TITLE OF THE INVENTION:

Recombinantly-Modified Adeno-Associated Virus Helper Vectors and Their Use to Improve the Packaging Efficiency of Recombinantly-Modified Adeno-Associated Virus

FIELD OF THE INVENTION:

[0001] The present invention is directed to recombinantly-modified adeno-associated virus (AAV) helper vectors that are capable of increasing the packaging efficiency of recombinantly-modified adeno-associated virus (rAAV) and their use to improve the packaging efficiency of such rAAV. The present invention is particularly directed to recombinantly-modified adeno-associated virus (AAV) helper vectors that have been further modified to replace (or augment) the **P5** and/or **P40** promoter sequences that are natively associated with the Rep proteins encoded by such rAAV with AAV **P5** and/or **P40** promoters that are associated with the Rep proteins of an rAAV of different serotype. The use of such substitute or additional promoter sequences causes increased production of recombinantly-modified adeno-associated virus.

REFERENCE TO SEQUENCE LISTING

[0002] This application includes one or more Sequence Listings pursuant to 37 C.F.R. 1.821 et seq., which are disclosed in computer-readable media (file name: 2650-0004US_ST25.txt, created on July 15, 2019, and having a size of 84,101 bytes), which file is herein incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION:

I. Adeno-Associated Virus (AAV)

[0003] Adeno-Associated Virus (**AAV**) is a small, naturally-occurring, non-pathogenic virus belonging to the *Dependovirus* genus of the *Parvoviridae* (Balakrishnan, B. *et al.* (2014) "*Basic Biology of Adeno-Associated Virus (AAV) Vectors Used in Gene Therapy*," Curr. Gene Ther. 14(2):86-100; Zinn, E. *et al.* (2014) "*Adeno-Associated Virus: Fit To Serve*," Curr. Opin. Virol. 0:90-97). Despite not

causing disease, AAV is known to be able to infect humans and other primates and is prevalent in human populations (Johnson, F.B. *et al.* (1972) "*Immunological Reactivity of Antisera Prepared Against the Sodium Dodecyl Sulfate-Treated Structural Polypeptides of Adenovirus-Associated Virus*," J. Virol. 9(6):1017-1026). AAV infect a broad range of different cell types (*e.g.*, cells of the central nervous system, heart, kidney, liver, lung, pancreas, retinal pigment epithelium or photoreceptor cells, or skeletal muscle cells). Twelve serotypes of the virus (*e.g.*, AAV2, AAV5, AAV6, *etc.*), exhibiting different tissue infection capabilities ("**tropisms**"), have been identified (Colella, P. *et al.* (2018) "*Emerging Issues in AAV-Mediated In Vivo Gene Therapy*," Molec. Ther. Meth. Clin. Develop. 8:87-104; Hocquemiller, M. *et al.* (2016) "*Adeno-Associated Virus-Based Gene Therapy for CNS Diseases*," Hum. Gene Ther. 27(7):478-496; Lisowski, L. *et al.* (2015) "*Adeno-Associated Virus Serotypes For Gene Therapeutics*," 24:59-67).

[0004] AAV is a single-stranded DNA virus that is composed of approximately 4,800 nucleotides. The viral genome may be described as having a 5' half and a 3' half which together comprise the genes that encode the virus' proteins (**Figure 1**). The 5' half of the AAV genome comprises the AAV ***rep*** gene, which, through the use of multiple reading frames, staggered initiating promoters (**P5**, **P19** and **P40**) and alternate splicing, encodes four non-structural **Rep** proteins (**Rep40**, **Rep52**, **Rep68** and **Rep78**) that are required for viral transcription control and replication and for the packaging of viral genomes into the viral capsule (Lackner, D.F. *et al.* (2002) "*Studies of the Mechanism of Transactivation of the Adeno-Associated Virus p19 Promoter by Rep Protein*," J. Virol. 76(16):8225-8235). In the presence of viral proteins (such as Ad proteins), the **P5** promoter becomes activated and mediates the transcription of the **Rep68** and **Rep78** proteins, which are involved in transcriptional control, in latency, in rescue, and in viral DNA replication and thus function as master controllers of the AAV life cycle (Murphy, M. *et al.* (2007) "*Adeno-Associated Virus Type 2 p5 Promoter: a Rep-Regulated DNA Switch Element Functioning in Transcription, Replication, and Site-Specific Integration*," J. Virol. 81(8):3721-3730). Expression of the **Rep68** and **Rep78** proteins activates the **P19** promoter, which is responsible for the transcription of the **Rep40** and **Rep52** proteins (Lackner, D.F. *et al.* (2002) "*Studies of the Mechanism of Transactivation of the Adeno-Associated Virus p19 Promoter by Rep Protein*," J. Virol.

76(16):8225-8235; Ogasawara, Y. *et al.* (1998) "*The Use of Heterologous Promoters for Adeno-Associated Virus (AAV) Protein Expression in AAV Vector Production*," Microbiol. Immunol. 42(3):177-185). The 3' half the AAV genome comprises the AAV capsid gene (***cap***), which encodes three **capsid** proteins (**VP**): **VP1**, **VP2** and **VP3**. The three capsid proteins are translated from a single mRNA transcript that is controlled by a single promoter (**P40** in case of AAV2). The 3' half of the AAV genome also comprises the **AAP** gene, which encodes the AAV assembly-activating protein (**AAP**). Sixty VP monomers (comprising approximately 5 copies of VP1, 5 copies of VP2, and 50 copies of VP3) self-assemble around the AAV genome to form the icosahedral protein shell (**capsid**) of the mature viral particle (Büning, H. *et al.* (2019) "*Capsid Modifications for Targeting and Improving the Efficacy of AAV Vectors*," Mol. Ther. Meth. Clin. Devel. 12:P248-P265; Van Vliet K.M. *et al.* (2008) *The Role of the Adeno-Associated Virus Capsid in Gene Transfer*. In: DRUG DELIVERY SYSTEMS, Jain, K.K. (eds.), Meth. Molec. Biol. 437:51-91). The AAV **AAP** protein is believed to be required for stabilizing and transporting newly produced VP proteins from the cytoplasm into the cell nucleus. The 3' half of the AAV genome also comprises the AAV ***X*** gene, which is believed to encode a protein that supports genome replication (Colella, P. *et al.* (2018) "*Emerging Issues in AAV-Mediated In Vivo Gene Therapy*," Molec. Ther. Meth. Clin. Develop. 8:87-104; Büning, H. *et al.* (2019) "*Capsid Modifications for Targeting and Improving the Efficacy of AAV Vectors*," Mol. Ther. Meth. Clin. Devel. 12:P248-P265; Cao, M. *et al.* (2014) "*The X Gene Of Adeno-Associated Virus 2 (AAV2) Is Involved In Viral DNA Replication*," PLoS ONE 9, e104596:1-10).

[0005] The above-described AAV gene-coding sequences are flanked by two AAV-specific palindromic inverted terminal repeated sequences (**ITR**) of 145 nucleotides (Balakrishnan, B. *et al.* (2014) "*Basic Biology of Adeno-Associated Virus (AAV) Vectors Used in Gene Therapy*," Curr. Gene Ther. 14(2):86-100; Colella, P. *et al.* (2018) "*Emerging Issues in AAV-Mediated In Vivo Gene Therapy*," Molec. Ther. Meth. Clin. Develop. 8:87-104).

[0006] AAV is an inherently defective virus, lacking the capacity to perform at least two critical functions: the ability to initiate the synthesis of viral-specific products and the ability to assemble such products to form the icosahedral protein shell (**capsid**) of

the mature infectious viral particle. It thus requires a co-infecting "helper" virus, such as adenovirus (**Ad**), herpes simplex virus (**HSV**), cytomegalovirus (**CMV**), vaccinia virus or human papillomavirus to provide the viral-associated (**VA**) RNA that is not encoded by the genes of the AAV genome. Such VA RNA is not translated, but plays a role in regulating the translation of other viral genes. Similarly, the AAV genome does not include genes that encode the viral proteins E1a, E1b, E2a, and E4; thus, these proteins must also be provided by a co-infecting "helper" virus. The E1a protein greatly stimulate viral gene transcription during the productive infection. The E1b protein block apoptosis in adenovirus-infected cells, and thus allow productive infection to proceed. The E2a protein plays a role in the elongation phase of viral strand displacement replication by unwinding the template and enhancing the initiation of transcription. The E4 protein has been shown to affect transgene persistence, vector toxicity and immunogenicity (see, Grieger, J.C. *et al.* (2012) "*Adeno-Associated Virus Vectorology, Manufacturing, and Clinical Applications*," Meth. Enzymol. 507:229-254; Dyson, N. *et al.* (1992) "*Adenovirus E1A Targets Key Regulators Of Cell Proliferation*," Canc. Surv. 12:161-195; Jones N.C. (1990) "*Transformation By The Human Adenoviruses*," Semin. Cancer Biol. 1(6):425-435; Ben-Israel, H. et al. (2002) "*Adenovirus and Cell Cycle Control*," Front. Biosci. 7:d1369-d1395; Hoeben, R.C. *et al.* (2013) "*Adenovirus DNA Replication*," Cold Spring Harb. Perspect. Biol. 5:a013003 (pages 1-11); Berk, A.J. (2013) "*Adenoviridae: The Viruses And Their Replication*, In: FIELDS VIROLOGY, 6th Edition (Knipe, D.M. *et al.*, Eds.), Vol. 2., Lippincott Williams & Wilkins, Philadelphia, pages 1704-1731; Weitzman, M.D. (2005) "*Functions Of The Adenovirus E4 Proteins And Their Impact On Viral Vectors*," Front. Biosci. 10:1106-1117).

[0007] AAV viruses infect both dividing and non-dividing cells, and persist as circular episomal molecules or can be integrated into the DNA of a host cell at specific chromosomic loci (**Adeno-Associated Virus Integration Sites** or **AAVS**) (Duan, D. (2016) "*Systemic Delivery Of Adeno-Associated Viral Vectors*," Curr. Opin. Virol. 21:16-25; Grieger, J.C. *et al.* (2012) "*Adeno-Associated Virus Vectorology, Manufacturing, and Clinical Applications*," Meth. Enzymol. 507:229-254). AAV remains latent in such infected cells unless a helper virus is present to provide the functions needed for AAV replication and maturation.

