A, E2A, E4 and VA RNA, all operably linked to a first promoter; and (ii) a second vector comprising Rep and Cap, operably linked to a second promoter, wherein a copy number ratio of E1A to each of E2A, E4, VA RNA, Rep, and Cap is

about 1:1 to about 3:1; (b) culturing the transfected E1 complementary producer cell under conditions suitable for producing the AAV; and (c) purifying the AAV from the cultured E1 complementary producer cell, thereby obtaining the AAV.

[00107] Embodiment 36 includes the method of embodiment 33 or 34, wherein one or more of the first, second, or third vectors further comprises a helper gene selected from E1B, NP1, NS2, or a combination thereof.

[00108] Embodiment 37 includes the method of embodiment 35, wherein one or both of the first or second vectors further comprises a helper gene selected from E1B, NP1, NS2, or a combination thereof.

[00109] Embodiment 38 includes the method of any one of embodiments 33 to 35, further comprising transfecting the E1 complementary producer cell with a vector comprising helper gene selected from E1B, NP1, NS2, or a combination thereof.

[00110] Embodiment 39 includes the method of any one of embodiments 36 to 38, wherein the E1B is E1B-19k or E1B-55k.

[00111] Embodiment 40 includes the method of any one of embodiments 36 to 38, wherein the helper gene comprises a combination of NP1 and NS2.

[00112] Embodiment 41 includes the method of any one of embodiments 33 to 40, further comprising transfecting the E1 complementary producer cell with a vector comprising a gene of interest.

[00113] Embodiment 42 includes the method of any one of embodiments 1 to 41, wherein the E1 complementary producer cell is a HEK293 cell, a 911 cell, a pTG6559 cell, a PER.C6 cell, a GH329 cell, an N52.E6 (CAP®) cell, a HeLa-E1 cell, an UR cell, a VLI-293 cell, an Ac51 cell, an Ac139 cell, or a variant or derivative thereof.

[00114] Embodiment 43 includes the method of embodiment 42, wherein the E1 complementary producer cell is a HEK293 cell, a PER.C6 cell, or a N52.E6 (CAP®) cell.

[00115] Embodiment 44 includes the method of embodiment 43, wherein the E1 complementary producer cell is a HEK293 cell.

[00116] Embodiment 45 includes the method of embodiment 44, wherein the HEK293 cell is a HEK293S cell, a HEK293T cell, a HEK293F cell, a HEK293FT cell, a HEK293FTM cell, a HEK293SG cell, a HEK293SGGD cell, a HEK293H cell, a HEK293E cell, a HEK293MSR cell, a HEK293A cell, or a combination thereof.

[00117] Embodiment 46 includes the method of embodiment 45, wherein the HEK293 cell is a HEK293T cell.

[00118] Embodiment 47 includes the method of embodiment 43, wherein the E1 complementary producer cell is a PER.C6 cell.

[00119] Embodiment 48 includes the method of embodiment 43, wherein the E1 complementary producer cell is a N52.E6 (CAP®) cell.

EXAMPLES

Example 1. Screening of candidate helper genes for AAV production

[00120] To identify new helper genes for improving AAV production by triple transfection, the full length cDNA for E1A, E1B-19k, E1B-55k, and human bocavirus NP1 and NS2 were synthesized. The cDNA for the additional helper genes were subcloned between the CMV promoter and the bovine growth hormone polyadenylation (BGH poly(A)) signal in the pcDNA3.1 vector to allow overexpression. NP1 and NS2 were linked with an internal ribosome entry site (IRES) for dual expression in a single mRNA transcript. The candidate plasmids contained: E1A alone (SEQ ID NO:1), E1B-19k alone (SEQ ID NO:2), E1B-55k alone (SEQ ID NO:3), NP1-NS2 (SEQ ID NO:4), E1A + E1B-19k, E1A + E1B-55k, E1B-19k + E1B-55k, or E1A + E1B-19k + E1B-55k.

[00121] To test these candidate plasmids, HEK293 cells were grown in 30 mL culture in 125 mL shaker flasks and transfected with the standard triple transfection plasmids shown in Figure 1 and a candidate helper plasmid. For the Rep/Cap plasmid, the pRep2-Cap9 was used, and for the pAAV plasmid, the pAAV-CMV-GFP was used for the production of AAV9.GFP viruses. The same amount of empty vector pcDNA3.1 was co-transfected as a control. Each candidate helper plasmid was tested in duplicate.