II. rAAV and Their Use in Gene Therapy

[0008] In light of AAV's properties, recombinantly-modified versions of AAV (**rAAV**) have found substantial utility as vectors for gene therapy (see, Naso, M.F. *et al.* (2017) "*Adeno-Associated Virus (AAV) as a Vector for Gene Therapy*," BioDrugs 31:317-334; Berns, K. I. *et al.* (2017) "*AAV: An Overview of Unanswered Questions*," Human Gene Ther. 28(4):308-313; Berry, G.E. *et al.* (2016) "*Cellular Transduction Mechanisms Of Adeno-Associated Viral Vectors*," Curr. Opin. Virol. 21:54-60; Blessing, D. *et al.* (2016) "*Adeno-Associated Virus And Lentivirus Vectors: A Refined Toolkit For The Central Nervous System*," 21:61-66; Santiago-Ortiz, J.L. (2016) "*Adeno-Associated Virus (AAV) Vectors in Cancer Gene Therapy*," J. Control Release 240:287-301; Salganik, M. *et al.* (2015) "*Adeno-Associated Virus As A Mammalian DNA Vector*," Microbiol. Spectr. 3(4):1-32; Hocquemiller, M. *et al.* (2016) "*Adeno-Associated Virus-Based Gene Therapy for CNS Diseases*," Hum. Gene Ther. 27(7):478-496; Lykken, E.A. *et al.* (2018) "*Recent Progress And Considerations For AAV Gene Therapies Targeting The Central Nervous System*," J. Neurodevelop. Dis. 10:16:1-10; Büning, H. *et al.* (2019) "*Capsid Modifications for Targeting and Improving the Efficacy of AAV Vectors*," Mol. Ther. Meth. Clin. Devel. 12:P248-P265; During, M.J. *et al.* (1998) "*In Vivo Expression Of Therapeutic Human Genes For Dopamine Production In The Caudates Of MPTP-Treated Monkeys Using An AAV Vector*," Gene The. 5:820-827; Grieger, J.C. *et al.* (2012) "*Adeno-Associated Virus Vectorology, Manufacturing, and Clinical Applications*," Meth. Enzymol. 507:229-254; Kotterman, M.A. *et al.* (2014) "*Engineering Adeno-Associated Viruses For Clinical Gene Therapy*," Nat. Rev. Genet. 15(7):445-451; Kwon, I. *et al.* (2007) "*Designer Gene Delivery Vectors: Molecular Engineering and Evolution of Adeno-Associated Viral Vectors for Enhanced Gene Transfer*," Pharm. Res. 25(3):489-499; US Patents No.: 10,266,845; 10,081,659; 9,890,396; 9,840,719; 9,839,696; 9,834,789; 9,803,218; 9,783,825; 9,777,291; 9,540,659; 9,527,904; 8,236,557; 7,972,593 and 7,943,374).

[0009] rAAV are typically produced using circular plasmids ("**rAAV plasmid vector**"). The AAV ***rep*** and ***cap*** genes are typically deleted from such constructs and replaced with a promoter, a β-globin intron, a cloning site into which a therapeutic gene of choice (**transgene**) has been inserted, and a poly-adenylation ("**polyA**") site. The inverted terminal repeated sequences (ITR) of the rAAV are, however, retained, so that

the **transgene expression cassette** of the rAAV plasmid vector is flanked by AAV ITR sequences (Colella, P. *et al.* (2018) "*Emerging Issues in AAV-Mediated In Vivo Gene Therapy*," Molec. Ther. Meth. Clin. Develop. 8:87-104; Büning, H. *et al.* (2019) "*Capsid Modifications for Targeting and Improving the Efficacy of AAV Vectors*," Mol. Ther. Meth. Clin. Devel. 12:P248-P265). Thus, in the 5' to 3' direction, the rAAV comprises a 5' ITR, the transgene expression cassette of the rAAV, and a 3' ITR.

[0010] rAAV have been used to deliver a transgene to patients suffering from any of a multitude of genetic diseases (*e.g.*, hereditary lipoprotein lipase deficiency (**LPLD**), Leber's congenital amaurosis (**LCA**), aromatic L-amino acid decarboxylase deficiency (**AADC**), choroideremia and hemophilia), and have utility in new clinical modalities, such as in interfering RNA (**RNAi**) therapy and gene-modifying strategies such as **Crispr/Cas9** (US Patents No. 8,697,359, 10,000,772, 10,113,167, 10,227,611; Lino, C.A. *et al.* (2018) "*Delivering CRISPR: A Review Of The Challenges And Approaches*," Drug Deliv. 25(1):1234-1237; Ferreira, V. *et al.* (2014) "*Immune Responses To AAV-Vectors, The Glybera Example From Bench To Bedside*" Front. Immunol. 5(82):1-15), Büning, H. *et al.* (2019) "*Capsid Modifications for Targeting and Improving the Efficacy of AAV Vectors*," Mol. Ther. Meth. Clin. Devel. 12:P248-P265; Rastall, D.P.W. (2017) "*Current and Future Treatments for Lysosomal Storage Disorders*," Curr. Treat Options Neurol. 19(12):45; Kay, M. *et al.* (2017) "*Future Of rAAV Gene Therapy: Platform For RNAi, Gene Editing And Beyond*," Human Gene Ther. 28:361-372); Berns, K. I. *et al.* (2017) "*AAV: An Overview of Unanswered Questions*," Human Gene Ther. 28(4):308-313). More than 150 clinical trials involving rAAV have been instituted (Büning, H. *et al.* (2019) "*Capsid Modifications for Targeting and Improving the Efficacy of AAV Vectors*," Mol. Ther. Meth. Clin. Devel. 12:P248-P265; Clément, N. *et al.* (2016) "*Manufacturing Of Recombinant Adeno-Associated Viral Vectors For Clinical Trials*," Meth. Clin. Develop. 3:16002:1-7). The most commonly used AAV serotype for such recombinantly-modified AAV is **AAV2**, which is capable of infecting cells of the central nervous system, kidney, retinal pigment epithelium and photoreceptor cells. AAV serotype is **AAV9**, which infects muscle cells, also has been widely used (Duan, D. (2016) "*Systemic Delivery Of Adeno-Associated Viral Vectors*," Curr. Opin. Virol. 21:16-25). AAV serotypes are described in US Patents No. 10,301,650; 10,266,846; 10,265,417; 10,214,785; 10,214,566; 10,202,657; 10,046,016;

9,884,071; 9,856,539; 9,737,618; 9,677,089; 9,458,517; 9,457,103; 9,441,244; 9,193,956; 8,846,389; 8,507,267; 7,906,111; 7,479,554; 7,186,552; 7,105,345; 6,984,517; 6,962,815; and 6,733,757.

III. Methods of rAAV Production

[0011] rAAV containing a desired transgene expression cassette are typically produced by human cells (such as **HEK293**) grown in suspension. Since, as described above, rAAV are defective viruses, additional functions must be provided in order to replicate and package rAAV.

[0012] rAAV can be produced by transiently transfecting cells with an rAAV plasmid vector and a second plasmid vector that comprises an AAV helper function-providing polynucleotide that provides the **Rep52** and **Rep78** genes that are required for vector transcription control and replication, and for the packaging of viral genomes into the viral capsule (**Rep40** and **Rep68** are not required for rAAV production) and the ***cap*** genes that were excised from the AAV in order to produce the rAAV. The second plasmid vector may additionally comprise a non-AAV helper function-providing polynucleotide that encodes the viral transcription and translation factors (E1a, E1b, E2a, VA and E4) required for AAV proliferation, so as to comprise, in concert with the rAAV, a **double plasmid transfection system** (Grimm, D. *et al.* (1998) "*Novel Tools For Production And Purification Of Recombinant Adeno-Associated Virus Vectors*," Hum. Gene Ther. 9:2745-2760; Penaud-Budloo, M. *et al.* (2018) *"Pharmacology of Recombinant Adeno-associated Virus Production*," Molec. Ther. Meth. Clin. Develop. 8:166-180).

[0013] However, it has become increasingly common to clone the AAV helper function-providing polynucleotide (which provides the required ***rep*** and ***cap*** genes) into an **AAV helper plasmid**, and to clone the non-AAV helper function-providing polynucleotide (which provides the genes that encode the viral transcription and translation factors) on a different plasmid (*e.g.*, an "**Ad helper plasmid**"), so that such plasmids, in concert with an rAAV plasmid vector, comprise a **triple plasmid transfection system** (**Figure 2**). Use of the triple plasmid transfection system has the advantage of permitting one to easily switch one ***cap*** gene for another, thereby facilitating changes in the rAAV's serotype. The use of helper plasmids, rather than

helper viruses, permits rAAV to be produced without additionally producing particles of the helper virus (François, A. *et al.* (2018) "*Accurate Titration of Infectious AAV Particles Requires Measurement of Biologically Active Vector Genomes and Suitable Controls*," Molec. Ther. Meth. Clin. Develop. 10:223-236; Matsushita, T. *et al.* (1998) "*Adeno-Associated Virus Vectors Can Be Efficiently Produced Without Helper Virus*," Gene Ther. 5:938-945).

[0014] The transient transfection of plasmid DNAs comprising the rAAV plasmid vector, the AAV ***rep*** and ***cap*** genes, and the trans-acting AAD helper genes into HEK293 cells by calcium phosphate coprecipitation has become the standard method to produce rAAV in the research laboratory (Grimm, D. *et al.* (1998) "*Novel Tools For Production And Purification Of Recombinant Adeno-Associated Virus Vectors*," Hum. Gene Ther. 9:2745-2760). However, the use of such a calcium phosphate-mediated transfection process with suspension-cultured transfected mammalian cells requires media exchanges, and is thus not considered ideal for the large-scale rAAV production that is required in order to produce therapeutic doses of rAAV (Lock, M. *et al.* (2010) "*Rapid, Simple, and Versatile Manufacturing of Recombinant Adeno-Associated Viral Vectors at Scale*," Hum. Gene Ther. 21:1259-1271). For this reason, polyethylenimine (**PEI**), has been used as a transfection reagent and has been found to provide yields of virus that are similar to those obtained using calcium phosphate-mediated transfection (Durocher, Y. *et al.* (2007) "*Scalable Serum-Free Production Of Recombinant Adeno-Associated Virus Type 2 By Transfection Of 293 Suspension Cells*," J. Virol. Meth. 144:32-40).

[0015] rAAV may alternatively be produced in insect cells (*e.g.*, sf9 cells) using baculoviral vectors (see, *e.g.*, US Patents No.: 9,879,282; 9,879,279; 8,945,918; 8,163,543; 7,271,002 and 6,723,551), or in HSV-infected baby hamster kidney (**BHK**) cells (*e.g.*, BHK21) (François, A. *et al.* (2018) "*Accurate Titration of Infectious AAV Particles Requires Measurement of Biologically Active Vector Genomes and Suitable Controls*," Molec. Ther. Meth. Clin. Develop. 10:223-236). Methods of rAAV production are reviewed in Grieger, J.C. *et al.* (2012) "*Adeno-Associated Virus Vectorology, Manufacturing, and Clinical Applications*," Meth. Enzymol. 507:229-254, and in Penaud-Budloo, M. *et al.* (2018) *"Pharmacology of Recombinant Adeno-associated Virus Production*," Molec. Ther. Meth. Clin. Develop. 8:166-180.

IV. Methods of rAAV Purification and Recovery

[0016] After production, rAAV are typically collected and purified by one or more overnight CsCl gradient centrifugations (Zolotukhin, S. *et al.* (1999) "*Recombinant Adeno-Associated Virus Purification Using Novel Methods Improves Infectious Titer And Yield*," Gene Ther. 6:973-985), followed by desalting to form a purified rAAV production stock. Titers of 10^{12}-10^{13} infectious rAAV capsids/mL are obtainable.