[00122] The crude viruses were harvested three days after transfection and subjected to droplet digital PCR (ddPCR) titer analysis to quantify the viral genome containing particles per milliliter

(vg/mL). The results in Figure 2 showed that the additional expression of E1A, or E1A plus E1B isoforms, significantly increased the titer by nearly 100% as compared to the control, while expression of the E1B isoforms or the bocavirus NP1 and NS2 had minimal improvement for the AAV titer.

Example 2. Confirmation of E1A over-expression for AAV production

[00123] The experiments in Example 1 were repeated with the following conditions: empty vector (control), E1A alone, E1B-19k alone, E1B-55k alone, NP1-NS2, and E1A + NP1-NS2, and the crude virus titer results were analyzed by ddPCR. As shown in Figure 3A, E1A alone or E1A plus the bocavirus NP1 and NS2 genes increased the AAV titer by about 50%, while bocavirus genes alone had no effect.

[00124] To confirm the over-expression of E1A, Western blot analysis was performed to evaluate E1A protein expression (transfection conditions shown in the Table of Figure 3B). As shown in Figure 3B, the additional co-transfection of E1A expressed higher levels of all three isoforms of E1A protein (289R, 243R, and 171R) in samples 3, 4, 11, and 12, which had plasmid-transfected E1A, as compared to samples 1 and 2, which only contained E1A proteins endogenously expressed by HEK293 cells. β -actin levels were measured as a control, and no significant differences were found between the different conditions. The protein expression levels of Rep (Rep78 and Rep52 isoforms) and Cap (VP1, VP2, and VP3) were also determined, and no significant difference was detected between the E1A-transfected samples and the controls. Thus, E1A overexpression may improve AAV titer through mechanisms other than regulation of Rep and Cap protein expression.

Example 3. Dosage-dependent improvement by E1A for AAV production

[00125] The E1A function for AAV titer improvement was further tested by titration of the E1A plasmids at 1:1, 2:1, or 3:1 molar ratio to the pHelper plasmid, indicated in the Table in Figure 4A as 1xE1A, 2xE1A, or 3xE1A, respectively. Transfection and AAV production were performed in the same manner as Example 1. The increase of E1A overexpression was confirmed by Western blot analysis, as shown in Figure 4A. The AAV9.GFP titer increased in an E1A-dosage dependent manner, as shown in Figure 4B.

Example 4. Novel and enhanced triple transfection for AAV production

[00126] To simplify the transfection experiment, the E1A expression cassette was subcloned from the pcDNA3.1-E1A plasmid of Example 1, into the standard pHelper vector shown in Figure 1. The new helper plasmid, termed “pLHI_Helper” (SEQ ID NO:5), was transfected into HEK293 cells in lieu of the conventional pHelper as shown in Figure 5A. Compared to the standard triple transfection, the pLHI_Helper-mediated triple transfection significantly improved the AAV2.GFP and AAV9.GFP production by 52.2% and 36.1%, respectively, over the control standard triple transfection using pHelper, as shown in Figure 5B.

SEQUENCES

[00127] SEQ ID NO:1 - Exemplary plasmid containing E1A (pcDNA3.1-E1A)

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[00128] SEQ ID NO:2 - Exemplary plasmid containing E1B-19k (pcDNA3.1-E1B-19K)

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[00129] SEQ ID NO:3 - Exemplary plasmid containing E1B-55k (pcDNA3.1-E1B-55K)

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[00130] SEQ ID NO:4 - Exemplary plasmid containing NP1 and NS2 (pcDNA3.1-NP1-NS2)

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[00131] SEQ ID NO:5 - Exemplary plasmid containing E1A, E2A, E4, and VA RNA

(pLHI_Helper)

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ggcgcgatggatcc

WHAT IS CLAIMED IS:

1. A method of producing an adeno-associated virus (AAV) in an E1 complementary producer cell, comprising:
 - a. transfecting the E1 complementary producer cell with one or more vectors comprising:
 - i. an E1A adenovirus helper gene;
 - ii. an adenovirus helper gene selected from E2A, E4, or both;
 - iii. a viral-associated, non-coding RNA (VA RNA); and
 - iv. an AAV gene selected from Rep, Cap, or both;
 - b. culturing the transfected E1 complementary producer cell under conditions suitable for producing the AAV; and
 - c. purifying the AAV from the cultured E1 complementary producer cell, thereby obtaining the AAV.
2. The method of claim 1, wherein (i), (ii), (iii), and (iv) are on a single vector.
3. The method of claim 1, wherein (i), (ii), (iii), and (iv) are on more than one vector.
4. The method of claim 3, wherein (i), (ii), and (iii) are on a first vector, and (iv) is a second vector.
5. The method of claim 3, wherein (i) is on a first vector, (ii) and (iii) are on a second vector, and (iv) is on a third vector.
6. The method of claim 3, wherein each of (i), (ii), (iii), and (iv) is on a separate vector.
7. The method of claim 5 or 6, wherein the vector comprising (i) is transfected into the E1 complementary producer cell at a ratio of about 1:1 to about 5:1 to each of the vectors comprising (ii), (iii), and (iv).
8. The method of claim 7, wherein the ratio of the vector comprising (i) to each of the vectors comprising (ii), (iii), and (iv) transfected into the E1 complementary producer cell is 1:1.

9. The method of claim 7, wherein the ratio of the vector comprising (i) to each of the vectors comprising (ii), (iii), and (iv) transfected into the E1 complementary producer cell is 2:1.
10. The method of claim 7, wherein the ratio of the vector comprising (i) to each of the vectors comprising of (ii), (iii), and (iv) transfected into the E1 complementary producer cell is 3:1.
11. The method of any one of claims 1 to 10, wherein (i), (ii), (iii), and (iv) are operably linked to one or more promoters.
12. The method of claim 11, wherein each of (i), (ii), (iii), and (iv) is operably linked to a separate promoter.
13. The method of claim 12, wherein the promoter operably linked to (i) provides substantially a same expression level as compared to the promoters operably linked to (ii), (iii), and (iv).
14. The method of claim 12, wherein the promoter operably linked to (i) provides at least 2-fold higher expression as compared to the promoters operably linked to (ii), (iii), and (iv).
15. The method of claim 12, wherein the promoter operably linked to (i) provides at least 3-fold higher expression as compared to the promoters operably linked to (ii), (iii), and (iv).
16. The method of any one of claims 11 to 15, wherein (i) is operably linked to a promoter and further operably linked to an enhancer, and wherein (ii), (iii), and (iv) are not operably linked to an enhancer.
17. The method of any one of claims 1 to 16, wherein a copy number ratio of (i) to each of (ii), (iii), and (iv) on the one or more vectors is about 1:1 to about 5:1.
18. The method of claim 17, wherein the copy number ratio of (i) to each of (ii), (iii), and (iv) on the one or more vectors is 1:1.

19. The method of claim 17, wherein the copy number ratio of (i) to each of (ii), (iii), and (iv) on the one or more vectors is 2:1.
20. The method of claim 17, wherein the copy number ratio of (i) to each of (ii), (iii), and (iv) on the one or more vectors is 3:1.
21. The method of any one of claims 1 to 20, wherein (ii) comprises E2A and E4.
22. The method of any one of claims 1 to 21, wherein (iv) comprises Rep and Cap.
23. The method of any one of claims 1 to 22, wherein the culturing comprises expressing E1A at a ratio of about 1:1 to about 5:1 to each of (ii), (iii), and (iv).
24. The method of claim 23, wherein the ratio of the expression of E1A to the expression of each of (ii), (iii), and (iv) is 1:1, 2:1, or 3:1.
25. The method of any one of claims 1 to 24, wherein the one or more vectors further comprises (v) a helper gene selected from E1B, NP1, NS2, or a combination thereof.
26. The method of claim 25, wherein the E1B is E1B-19k or E1B-55k.
27. The method of claim 25, wherein (v) comprises NP1 and NS2.
28. The method of any one of claims 25 to 27, wherein (i) and (v) are on a single vector.
29. The method of any one of claims 25 to 27, wherein (v) is on a separate vector from (i), (ii), (iii), and (iv).
30. The method of any one of claims 1 to 29, wherein the one or more vectors further comprises a gene of interest.
31. The method of any one of claims 1 to 29, further comprising transfecting the E1 complementary producer cell with a vector comprising a gene of interest, wherein the gene of interest is on a separate vector from (i), (ii), (iii), and (iv).