[0017] Because rAAV infection does not cause a cytopathic effect, plaque assays cannot be used to determine the infectious titer of an rAAV preparation. Infectious titer is thus typically measured as the median tissue culture infective dose (**TCID50**). In this method, a HeLa-derived AAV2 ***rep***- and ***cap***-expressing cell line is grown in a 96-well plate and infected with replicate 10-fold serial dilutions of the rAAV preparation, in the presence of adenovirus of serotype 5. After infection, vector genome replication is determined by quantitative PCR (qPCR) (Zen, Z. *et al.* (2004) "*Infectious Titer Assay For Adeno-Associated Virus Vectors With Sensitivity Sufficient To Detect Single Infectious Events*," Hum. Gene Ther. 15:709-715). Alternatively, the infectious titer of an rAAV preparation can be measured using the infectious center assay (**ICA**). This assay uses HeLa ***rep-cap*** cells and Ad, but, after incubation, involves transferring the cells to a membrane. A labeled probe that is complementary to a portion of the employed transgene is used to detect infectious centers (representing individual infected cells) via hybridization. Although more widely used, the TCID50 assay has been reported to lead to a higher background than the ICA and to overestimate vector infectivity relative to the ICA (François, A. *et al.* (2018) "*Accurate Titration of Infectious AAV Particles Requires Measurement of Biologically Active Vector Genomes and Suitable Controls*," Molec. Ther. Meth. Clin. Develop. 10:223-236). Methods of producing and purifying rAAV are described *inter alia* in US Patents No. 10,294,452; 10,161,011; 10,017,746; 9,598,703; 7,625,570; 7,439,065; 7,419,817; 7,208,315; 6,995,006; 6,989,264; 6,846,665 and 6,841,357.

[0018] Despite all such prior advances, a need remains to develop methods capable of addressing problems that presently limit the applicability of rAAV to gene therapy (Grieger, J.C. *et al.* (2012) "*Adeno-Associated Virus Vectorology, Manufacturing, and Clinical Applications*," Meth. Enzymol. 507:229-254; Kotterman, M.A. *et al.* (2014)

"*Engineering Adeno-Associated Viruses For Clinical Gene Therapy*," Nat. Rev. Genet. 15(7):445-451; Kwon, I. *et al.* (2007) "*Designer Gene Delivery Vectors: Molecular Engineering and Evolution of Adeno-Associated Viral Vectors for Enhanced Gene Transfer*," Pharm. Res. 25(3):489-499; Naso, M.F. *et al.* (2017) "*Adeno-Associated Virus (AAV) as a Vector for Gene Therapy*," BioDrugs 31:317-334).

[0019] The present invention is directed to improved methods for increasing the efficiency of AAV and rAAV packaging through regulation of the expression of the AAV ***rep*** and ***cap*** genes.

SUMMARY OF THE INVENTION:

[0020] The present invention is directed to recombinantly-modified adeno-associated virus (AAV) helper vectors that are capable of increasing the packaging efficiency of recombinantly-modified adeno-associated virus (rAAV) and their use to improve the packaging efficiency of such rAAV. The present invention is particularly directed to recombinantly-modified adeno-associated virus (AAV) helper vectors that have been further modified to replace (or augment) the **P5** and/or **P40** promoter sequences that are natively associated with the Rep proteins encoded by such rAAV with AAV **P5** and/or **P40** promoters that are associated with the Rep proteins of an rAAV of different serotype. The use of such substitute or additional promoter sequences causes increased production of recombinantly-modified adeno-associated virus.

[0021] In detail, the invention provides a recombinantly-modified adeno-associated virus (AAV) helper vector that comprises an AAV helper function-providing polynucleotide, and especially an AAV helper function-providing polynucleotide that is a plasmid vector, wherein the polynucleotide comprises a non-native AAV serotype **P5** or **P40** promoter sequence.

[0022] The invention particularly includes the embodiment of such recombinantly-modified adeno-associated virus (AAV) helper vector wherein the AAV helper function-providing polynucleotide vector comprises a non-native AAV serotype **P5** promoter sequence and/or a non-native AAV serotype **P40** promoter sequence.

[0023] The invention also particularly includes the embodiment of such recombinantly-modified adeno-associated virus (AAV) helper vector wherein the non-native AAV serotype **P5** or **P40** promoter sequence replaces a native AAV serotype promoter sequence.

[0024] The invention also particularly includes the embodiment of such recombinantly-modified adeno-associated virus (AAV) helper vector wherein the vector additionally comprises a non-AAV helper function-providing polynucleotide.

[0025] The invention additionally provides a method for increasing the production titer of a recombinantly-modified adeno-associated virus (rAAV) that comprises a transgene cassette, wherein the method comprises culturing cells that have been transfected with:

(1) the rAAV;

(2) the above-described recombinantly-modified adeno-associated virus (AAV) helper vector that additionally comprises a non-AAV helper function-providing polynucleotide;

wherein the culturing is conducted in a culture medium under conditions sufficient to permit the production of the rAAV, and wherein the presence of the non-native AAV serotype **P5** or **P40** promoter sequence causes the cells to produce the rAAV at an increased production titer relative to that which would be attained if the AAV helper function-providing polynucleotide contained native serotype **P5** and **P40** promoters.

[0026] The invention additionally provides a method for increasing the production titer of recombinantly-modified adeno-associated virus (rAAV) that comprises a transgene cassette, wherein the method comprises culturing cells that have been transfected with:

(1) the rAAV;

(2) any of the above-described recombinantly-modified adeno-associated virus (AAV) helper vectors; and

(3) an additional vector, especially a plasmid vector, that comprises a non-AAV helper function-providing polynucleotide;

wherein the culturing is conducted in a culture medium under conditions sufficient to permit the production of the rAAV, and wherein the presence of the non-native AAV

serotype **P5** or **P40** promoter sequence causes the cells to produce the rAAV at an increased production titer relative to that which would be attained if the AAV helper function-providing polynucleotide contained native serotype **P5** and **P40** promoters.

[0027] The invention particularly includes the embodiment of such methods, wherein the transgene cassette encodes a protein, or comprises a transcribed nucleic acid, that is therapeutic for a genetic or heritable disease or condition.

[0028] The invention also particularly includes the embodiment of such methods, wherein:

(A) the AAV helper function-providing polynucleotide of the vector encodes an AAV1 **Cap** protein, and the non-native AAV serotype promoter sequence is a promoter sequence of an AAV of serotype AAV3, AAV4, AAV5, AAV6, AAV7 or AAV8, or a hybrid of one or more of the serotypes;

(B) the AAV helper function-providing polynucleotide of the vector encodes an AAV2 **Cap** protein, and the non-native AAV serotype promoter sequence is a promoter sequence of an AAV of serotype AAV1, AAV3, AAV4, AAV5, AAV6, AAV7 or AAV8, or a hybrid of one or more of the serotypes;

(C) the AAV helper function-providing polynucleotide of the vector encodes an AAV3 **Cap** protein, and the non-native AAV serotype promoter sequence is a promoter sequence of an AAV of serotype AAV1, AAV4, AAV5, AAV6, AAV7 or AAV8, or a hybrid of one or more of the serotypes;

(D) the AAV helper function-providing polynucleotide of the vector encodes an AAV4 **Cap** protein, and the non-native AAV serotype promoter sequence is a promoter sequence of an AAV of serotype AAV1, AAV3, AAV5, AAV6, AAV7 or AAV8, or a hybrid of one or more of the serotypes;

(E) the AAV helper function-providing polynucleotide of the vector encodes an AAV5 **Cap** protein, and the non-native AAV serotype promoter sequence is a promoter sequence of an AAV of serotype

AAV1, AAV3, AAV4, AAV6, AAV7 or AAV8, or a hybrid of one or more of the serotypes;

(F) the AAV helper function-providing polynucleotide of the vector encodes an AAV6 **Cap** protein, and the non-native AAV serotype promoter sequence is a promoter sequence of an AAV of serotype AAV1, AAV3, AAV4, AAV5, AAV7 or AAV8, or a hybrid of one or more of the serotypes;

(G) the AAV helper function-providing polynucleotide of the vector encodes an AAV7 **Cap** protein, and the non-native AAV serotype promoter sequence is a promoter sequence of an AAV of serotype AAV1, AAV3, AAV4, AAV5, AAV6 or AAV8, or a hybrid of one or more of the serotypes;

(H) the AAV helper function-providing polynucleotide of the vector encodes an AAV8 **Cap** protein, and the non-native AAV serotype promoter sequence is a promoter sequence of an AAV of serotype AAV1, AAV3, AAV4, AAV5, AAV6 or AAV7, or a hybrid of one or more of the serotypes.

[0029] The invention also particularly includes the embodiment of such methods, wherein the cells are human embryonic kidney cells, baby hamster kidney cells or sf9 insect cells.

[0030] The invention additionally provides a pharmaceutical composition that comprises the recombinantly-modified adeno-associated virus (rAAV) produced by any of the above-listed methods, and a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS:

[0031] **Figure 1** provides a schematic genetic map of the wild-type (**Wt**) AAV genome.

[0032] **Figure 2** provides a schematic of the structural domain of the wild-type AAV2 genome **(1)**, a recombinant AAV (rAAV) **(2)**, complementing "AAV helper plasmid" **(3)** and an adenovirus helper plasmid ("Ad helper plasmid") **(4)**. The wild-type (Wt) AAV2 **(1)** is composed of AAV-specific palindromic inverted terminal repeated

sequences (**ITR**), a 5' half containing genes that encode the **Rep** proteins and a 3' half containing genes that encode the **Cap** proteins. The rAAV **(2)** is formed by replacing the **Rep**- and **Cap**-encoding genes of the wild-type (Wt) AAV2 **(1)** with a transgene cassette that comprises a promoter (**Pro**), the exogenous transgene of interest, and a polyadenylation site (**pA**). In order to produce the rAAV **(2)**, a complementing "AAV helper" plasmid vector **(3)** and an adenovirus helper plasmid vector (Ad helper plasmid) **(4)** are provided. The complementing AAV helper plasmid **(3)** provides **Rep** and **Cap** proteins. The Ad helper plasmid **(4)** provides adenovirus proteins E1a, E1b, E2a, VA and E4.

[0033] **Figure 3** shows a map of the AAV helper plasmid vector **pAAV-RC1** (**SEQ ID NO:1**).

[0034] **Figure 4** shows a map of the AAV helper plasmid vector **pAAV-RC2** (**SEQ ID NO:2**).

[0035] **Figure 5** shows a map of the AAV helper plasmid vector **pAAV-RC5** (**SEQ ID NO:3**).

[0036] **Figure 6** shows a map of the AAV helper plasmid vector **pAAV-RC6** (**SEQ ID NO:4**).

[0037] **Figure 7** shows a map of the AAV helper plasmid vector **pAAV-RC7** (**SEQ ID NO:5**).

[0038] **Figure 8** shows a map of the non-AAV helper plasmid vector **pHelper-Kan** (**SEQ ID NO:6**).

[0039] **Figure 9** shows a map of the rAAV plasmid vector **pAV-CMV-EGFP** (**SEQ ID NO:7**).

[0040] **Figure 10** shows a map of the rAAV plasmid vector **pAV-TBG-EGFP** (**SEQ ID NO:8**).

[0041] **Figure 11** shows the overall structure and approach followed for the development of the exemplary AAV helper constructs described herein. The parent construct (**pAAV-RC2**; **Parent-RC**) comprises AAV2 serotype promoter sequences

for the **P5** and **P19** promoters (solid black boxes) that direct expression of the native AAV2 ***rep*** gene (white boxed gene), which encodes the **Rep** proteins, as well as the AAV2 serotype promoter sequence of the **P40** promoter (solid black box) that directs expression of the native AAV2 ***cap*** gene (gray boxed gene), which encodes the **Cap** proteins.

[0042] **P5-RC** constructs are derivatives of parental plasmid **AAV RC** that have been modified to direct expression of the AAV ***rep*** gene using a non-native **P5** promoter (*i.e.*, an AAV **P5** promoter that is not natively present within the AAV ***rep*** gene of the vector (downward striped box)) in lieu of the native AAV serotype **P5** promoter (solid black box); **P5-RC** constructs direct expression of the AAV ***rep*** and ***cap*** genes using the native AAV serotype **P19** and **P40** promoter sequences (solid black boxes) of the parent vector. **P40-RC** constructs are derivatives of parental plasmid **AAV RC** that have been modified to direct expression of the AAV ***cap*** gene using a non-native **P40** promoter (*i.e.*, an AAV **P40** promoter that is not natively present within the AAV ***rep*** gene (upward striped box)) of the vector in lieu of the native AAV serotype **P40** promoter (solid black box); **P40-RC** constructs direct expression of the AAV ***rep*** gene using the native AAV serotype **P5** and **P19** promoter sequences (solid black boxes) of the parent vector. **P5/P40-RC** constructs are derivatives of parental plasmid **AAV RC** that have been modified to direct expression of the AAV ***rep*** gene using a non-native **P5** promoter (*i.e.*, an AAV **P5** promoter that is not natively present within the AAV ***rep*** gene of the vector (downward striped box)) in lieu of the native AAV serotype **P5** promoter (solid black box). **P5/P40-RC** constructs have additionally been modified to direct expression to direct expression of the AAV ***cap*** gene using a non-native **P40** promoter (*i.e.*, an AAV **P40** promoter that is not natively present within the AAV ***rep*** gene (upward striped box)) of the vector in lieu of the native AAV serotype **P40** promoter (solid black box). **P40-RC** constructs direct expression of the AAV ***rep*** gene using the native AAV serotype **P19** promoter sequences (solid black boxes) of the parent vector. The sequences of the promoter regions are shown in **Table 1**.

[0043] **Figures 12A-12B** show the production titers of rAAV obtained by modifying a parental **RC2** vector to comprise a non-native **P5** promoter sequence (**Figure 11**; **Figure 12A**; downward striped rectangle) in lieu of the AAV2 **P5** promoter that is natively associated with the ***rep*** gene of such vector. The **P19** and **P40** promoters are

both native AAV2 serotype promoter sequences (solid black rectangles). **Figure 12B** shows the production titers of rAAV obtained using such AAV helper plasmid vectors. The following constructs were employed: **Parent-RC2**, **P5(2)-RC2**, **P5(1)-RC2**, **P5(3)-RC2**, **P5(4)-RC2**, **P5(5)-RC2**, **P5(7)-RC2**, and **P5(8)-RC2**. The sequences of the promoter regions are shown in **Table 1**. The production titers of rAAV were obtained using a triple plasmid transfection system with an rAAV, and an Ad helper plasmid that provided the required adenoviral functions.

[0044] **Figures 13A-13B** show the production titers of rAAV obtained by modifying a parental **RC2** vector to comprise a non-native **P40** promoter sequence (**Figure 11**; **Figure 13A**; upward striped rectangle) in lieu of the AAV2 serotype **P40** promoter of the parental vector. The **P5** and **P19** promoters are both native AAV2 serotype promoter sequences (solid black rectangle). **Figure 13B** shows the production titers of rAAV obtained using such AAV helper plasmid vectors. The following constructs were employed: **Parent-RC2**, **P40(1)-RC2**, **P40(3)-RC2**, **P40(4)-RC2**, **P40(5)-RC2**, **P40(6)-RC2**, **P40(7)-RC2**, and **P40(8)-RC2**. The sequences of the promoter regions are shown in **Table 1**. The production titers of rAAV were obtained using a triple plasmid transfection system with an rAAV, and an Ad helper plasmid that provided the required adenoviral functions.

[0045] **Figures 14A-14B** show the production titers of rAAV obtained by modifying a parental **RC2** vector to comprise a non-native **P5** promoter sequence and/or a non-native **P40** promoter sequence (**Figure 11**; **Figure 14A**; **P5**, downward striped rectangle; **P40**, upward striped rectangle) in lieu of the AAV2 serotype **P5** and **P40** promoters of the parental vector. The **P19** promoter is a native AAV2 serotype promoter sequences (solid black rectangle). The following constructs were employed: **Parent-RC2**, **P5(2)-RC2**, **P5(3)-RC2**, **P5(5)-RC2**, **P40(1)-RC2**, **P5(2)/P40(1)-RC2**, **P5(3)/P40(1)-RC2**, and **P5(5)/P40(1)-RC2**. The sequences of the promoter regions are shown in **Table 1**. **Figure 14B** shows the production titers of rAAV obtained using such AAV helper plasmid vectors. The production titers of rAAV were obtained using a triple plasmid transfection system with an rAAV, and an Ad helper plasmid that provided the required adenoviral functions.

[0046] **Figures 15A-15C** show the production titers of rAAV obtained by modifying a parental **RC6** vector to comprise a non-native **P5** promoter sequence (**Figure 11**; **Figure 15A**; downward striped rectangle) in lieu of the AAV2 serotype **P5** promoter that is natively associated with the ***rep*** gene of such vector. The **P19** and **P40** promoters are both native AAV2 serotype promoter sequences (solid black rectangles). The following constructs were employed: **Parent-RC6**, **P5(1)-RC6**, **P5(2)-RC6**, **P5(3)-RC6**, **P5(7)-RC6** and **P5(8)-RC6**. The sequences of the promoter regions are shown in **Table 1**. The production titers of rAAV were obtained (**Figures 15B-15C**) using a triple plasmid transfection system with an rAAV, and an Ad helper plasmid that provided the required adenoviral functions.

[0047] **Figures 16A-16B** show the production titers of rAAV obtained by modifying a parental **RC1**, **RC5**, or **RC7** vector to comprise a non-native **P5** promoter sequence (**Figure 11**; **Figure 16A**; downward striped rectangle) in lieu of the AAV2 serotype **P5** promoter that is natively associated with the ***rep*** gene of such vectors. The **P19** and **P40** promoters are both native AAV2 serotype promoter sequences (solid black rectangles). The following constructs were employed: **Parent-RC1**, **Parent-RC5**, **Parent-RC7**, **P5(2)-RC1**, **P5(7)-RC1**, **P5(8)-RC1**, **P5(7)-RC5**, **P5(2)-RC7**, **P5(7)-RC7** and **P5(8)-RC7**. The sequences of the promoter regions are shown in **Table 1**. The production titers of rAAV (**Figure 16B**) were obtained using a triple plasmid transfection system with an rAAV, and an Ad helper plasmid that provided the required adenoviral functions.

DETAILED DESCRIPTION OF THE INVENTION:

I. The Methods of the Present Invention

[0048] The present invention is directed to recombinantly-modified adeno-associated virus (AAV) helper vectors that are capable of increasing the packaging efficiency of recombinantly-modified adeno-associated virus (rAAV) and their use to improve the packaging efficiency of such rAAV. The present invention is particularly directed to recombinantly-modified adeno-associated virus (AAV) helper vectors that have been further modified to replace (or augment) the **P5** and/or **P40** promoter sequences that are natively associated with the Rep proteins encoded by such rAAV with AAV **P5** and/or **P40** promoters that are associated with the Rep proteins of an rAAV of different

serotype. The use of such substitute or additional promoter sequences causes increased production of recombinantly-modified adeno-associated virus.

[0049] The present invention is based in part on the recognition that high levels of **Rep** and **Cap** proteins increase the amount of rAAV genomes particles produced and, consequently, the efficiency of rAAV packaging, and thus result in high production titers of rAAV stocks. It has been unexpectedly found that by replacing the AAV **P5** and/or **P40** promoters that direct the expression of the **Cap** proteins with different AAV **P5** and/or **P40** promoters, or by adding such different AAV **P5** and/or **P40** promoters in addition to those initially present, causes the desired high levels of rAAV to be attained. AAV Rep proteins are described in US Patents No. 10,214,730; 7,122,348; 6,821,511; 6,753,419; 9,441,206; and 7,115,391.

[0050] As discussed above, AAV and rAAV are characterized based on their serotype, which is determined by their capsid proteins (Colella, P. *et al.* (2018) "*Emerging Issues in AAV-Mediated In Vivo Gene Therapy*," Molec. Ther. Meth. Clin. Develop. 8:87-104; Hocquemiller, M. *et al.* (2016) "*Adeno-Associated Virus-Based Gene Therapy for CNS Diseases*," Hum. Gene Ther. 27(7):478-496; Lisowski, L. *et al.* (2015) "*Adeno-Associated Virus Serotypes For Gene Therapeutics*," 24:59-67; US Patents No. 10,301,650; 10,266,846; 10,265,417; 10,214,785; 10,214,566; 10,202,657; 10,046,016; 9,884,071; 9,856,539; 9,737,618; 9,677,089; 9,458,517; 9,457,103; 9,441,244; 9,193,956; 8,846,389; 8,507,267; 7,906,111; 7,479,554; 7,186,552; 7,105,345; 6,984,517; 6,962,815; and 6,733,757). By forming AAV and rAAV in the presence of AAV helper function-providing polynucleotides that encode two or more capsid proteins of different serotype, one can produce AAV and rAAV having "**hybrid**" serotypes. Such AAV and rAAV exhibit the combined trophism of AAV and rAAV having each of such capsid proteins.

[0051] The **Rep** proteins of the different AAV serotypes differ, however, since such proteins are not structural proteins, the differences do not contribute to the observed serotype of an AAV.

[0052] As used herein, the term "**AAV**" is intended to denote adeno-associated virus, and may be used to refer to the virus itself or derivatives thereof. The term covers all subtypes and both naturally-occurring and recombinant forms. As used herein, the term

"**rAAV**" is intended to denote a recombinantly-modified version of AAV that comprises a polynucleotide sequence not of AAV origin (*i.e.*, a polynucleotide heterologous to AAV). The rAAV may be single-stranded or double-stranded, and may be composed of deoxyribonucleotides or ribonucleotides. As discussed above, rAAV typically lack certain AAV genes and thus are produced using a double plasmid transfection system, or more preferably a triple plasmid transfection system that comprises a plasmid vector that comprises an **AAV helper function-providing polynucleotide**, a plasmid vector that comprises a **non-AAV helper function-providing polynucleotide**, and the **rAAV plasmid vector** (**Figure 2**). In one embodiment, the **AAV helper function-providing polynucleotide** of such double or triple transfection systems may comprise more than one ***rep*** and/or ***cap*** gene, so as to be capable of forming rAAV having hybrid serotypes. In another embodiment, a second or additional **AAV helper function-providing polynucleotide** (for example on a second or additional plasmid vector) may be provided to permit the formation of rAAV having hybrid serotypes.