32. The method of any one of claims 1 to 31, wherein the method produces at least 1.5-fold or higher titer of the AAV as compared to a method that does not comprise transfecting a E1 complementary producer cell with an E1A adenovirus helper gene.
33. A method of producing an adeno-associated virus (AAV) in an E1 complementary producer cell, comprising:
- a. transfecting the E1 complementary producer cell with:
 - i. a first vector comprising E1A operably linked to a first promoter;
 - ii. a second vector comprising E2A, E4, and a viral-associated, non-coding RNA (VA RNA), all operably linked to a second promoter; and
 - iii. a third vector comprising Rep and Cap, both operably linked to a third promoter,wherein a transfection ratio of (i) to each of (ii) and (iii) is about 1:1 to about 3:1;
 - b. culturing the transfected E1 complementary producer cell under conditions suitable for producing the AAV; and
 - c. purifying the AAV from the cultured E1 complementary producer cell, thereby obtaining the AAV.
34. A method of producing an adeno-associated virus (AAV) in an E1 complementary producer cell, comprising:
- a. transfecting the E1 complementary producer cell with:
 - i. a first vector comprising E1A operably linked to a first promoter and an enhancer;
 - ii. a second vector comprising E2A, E4, and a viral-associated, non-coding RNA (VA RNA), all operably linked to a second promoter; and
 - iii. a third vector comprising Rep and Cap, each operably linked to a third promoter;
 - b. culturing the transfected E1 complementary producer cell under conditions suitable for producing the AAV, wherein E1A is expressed at a ratio of about 1:1 to about 3:1 to each of E2A, E4, VA RNA, Rep, and Cap; and
 - c. purifying the AAV from the cultured E1 complementary producer cell, thereby obtaining the AAV.

35. A method of producing an adeno-associated virus (AAV) in an E1 complementary producer cell, comprising:
- a. transfecting the E1 complementary producer cell with:
 - i. a first vector comprising E1A, E2A, E4 and VA RNA, all operably linked to a first promoter; and
 - ii. a second vector comprising Rep and Cap, operably linked to a second promoter,wherein a copy number ratio of E1A to each of E2A, E4, VA RNA, Rep, and Cap is about 1:1 to about 3:1;
 - b. culturing the transfected E1 complementary producer cell under conditions suitable for producing the AAV; and
 - c. purifying the AAV from the cultured E1 complementary producer cell, thereby obtaining the AAV.
36. The method of claim 33 or 34, wherein one or more of the first, second, or third vectors further comprises a helper gene selected from E1B, NP1, NS2, or a combination thereof.
37. The method of claim 35, wherein one or both of the first or second vectors further comprises a helper gene selected from E1B, NP1, NS2, or a combination thereof.
38. The method of any one of claims 33 to 35, further comprising transfecting the E1 complementary producer cell with a vector comprising helper gene selected from E1B, NP1, NS2, or a combination thereof.
39. The method of any one of claims 36 to 38, wherein the E1B is E1B-19k or E1B-55k.
40. The method of any one of claims 36 to 38, wherein the helper gene comprises a combination of NP1 and NS2.
41. The method of any one of claims 33 to 40, further comprising transfecting the E1 complementary producer cell with a vector comprising a gene of interest.
42. The method of any one of claims 1 to 41, wherein the E1 complementary producer cell is a HEK293 cell, a 911 cell, a pTG6559 cell, a PER.C6 cell, a GH329 cell, an N52.E6

(CAP®) cell, a HeLa-E1 cell, an UR cell, a VLI-293 cell, an Ac51 cell, an Ac139 cell, or a variant or derivative thereof.

43. The method of claim 42, wherein the E1 complementary producer cell is a HEK293 cell, a PER.C6 cell, or a N52.E6 (CAP®) cell.
44. The method of claim 43, wherein the E1 complementary producer cell is a HEK293 cell.
45. The method of claim 44, wherein the HEK293 cell is a HEK293S cell, a HEK293T cell, a HEK293F cell, a HEK293FT cell, a HEK293FTM cell, a HEK293SG cell, a HEK293SGGD cell, a HEK293H cell, a HEK293E cell, a HEK293MSR cell, a HEK293A cell, or a combination thereof.
46. The method of claim 45, wherein the HEK293 cell is a HEK293T cell.
47. The method of claim 43, wherein the E1 complementary producer cell is a PER.C6 cell.
48. The method of claim 43, wherein the E1 complementary producer cell is a N52.E6 (CAP®) cell.