A. Illustrative AAV Helper Function-Providing Polynucleotides

[0053] As used herein, the term "**AAV helper functions**" denotes AAV proteins (*e.g.*, **Rep** and **Cap**) and/or polynucleotides of AAV that are required for the replication and packaging of an rAAV. Such AAV helper functions are provided by an "**AAV helper function-providing polynucleotide**," which as such term is used herein is a virus, plasmid vector, a non-plasmid vector, or a polynucleotide that has been integrated into a cellular chromosome, that provides AAV helper functions. AAV helper plasmids that may be used in accordance with the present invention to provide **AAV helper functions** include **pAAV-RC** (Agilent; Addgene; Cell Biolabs), **pAAV-RC1**, **pAAV-RC2**, **pAAV-RC5**, **pAAV-RC6**, and **pAAV-RC7**.

1. Plasmid pAAV-RC1

[0054] Plasmid **pAAV-RC1** (**SEQ ID NO:1; Figure 3**) is an AAV helper plasmid that expresses AAV1 serotype capsid proteins that may be used in accordance with the present invention to provide **AAV helper functions**. The **P5** and **P40** promoters of **pAAV-RC1** are AAV2 serotype promoters (**SEQ ID NO:10** and **SEQ ID NO:18**, respectively).

Coding Strand of Plasmid pAAV-RC1 (SEQ ID NO:1):

catggttttg ggacgtttcc tgagtcagat tcgcgaaaaa ctgattcaga
gaatttaccg cgggatcgag ccgactttgc caaactggtt cgcggtcaca
aagaccagaa atggcgccgg aggcgggaac aaggtggtgg atgagtgcta
catccccaat tacttgctcc ccaaaaccca gcctgagctc cagtgggcgt
ggactaatat ggaacagtat ttaagcgcct gtttgaatct cacggagcgt
aaacggttgg tggcgcagca tctgacgcac gtgtcgcaga cgcaggagca
gaacaaagag aatcagaatc ccaattctga tgcgccggtg atcagatcaa
aaacttcagc caggtacatg gagctggtcg ggtggctcgt ggacaagggg
attacctcgg agaagcagtg gatccaggag gaccaggcct catacatctc
cttcaatgcg gcctccaact cgcggtccca aatcaaggct gccttggaca
atgcgggaaa gattatgagc ctgactaaaa ccgcccccga ctacctggtg
ggccagcagc ccgtggagga catttccagc aatcggattt ataaaatttt
ggaactaaac gggtacgatc cccaatatgc ggcttccgtc tttctgggat
gggccacgaa aaagttcggc aagaggaaca ccatctggct gtttgggcct
gcaactaccg ggaagaccaa catcgcggag gccatagccc acactgtgcc
cttctacggg tgcgtaaact ggaccaatga gaactttccc ttcaacgact
gtgtcgacaa gatggtgatc tggtgggagg aggggaagat gaccgccaag
gtcgtggagt cggccaaagc cattctcgga ggaagcaagg tgcgcgtgga
ccagaaatgc aagtcctcgg cccagataga cccgactccc gtgatcgtca
cctccaacac caacatgtgc gccgtgattg acgggaactc aacgaccttc
gaacaccagc agccgttgca agaccggatg ttcaaatttg aactcacccg
ccgtctggat catgactttg ggaaggtcac caagcaggaa gtcaaagact
ttttccggtg ggcaaaggat cacgtggttg aggtggagca tgaattctac
gtcaaaaagg gtggagccaa gaaaagaccc gcccccagtg acgcagatat
aagtgagccc aaacgggtgc gcgagtcagt tgcgcagcca tcgacgtcag
acgcggaagc ttcgatcaac tacgcagaca ggtaccaaaa caaatgttct
cgtcacgtgg gcatgaatct gatgctgttt ccctgcagac aatgcgagag
aatgaatcag aattcaaata tctgcttcac tcacggacag aaagactgtt
tagagtgctt tcccgtgtca gaatctcaac ccgtttctgt cgtcaaaaag
gcgtatcaga aactgtgcta cattcatcat atcatgggaa aggtgccaga
cgcttgcact gcctgcgatc tggtcaatgt ggatttggat gactgcatct
ttgaacaata aatgatttaa atcaggtatg gctgccgatg gttatcttcc
agattggctc gaggacaacc tctctgaggg cattcgcgag tggtgggact
tgaaacctgg agccccgaag cccaaagcca accagcaaaa gcaggacgac
ggccggggtc tggtgcttcc tggctacaag tacctcggac ccttcaacgg
actcgacaag ggggagcccg tcaacgcggc ggacgcagcg gccctcgagc
acgacaaggc ctacgaccag cagctcaaag cgggtgacaa tccgtacctg
cggtataacc acgccgacgc cgagtttcag gagcgtctgc aagaagatac
gtcttttggg ggcaacctcg ggcgagcagt cttccaggcc aagaagcggg
ttctcgaacc tctcggtctg gttgaggaag gcgctaagac ggctcctgga
aagaaacgtc cggtagagca gtcgccacaa gagccagact cctcctcggg
catcggcaag acaggccagc agcccgctaa aaagagactc aattttggtc
agactggcga ctcagagtca gtccccgatc cacaacctct cggagaacct
ccagcaaccc ccgctgctgt gggacctact acaatggctt caggcggtgg
cgcaccaatg gcagacaata acgaaggcgc cgacggagtg ggtaatgcct
caggaaattg gcattgcgat tccacatggc tgggcgacag agtcatcacc
accagcaccc gcacctgggc cttgcccacc tacaataacc acctctacaa
gcaaatctcc agtgcttcaa cgggggccag caacgacaac cactacttcg
gctacagcac cccctggggg tattttgatt tcaacagatt ccactgccac

ttttcaccac gtgactggca gcgactcatc aacaacaatt ggggattccg
gcccaagaga ctcaacttca aactcttcaa catccaagtc aaggaggtca
cgacgaatga tggcgtcaca accatcgcta ataaccttac cagcacggtt
caagtcttct cggactcgga gtaccagctt ccgtacgtcc tcggctctgc
gcaccagggc tgcctccctc cgttcccggc ggacgtgttc atgattccgc
aatacggcta cctgacgctc aacaatggca gccaagccgt gggacgttca
tccttttact gcctggaata tttcccttct cagatgctga gaacgggcaa
caactttacc ttcagctaca cctttgagga agtgcctttc cacagcagct
acgcgcacag ccagagcctg gaccggctga tgaatcctct catcgaccaa
tacctgtatt acctgaacag aactcaaaat cagtccggaa gtgcccaaaa
caaggacttg ctgtttagcc gtgggtctcc agctggcatg tctgttcagc
ccaaaaactg gctacctgga ccctgttatc ggcagcagcg cgtttctaaa
acaaaaacag acaacaacaa cagcaatttt acctggactg gtgcttcaaa
atataacctc aatgggcgtg aatccatcat caaccctggc actgctatgg
cctcacacaa agacgacgaa gacaagttct ttcccatgag cggtgtcatg
atttttggaa aagagagcgc cggagcttca aacactgcat tggacaatgt
catgattaca gacgaagagg aaattaaagc cactaaccct gtggccaccg
aaagatttgg gaccgtggca gtcaatttcc agagcagcag cacagaccct
gcgaccggag atgtgcatgc tatgggagca ttacctggca tggtgtggca
agatagagac gtgtacctgc agggtcccat ttgggccaaa attcctcaca
cagatggaca ctttcacccg tctcctctta tgggcggctt tggactcaag
aacccgcctc ctcagatcct catcaaaaac acgcctgttc ctgcgaatcc
tccggcggag ttttcagcta caaagtttgc ttcattcatc acccaatact
ccacaggaca agtgagtgtg gaaattgaat gggagctgca gaaagaaaac
agcaagcgct ggaatcccga agtgcagtac acatccaatt atgcaaaatc
tgccaacgtt gattttactg tggacaacaa tggactttat actgagcctc
gccccattgg cacccgttac cttacccgtc ccctgtaagg cgcgccaccg
gttgcttgtt aatcaataaa ccgtttaatt cgtttcagtt gaactttggt
ctctgcgtat ttctttctta tctagtttcc atgctctagg atccactagt
aacggccgcc agtgtgctgg aattcggctt tgtagttaat gattaacccg
ccatgctact tatctacgta gccatgctct agaggtcctg tattagaggt
cacgtgagtg ttttgcgaca ttttgcgaca ccatgtggtc acgctgggta
tttaagcccg agtgagcacg cagggtctcc attttgaagc gggaggtttg
aacgcgcagc cgccaagccg aattctgcag atatccaaac actggcggcc
gctcgactag agcggccgcc accgcggtgg agctccagct tttgttccct
ttagtgaggg ttaattgcgc gcttggcgta atcatggtca tagctgtttc
ctgtgtgaaa ttgttatccg ctcacaattc cacacaacat acgagccgga
agcataaagt gtaaagcctg gggtgcctaa tgagtgagct aactcacatt
aattgcgttg cgctcactgc ccgctttcca gtcgggaaac ctgtcgtgcc
agctgcatta atgaatcggc caacgcgcgg ggagaggcgg tttgcgtatt
gggcgctctt ccgcttcctc gctcactgac tcgctgcgct cggtcgttcg
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cagaatcagg ggataacgca ggaaagaaca tgtgagcaaa aggccagcaa
aaggccagga accgtaaaaa ggccgcgttg ctggcgtttt tccataggct
ccgccccccct gacgagcatc acaaaaatcg acgctcaagt cagaggtggc
gaaacccgac aggactataa agataccagg cgtttccccc tggaagctcc
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ctttctccct tcgggaagcg tggcgctttc tcatagctca cgctgtaggt
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tggtggccta actacggcta cactagaaga acagtatttg gtatctgcgc
tctgctgaag ccagttacct tcggaaaaag agttggtagc tcttgatccg
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ggggtctgac gctcagtgga acgaaaactc acgttaaggg attttggtca
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agttttaaat caatctaaag tatatatgag taaacttggt ctgacagtta
ccaatgctta atcagtgagg cacctatctc agcgatctgt ctatttcgtt
catccatagt tgcctgactc cccgtcgtgt agataactac gatacgggag
ggcttaccat ctggccccag tgctgcaatg ataccgcgag acccacgctc
accggctcca gatttatcag caataaacca gccagccgga agggccgagc
gcagaagtgg tcctgcaact ttatccgcct ccatccagtc tattaattgt
tgccgggaag ctagagtaag tagttcgcca gttaatagtt tgcgcaacgt
tgttgccatt gctacaggca tcgtggtgtc acgctcgtcg tttggtatgg
cttcattcag ctccggttcc caacgatcaa ggcgagttac atgatccccc
atgttgtgca aaaaagcggt tagctccttc ggtcctccga tcgttgtcag
aagtaagttg gccgcagtgt tatcactcat ggttatggca gcactgcata
attctcttac tgtcatgcca tccgtaagat gcttttctgt gactggtgag
tactcaacca agtcattctg agaatagtgt atgcggcgac cgagttgctc
ttgcccggcg tcaatacggg ataataccgc gccacatagc agaactttaa
aagtgctcat cattggaaaa cgttcttcgg ggcgaaaact ctcaaggatc
ttaccgctgt tgagatccag ttcgatgtaa cccactcgtg cacccaactg
atcttcagca tcttttactt tcaccagcgt ttctgggtga gcaaaaacag
gaaggcaaaa tgccgcaaaa aagggaataa gggcgacacg gaaatgttga
atactcatac tcttcctttt tcaatattat tgaagcattt atcagggtta
ttgtctcatg agcggataca tatttgaatg tatttagaaa aataaacaaa
taggggttcc gcgcacattt ccccgaaaag tgccacctaa attgtaagcg
ttaatatttt gttaaaattc gcgttaaatt tttgttaaat cagctcattt
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gatggcccac tacgtgaacc atcaccctaa tcaagttttt tggggtcgag
gtgccgtaaa gcactaaatc ggaaccctaa agggagcccc cgatttagag
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aaaggagcgg gcgctagggc gctggcaagt gtagcggtca cgctgcgcgt
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gtgagcgcgc gtaatacgac tcactatagg gcgaattggg taccgggccc
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ctttgtgaac tgggtggccg agaaggaatg ggagttgccg ccagattctg
acatggatct gaatctgatt gagcaggcac ccctgaccgt ggccgagaag
ctgcagcgcg actttctgac ggaatggcgc cgtgtgagta aggccccgga
ggctcttttc tttgtgcaat ttgagaaggg agagagctac ttccacatgc
acgtgctcgt ggaaaccacc ggggtgaaat c

[0055] In **SEQ ID NO:1**, residues 1-1561 of **pAAV-RC1** encode the **Rep** protein, **Rep78** (with residues 95-221 corresponding to the AAV2 **P19** promoter and residues 1075-1254 corresponding to the AAV2 **P40** promoter (**SEQ ID NO:18**)); residues 1578-3788 encode the AAV1 **VP1** capsid protein; residues 7127-7431 encode a portion of the **Rep68** protein; residues 3984-4114 correspond to AAV2 **P5** promoter sequences (**SEQ ID NO:10**); residues 4237-4253 are *M13 Rev* sequences; residues 4261-4277 are *Lac* operator sequences; 4285-4315 are *Lac* promoter sequences; residues 4578-5302 correspond to pMB ori sequences, residues 5398-6258 encode an ampicillin resistance determinant; and residues 6259-6357 are *bla* promoter sequences (**Figure 3**).

2. Plasmid pAAV-RC2

[0056] Plasmid **pAAV-RC2** (**SEQ ID NO:2**; **Figure 4**) is an AAV helper plasmid that expresses AAV2 serotype capsid proteins that may be used in accordance with the present invention to provide AAV helper functions. The **P5** and **P40** promoters of **pAAV-RC2** are AAV2 serotype promoters (**SEQ ID NO:10** and **SEQ ID NO:18**, respectively).

Coding Strand of Plasmid pAAV-RC2 (SEQ ID NO:2):

ccgggccccc cctcgaggtc gacggtatcg ggggagctcg cagggtctcc
attttgaagc gggaggtttg aacgcgcagc cgccatgccg gggttttacg
agattgtgat taaggtcccc agcgaccttg acgagcatct gcccggcatt
tctgacagct ttgtgaactg ggtggccgag aaggaatggg agttgccgcc
agattctgac atggatctga atctgattga gcaggcaccc ctgaccgtgg
ccgagaagct gcagcgcgac tttctgacgg aatggcgccg tgtgagtaag
gccccggagg ctcttttctt tgtgcaattt gagaagggag agagctactt
ccacatgcac gtgctcgtgg aaaccaccgg ggtgaaatcc atggttttgg
gacgtttcct gagtcagatt cgcgaaaaac tgattcagag aatttaccgc
gggatcgagc cgactttgcc aaactggttc gcggtcacaa agaccagaaa
tggcgccgga ggcgggaaca aggtggtgga tgagtgctac atccccaatt
acttgctccc caaaacccag cctgagctcc agtgggcgtg gactaatatg
gaacagtatt taagcgcctg tttgaatctc acggagcgta aacggttggt
ggcgcagcat ctgacgcacg tgtcgcagac gcaggagcag aacaaagaga
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ggcgaaaaac cgtctatcag ggcgatggcc cactacgtga accatcaccc
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aagggcgatc ggtgcgggcc tcttcgctat tacgccagct ggcgaaaggg
ggatgtgctg caaggcgatt aagttgggta acgccagggt tttcccagtc
acgacgttgt aaaacgacgg ccagtgagcg cgcgtaatac gactcactat
agggcgaatt gggta

[0057] In **SEQ ID NO:2**, residues 85-1950 of **pAAV-RC2** encode the **Rep** protein, **Rep78** (with residues 484-663 corresponding to the AAV2 **P19** promoter, residues 1464-1643 corresponding to the AAV2 **P40** promoter (**SEQ ID NO:18**) and residues 1668-1676 being a donor site); residues 1967-4174 encode the AAV2 **VP1** capsid protein; residues 1992-2016 encode a portion of the **Rep68** protein; residues 4175-4256 encode a polyA sequence; residues 4357-4487 correspond to the AAV2 **P5** promoter sequences of **SEQ ID NO:10**); residues 4610-4626 are *M13 Rev* sequences; residues 4634-4650 are *Lac* operator sequences; 4658-4688 are *Lac* promoter sequences; residues 4951-5675 correspond to pMB ori sequences, residues 5771-6631 encode an ampicillin resistance determinant; and residues 6632-6730 are *bla* promoter sequences (**Figure 4**).

3. Plasmid pAAV-RC5

[0058] Plasmid **pAAV-RC5** (**SEQ ID NO:3**; **Figure 5**) is an AAV helper plasmid that expresses AAV5 serotype capsid proteins that may be used in accordance with the present invention to provide AAV helper functions. The **P5** and **P40** promoters of

pAAV-RC5 are AAV2 serotype promoters (**SEQ ID NO:10** and **SEQ ID NO:18**, respectively).

Coding Strand of Plasmid pAAV-RC5 (SEQ ID NO:3):

catggttttg ggacgtttcc tgagtcagat tcgcgaaaaa ctgattcaga
gaatttaccg cgggatcgag ccgactttgc caaactggtt cgcggtcaca
aagaccagaa atggcgccgg aggcgggaac aaggtggtgg atgagtgcta
catccccaat tacttgctcc ccaaaaccca gcctgagctc cagtgggcgt
ggactaatat ggaacagtat ttaagcgcct gtttgaatct cacggagcgt
aaacggttgg tggcgcagca tctgacgcac gtgtcgcaga cgcaggagca
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aaacttcagc caggtacatg gagctggtcg ggtggctcgt ggacaagggg
attacctcgg agaagcagtg gatccaggag gaccaggcct catacatctc
cttcaatgcg gcctccaact cgcggtccca aatcaaggct gccttggaca
atgcgggaaa gattatgagc ctgactaaaa ccgcccccga ctacctggtg
ggccagcagc ccgtggagga catttccagc aatcggattt ataaaatttt
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gggccacgaa aaagttcggc aagaggaaca ccatctggct gtttgggcct
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gtcacgctgc gcgtaaccac cacacccgcc gcgcttaatg cgccgctaca
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cggtgcgggc ctcttcgcta ttacgccagc tggcgaaagg gggatgtgct
gcaaggcgat taagttgggt aacgccaggg ttttcccagt cacgacgttg
taaaacgacg gccagtgagc gcgcgtaata cgactcacta tagggcgaat
tgggtaccgg gccccccctc gaggtcgacg gtatcggggg agctcgcagg
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gccgccagat tctgacatgg atctgaatct gattgagcag gcacccctga
ccgtggccga gaagctgcag cgcgactttc tgacggaatg gcgccgtgtg

agtaaggccc cggaggctct tttctttgtg caatttgaga agggagagag
ctacttccac atgcacgtgc tcgtggaaac caccggggtg aaatc

[0059] In **SEQ ID NO:3**, residues 1-1561 of **pAAV-RC5** encode the **Rep** protein, **Rep78** (with residues 91-221 corresponding to the AAV2 **P19** promoter, and residues 1075-1254 corresponding to the **P40** promoter (**SEQ ID NO:18**)); residues 1578-3749 encode the AAV5 **VP1** capsid protein; residues 7091-7395 encode a portion of the **Rep68** protein; residues 3948-4078 correspond to the AAV2 **P5** promoter sequences of **SEQ ID NO:10**); residues 4201-4217 are *M13 Rev* sequences; residues 4225-4241 are *Lac* operator sequences; 4249-4279 are *Lac* promoter sequences; residues 4542-5266 correspond to pMB ori sequences, residues 5362-6222 encode an ampicillin resistance determinant; and residues 6223-6321 are *bla* promoter sequences (**Figure 5**).

4. Plasmid pAAV-RC6

[0060] Plasmid **pAAV-RC6** (**SEQ ID NO:4**; **Figure 6**) is an AAV helper plasmid that expresses AAV6 serotype capsid proteins that may be used in accordance with the present invention to provide AAV helper functions. The **P5** and **P40** promoters of **pAAV-RC6** are AAV2 serotype promoters (**SEQ ID NO:10** and **SEQ ID NO:18**, respectively).

Coding Strand of Plasmid pAAV-RC6 (SEQ ID NO:4):

catggttttg ggacgtttcc tgagtcagat tcgcgaaaaa ctgattcaga
gaatttaccg cgggatcgag ccgactttgc caaactggtt cgcggtcaca
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catccccaat tacttgctcc ccaaaaccca gcctgagctc cagtgggcgt
ggactaatat ggaacagtat ttaagcgcct gtttgaatct cacggagcgt
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ggaactaaac gggtacgatc cccaatatgc ggcttccgtc tttctgggat
gggccacgaa aaagttcggc aagaggaaca ccatctggct gtttgggcct
gcaactaccg ggaagaccaa catcgcggag gccatagccc acactgtgcc
cttctacggg tgcgtaaact ggaccaatga gaactttccc ttcaacgact
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ggctcttttc tttgtgcaat ttgagaaggg agagagctac ttccacatgc
acgtgctcgt ggaaaccacc ggggtgaaat c

[0061] In **SEQ ID NO:4**, residues 1-1561 of **pAAV-RC6** encode the **Rep** protein, **Rep78** (with residues 91-221 corresponding to the AAV2 **P19** promoter, and residues 1075-1254 corresponding to the **P40** promoter (**SEQ ID NO:18**)); residues 1578-3788 encode the AAV6 **VP1** capsid protein; residues 736-1281 encode a portion of the **Rep68** protein; residues 3984-4114 correspond to the AAV2 **P5** promoter sequences of **SEQ ID NO:10**); residues 4237-4253 are *M13 Rev* sequences; residues 4261-4277 are *Lac* operator sequences; 4285-4315 are *Lac* promoter sequences; residues 4578-5302 correspond to pMB ori sequences, residues 5398-6258 encode an ampicillin resistance determinant; and residues 6259-6357 are *bla* promoter sequences (**Figure 6**).

5. Plasmid pAAV-RC7

[0062] Plasmid **pAAV-RC7** (**SEQ ID NO:5**; **Figure 7**) is an AAV helper plasmid that expresses AAV6 serotype capsid proteins that may be used in accordance with the present invention to provide AAV helper functions. The **P5** and **P40** promoters of

pAAV-RC7 are AAV2 serotype promoters (**SEQ ID NO:10** and **SEQ ID NO:18**, respectively).

Coding Strand of Plasmid pAAV-RC7 (SEQ ID NO:5):

catggttttg ggacgtttcc tgagtcagat tcgcgaaaaa ctgattcaga
gaatttaccg cgggatcgag ccgactttgc caaactggtt cgcggtcaca
aagaccagaa atggcgccgg aggcgggaac aaggtggtgg atgagtgcta
catccccaat tacttgctcc ccaaaaccca gcctgagctc cagtgggcgt
ggactaatat ggaacagtat ttaagcgcct gtttgaatct cacggagcgt
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ggccagcagc ccgtggagga catttccagc aatcggattt ataaaatttt
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tgcacgtgct cgtggaaacc accggggtga aatc

[0063] In **SEQ ID NO:5**, residues 1-1561 of **pAAV-RC7** encode the **Rep** protein, **Rep78** (with residues 91-221 corresponding to the AAV2 **P19** promoter, and residues 1075-1254 corresponding to the **P40** promoter (**SEQ ID NO:18**)); residues 1578-3791 encode the AAV7 **VP1** capsid protein; residues 736-1281 encode a portion of the **Rep68** protein; residues 3987-4117 correspond to the AAV2 **P5** promoter sequences of **SEQ ID NO:10);** residues 4240-4256 are *M13 Rev* sequences; residues 4264-4280 are *Lac* operator sequences; 4288-4318 are *Lac* promoter sequences; residues 4581-5305 correspond to pMB ori sequences, residues 5401-6261 encode an ampicillin resistance determinant; and residues 6262-6360 are *bla* promoter sequences (**Figure 7**).

B. Illustrative Non-AAV Helper Function-Providing Polynucleotides

[0064] As used herein, the term "**non-AAV helper functions**" denotes proteins of Ad, CMV, HSV or other non-AAD viruses (*e.g.*, E1a, E1b, E2a, VA and E4) and/or polynucleotides of Ad, CMV, HSV or other non-AAD viruses that are required for the replication and packaging of an rAAV. Such non-AAV helper functions are provided by a "**non-AAV helper function-providing polynucleotide**," which as such term is used herein is a virus, plasmid vector, a non-plasmid vector, or a polynucleotide that has been integrated into a cellular chromosome, that provides non-AAV helper functions. The vector, **pHelper**, and derivatives thereof (such as those commercially available from Cell Biolabs, Inc., Invitrogen, Stratagene and other sources), are suitable non-AAV helper function-providing polynucleotide (see, *e.g.*, Matsushita, T. *et al.* (1998) "*Adeno-Associated Virus Vectors Can Be Efficiently Produced Without Helper Virus*," Gene Ther. 5:938-945; Sharma, A. *et al.* (2010) "*Transduction Efficiency Of AAV 2/6, 2/8 And 2/9 Vectors For Delivering Genes In Human Corneal Fibroblasts*," Brain Res. Bull. 81(2-3):273-278).

[0065] Plasmid **pHelper-Kan (SEQ ID NO:6; Figure 8)** is a **non-AAV helper function-providing polynucleotide** that may be used in accordance with the present invention to provide **non-AAV helper functions**.

Coding Strand of Plasmid pHelper-Kan (SEQ ID NO:6):

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gcaaccagga acagctctac agcttcctgg agcgccactc gccctacttc

cgcagccaca gtgcgcagat taggagcgcc acttcttttt gtcacttgaa
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tatcctgatg cacgattatg actctacccc catgtaggcg tggacttccc
cttcgccgcc cgttgagcaa ccgcaagttg gacagcagcc tgtggctcag
cagctggaca gcgacatgaa cttaagcgag ctgcccgggg agtttattaa
tatcactgat gagcgtttgg ctcgacagga aaccgtgtgg aatataacac
ctaagaatat gtctgttacc catgatatga tgctttttaa ggccagccgg
ggagaaagga ctgtgtactc tgtgtgttgg gagggaggtg gcaggttgaa
tactagggtt ctgtgagttt gattaaggta cggtgatcaa tataagctat
gtggtggtgg ggctatacta ctgaatgaaa aatgacttga aattttctgc
aattgaaaaa taaacacgtt gaaacataac atgcaacagg ttcacgattc
tttattcctg ggcaatgtag gagaaggtgt aagagttggt agcaaaagtt
tcagtggtgt attttccact ttcccaggac catgtaaaag acatagagta
agtgcttacc tcgctagttt ctgtggattc actagaatcg atgtaggatg
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gctctccttt tgcacggtct agagcgtcaa cgactgcgca cgcctcaccg
gccagagcgt cccgaccatg gagcactttt tgccgctgcg caacatctgg
aaccgcgtcc gcgactttcc gcgcgcctcc accaccgccg ccggcatcac
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cgcaggaaag aacatgtgag caaaaggcca gcaaaaggcc aggaaccgta
aaaaggccgc gttgctggcg tttttccata ggctccgccc ccctgacgag
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ataaagatac caggcgtttc cccctggaag ctccctcgtg cgctctcctg
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ggtcgttcgc tccaagctgg gctgtgtgca cgaaccccc gttcagcccg
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cacgacttat cgccactggc agcagccact ggtaacagga ttagcagagc
gaggtatgta ggcggtgcta cagagttctt gaagtggtgg cctaactacg
gctacactag aagaacagta tttggtatct gcgctctgct gaagccagtt
accttcggaa aaagagttgg tagctcttga tccggcaaac aaaccaccgc
tggtagcggt ggtttttttg tttgcaagca gcagattacg cgcagaaaaa
aaggatctca agaagatcct ttgatctttt ctacggggtc tgacgctcag
tggaacgaaa actcacgtta agggattttg gtcatgagat tatcaaaaag

gatcttcacc tagatccttt taaattaaaa atgaagtttt aaatcaatct
aaagtatata tgagtaaact tggtctgaca gtcagaagaa ctcgtcaaga
aggcgataga aggcgatgcg ctgcgaatcg ggagcggcga taccgtaaag
cacgaggaag cggtcagccc attcgccgcc aagctcttca gcaatatcac
gggtagccaa cgctatgtcc tgatagcggt ccgccacacc cagccggcca
cagtcgatga atccagaaaa gcggccattt tccaccatga tattcggcaa
gcaggcatcg ccatgggtca cgacgagatc ctcgccgtcg ggcatgctcg
ccttgagcct ggcgaacagt tcggctggcg cgagcccctg atgctcttcg
tccagatcat cctgatcgac aagaccggct tccatccgag tacgtgctcg
ctcgatgcga tgtttcgctt ggtggtcgaa tgggcaggta gccggatcaa
gcgtatgcag ccgccgcatt gcatcagcca tgatggatac tttctcggca
ggagcaaggt gagatgacag gagatcctgc cccggcactt cgcccaatag
cagccagtcc cttcccgctt cagtgacaac gtcgagtaca gctgcgcaag
gaacgcccgt cgtggccagc cacgatagcc gcgctgcctc gtcttgcagt
tcattcaggg caccggacag gtcggtcttg acaaaaagaa ccgggcgccc
ctgcgctgac agccggaaca cggcggcatc agagcagccg attgtctgtt
gtgcccagtc atagccgaat agcctctcca cccaagcggc cggagaacct
gcgtgcaatc catcttgttc aatcatactc ttccttttc aatattattg
aagcatttat cagggttatt gtctcatgag cggatacata tttgaatgta
tttagaaaaa taaacaaata ggggttccgc gcacatttcc ccgaaaagtg
ccacctaaat tgtaagcgtt aatattttgt taaaattcgc gttaaatttt
tgttaaatca gctcatttt taaccaatag gccgaaatcg gcaaaatccc
ttataaatca aaagaataga ccgagatagg gttgagtgtt gttccagttt
ggaacaagag tccactatta aagaacgtgg actccaacgt caaagggcga
aaaaccgtct atcagggcga tggcccacta cgtgaaccat caccctaatc
aagtttttg gggtcgaggt gccgtaaagc actaaatcgg aaccctaaag
ggagcccccg atttagagct tgacggggaa agccggcgaa cgtggcgaga
aaggaaggga agaaagcgaa aggagcgggc gctagggcgc tggcaagtgt
agcggtcacg ctgcgcgtaa ccaccacacc cgccgcgctt aatgcgccgc
tacagggcgc gatggatcc

[0066] In **SEQ ID NO:6**, residues 1-5343 of **pHelper-Kan** are derived from adenovirus, and include a polynucleotide encoding the **E2A** protein (residues 258-1847); residues 5344-8535 are derived from adenovirus, and include a polynucleotide encoding the **E4orf6** protein; residues 9423-10011 correspond to ori sequences; residues 10182-10976 encode a kanamycin resistance determinant expressed by a *bla* promoter sequence (residues 10977-11081); residues 11107-11561 correspond to f1 ori sequences (**Figure 8**).

C. Illustrative rAAV Plasmid Vectors

[0067] As discussed above, **AAV helper function-providing polynucleotides** and **non-AAV helper function-providing polynucleotides** are typically employed in concert with an rAAV plasmid vector to comprise a triple plasmid transfection system. Multiple commercially available rAAV plasmid vectors (*e.g.*, **pAV-CMV-EGFP**,

pGOI, *etc.* (Cell Biolabs, Inc., Invitrogen and Stratagene)) may be used in accordance with the present invention. An illustrative rAAV plasmid vector that may be used in accordance with the present invention is **pAV-CMV-EGFP** (**SEQ ID NO:7**; **Figure 9**) which comprises a 5' ITR, a U6 promoter, CMV enhancer and promoter sequences, a polynucleotide encoding the enhanced green fluorescent protein (**EGFP**) (Gambotto, A. *et al.* (2000) "*Immunogenicity Of* Enhanced *Green Fluorescent Protein (EGFP) In BALB/C Mice: Identification Of An H2-Kd-Restricted CTL Epitope*," Gene Ther. 7(23):2036-2040; Tsien, R.Y. (1998) "*The Green Fluorescent Protein*," Annu. Rev. Biochem. 67:509-544; Cinelli, R.A. *et al.* (2000) "*The Enhanced Green Fluorescent Protein As A Tool For The Analysis Of Protein Dynamics And Localization: Local Fluorescence Study At The Single-Molecule Level*," Photochem. Photobiol. 71(6):771-776; Chopra A. (2008) "*Recombinant Adenovirus With Enhanced Green Fluorescent Protein*," In: MOLECULAR IMAGING AND CONTRAST AGENT DATABASE (MICAD), National Center for Biotechnology Information, Bethesda MD), FLAG-tag and 6xHis-tag sites for facilitating recovery or localization of expressed proteins, an SV40 poly(A) site and a 3' ITR.

Coding Strand of Plasmid pAV-CMV-EGFP (SEQ ID NO:7):

cctgcaggca gctgcgcgct cgctcgctca ctgaggccgc ccgggcgtcg
ggcgaccttt ggtcgcccgg ccctccagtg agcgagcgcg cagagaggga
gtggccaact ccatcactag gggttcctgc ggccgcacgc gtctagttat
taatagtaat cgaattcgtg ttactcataa ctagtaaggt cgggcaggaa
gagggcctat ttcccatgat tccttcatat ttgcatatac gatacaaggc
tgttagagag ataattagaa ttaatttgac tgtaaacaca aagatattag
tacaaaatac gtgacgtaga aagtaataat ttcttgggta gtttgcagtt
ttaaaattat gttttaaaat ggactatcat atgcttaccg taacttgaaa
gtatttcgat ttcttgggtt tatatatctt gtggaaagga cgcgggatcc
actggaccag gcagcagcgt cagaagactt ttttggaaaa gcttgactag
taatactgta atagtaatca attacggggt cattagttca tagcccatat
atggagttcc gcgttacata acttacggta aatggcccgc ctggctgacc
gcccaacgac ccccgcccat tgacgtcaat aatgacgtat gttcccatag
taacgccaat agggactttc cattgacgtc aatgggtgga gtatttacgg
taaactgccc acttggcagt acatcaagtg tatcatatgc caagtacgcc
ccctattgac gtcaatgacg gtaaatggcc cgcctggcat tatgcccagt
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ctgcccgtgc cctggcccac cctcgtgacc accctgacct acggcgtgca
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ccgccatgcc cgaaggctac gtccaggagc gcaccatctt cttcaaggac
gacggcaact acaagacccg cgccgaggtg aagttcgagg gcgacaccct
ggtgaaccgc atcgagctga agggcatcga cttcaaggag gacggcaaca
tcctggggca caagctggag tacaactaca acagccacaa cgtctatatc
atggccgaca agcagaagaa cggcatcaag gtgaacttca agatccgcca
caacatcgag gacggcagcg tgcagctcgc cgaccactac cagcagaaca
cccccatcgg cgacggcccc gtgctgctgc ccgacaacca ctacctgagc
acccagtccg ccctgagcaa agaccccaac gagaagcgcg atcacatggt
cctgctggag ttcgtgaccg ccgccgggat cactctcggc atggacgagc
tgtacaagta agtcgaggat tataaggatg acgacgataa attcgtcgag
caccaccacc accaccacta ataaggttta tccgatccac cggatctaga
taagatatcc gatccaccgg atctagataa ctgatcataa tcagccatac
cacatttgta gaggttttac ttgctttaaa aaacctccca cacctccccc
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gcagcttata atggttacaa ataaagcaat agcatcacaa atttcacaaa
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acgtttacaa ttttatggtg cactctcagt acaatctgct ctgatgccgc
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taaaacttca tttttaattt aaaaggatct aggtgaagat cctttttgat
aatctcatga ccaaaatccc ttaacgtgag ttttcgttcc actgagcgtc
agaccccgta gaaaagatca aaggatcttc ttgagatcct ttttttctgc
gcgtaatctg ctgcttgcaa acaaaaaaac caccgctacc agcggtggtt
tgtttgccgg atcaagagct accaactctt tttccgaagg taactggctt
cagcagagcg cagataccaa atactgtcct tctagtgtag ccgtagttag
gccaccactt caagaactct gtagcaccgc ctacatacct cgctctgcta
atcctgttac cagtggctgc tgccagtggc gataagtcgt gtcttaccgg
gttggactca agacgatagt taccggataa ggcgcagcgg tcgggctgaa
cgggggttc gtgcacacag cccagcttgg agcgaacgac ctacaccgaa
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gagaaaggcg gacaggtatc cggtaagcgg cagggtcgga acaggagagc
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gggtttcgcc acctctgact tgagcgtcga tttttgtgat gctcgtcagg
ggggcggagc ctatggaaaa acgccagcaa cgcggccttt ttacggttcc
tggccttttg ctggcctttt gctcacatgt

[0068] In **SEQ ID NO:7**, residues 1-128 of **pAV-CMV-EGFP** correspond to the 5' ITR; residues 201-441 are U6 promoter sequences; residues 562-865 are human cytomegalovirus (CMV) immediate early enhancer sequences; residues 866-1068 comprise the CMV immediate early promoter; residues 1192-1911 comprise a mammalian codon-optimized polynucleotide that encodes the EGFP; residues 1918-1941 encode the FLAG-tag; residues 1951-1968 encode the 6xHis-tag; residues 2139-2260 encode the SV40 poly(A) sequence; residues 2293-2433 correspond to the 3' ITR; residues 2508-22963 correspond to F1 ori sequences; residues 3350-4210 encode an ampicillin resistance determinant and its signal sequence (residues 3350-3418) expressed by a *bla* promoter sequence (residues 3245-3349); residues 4381-4969 correspond to an ori sequence (**Figure 9**).

[0069] A second illustrative rAAV plasmid vector that may be used in accordance with the present invention is **pAV-TBG-EGFP** (**SEQ ID NO:8**; **Figure 10**) which comprises a 5' ITR, a thyroid hormone-binding globulin (TBG) promoter, a

polynucleotide encoding the enhanced green fluorescent protein (EGFP), FLAG-tag and 6xHis-tag sites for facilitating recovery or localization of expressed proteins, an SV40 poly(A) site and a 3' ITR.

Coding Strand of Plasmid pAV-TBG-EGFP (SEQ ID NO:8):

cctgcaggca gctgcgcgct cgctcgctca ctgaggccgc ccgggcgtcg
ggcgaccttt ggtcgcccgg cctcagtgag cgagcgagcg cgcagagagg
gagtggccaa ctccatcact aggggttcct gcggccggtc gcgtctagta
ctagtaggtt aatttttaaa aagcagtcaa aagtccaagt ggcccttggc
agcatttact ctctctgttt gctctggtta ataatctcag gagcacaaac
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aaattcgtcg agcaccacca ccaccaccac taataaggtt tatccgatcc
accggatcta gataagatat ccgatccacc ggatctagat aactgatcat
aatcagccat accacatttg tagaggtttt acttgcttta aaaaacctcc
cacacctccc cctgaacctg aaacataaaa tgaatgcaat tgttgttgtt
aacttgttta ttgcagctta taatggttac aaataaagca atagcatcac
aaatttcaca aataaagcat ttttttcact gcattctagt tgtggtttgt
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ccgcaggaac ccctagtgat ggagttggcc actccctctc tgcgcgctcg
ctcgctcact gaggccgggc gaccaaaggt cgcccgacgc ccgggctttg
cccgggcggc ctcagtgagc gagcgagcgc gcagctgcct gcaggggcgc
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acgtcaaagc aaccatagta cgcgccctgt agcggcacat taagcgcggc
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cgcccgctcc tttcgctttc ttcccttcct ttctcgccac gttcgccggc
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gtagtgggcc atcgccctga tagacggttt ttcgcccttt gacgttggag
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ctctatctcg ggctattctt ttgatttata agggattttg ccgatttcgg
tctattggtt aaaaaatgag ctgatttaac aaaaatttaa cgcgaatttt
aacaaaatat taacgtttac aattttatgg tgcactctca gtacaatctg
ctctgatgcc gcatagttaa gccagccccg acacccgcca acacccgctg
acgcgccctg acgggcttgt ctgctcccgg catccgctta cagacaagct
gtgaccgtct ccgggagctg catgtgtcag aggttttcac cgtcatcacc
gaaacgcgcg agacgaaagg gcctcgtgat acgcctattt ttataggtta
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aatgtgcgcg gaacccctat ttgtttattt ttctaaatac attcaaatat
gtatccgctc atgagacaat aaccctgata aatgcttcaa taatattgaa
aaaggaagag tatgagtatt caacatttcc gtgtcgccct tattcccttt
tttgcggcat tttgccttcc tgtttttgct cacccagaaa cgctggtgaa
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tggatctcaa cagcggtaag atccttgaga gttttcgccc cgaagaacgt
tttccaatga tgagcacttt taaagttctg ctatgtggcg cggtattatc
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agaatgactt ggttgagtac tcaccagtca cagaaaagca tcttacggat
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cactgcggcc aacttacttc tgacaacgat cggaggaccg aaggagctaa
ccgctttttt gcacaacatg ggggatcatg taactcgcct tgatcgttgg
gaaccggagc tgaatgaagc cataccaaac gacgagcgtg acaccacgat
gcctgtagca atggcaacaa cgttgcgcaa actattaact ggcgaactac
ttactctagc ttcccggcaa caattaatag actggatgga ggcggataaa
gttgcaggac cacttctgcg ctcggccctt ccggctggct ggtttattgc
tgataaatct ggagccggtg agcgtgggtc tcgcggtatc attgcagcac
tggggccaga tggtaagccc tcccgtatcg tagttatcta cacgacgggg
agtcaggcaa ctatggatga acgaaataga cagatcgctg agataggtgc
ctcactgatt aagcattggt aactgtcaga ccaagtttac tcatatatac
tttagattga tttaaaactt cattttaat ttaaaaggat ctaggtgaag
atccttttg ataatctcat gaccaaaatc ccttaacgtg agttttcgtt
ccactgagcg tcagaccccg tagaaaagat caaaggatct tcttgagatc
ctttttttct gcgcgtaatc tgctgcttgc aaacaaaaaa accaccgcta
ccagcggtgg tttgtttgcc ggatcaagag ctaccaactc tttttccgaa
ggtaactggc ttcagcagag cgcagatacc aaatactgtt cttctagtgt
agccgtagtt aggccaccac ttcaagaact ctgtagcacc gcctacatac
ctcgctctgc taatcctgtt accagtggct gctgccagtg gcgataagtc
gtgtcttacc gggttggact caagacgata gttaccggat aaggcgcagc
ggtcgggctg aacgggggt tcgtgcacac agcccagctt ggagcgaacg
acctacaccg aactgagata cctacagcgt gagctatgag aaagcgccac
gcttcccgaa gggagaaagg cggacaggta tccggtaagc ggcagggtcg
gaacaggaga gcgcacgagg gagcttccag ggggaaacgc ctggtatctt
tatagtcctg tcgggtttcg ccacctctga cttgagcgtc gatttttgtg

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atgctcgtca gggggggcgga gcctatggaa aaacgccagc aacgcggcct
ttttacggtt cctggccttt tgctggcctt ttgctcacat gt
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[0070] In **SEQ ID NO:8**, residues 1-130 of **pAV-TBG-EGFP** correspond to the 5' ITR; residues 150-854 are TBG promoter sequences, with residues 415-824 comprising the TBG promoter; residues 886-1608 encode the EGFP; residues 1630-1653 encode the FLAG-tag; residues 1663-1680 encode the 6xHis-tag; residues 1851-1972 encode the poly(A) sequence; residues 2005-2145 corresponds to the 3' ITR; residues 2220-2675 correspond to F1 ori sequences; residues 3062-3922 encode an ampicillin resistance determinant and its signal sequence (residues 3062-3130) expressed by a *bla* promoter sequence (residues 2957-3061); residues 4093-4681 correspond to an ori sequence (**Figure 10**).

[0071] As used herein, the term "**native AAV serotype promoter sequence**" is intended to denote a promoter sequence that natively controls the transcription of an AAV ***rep*** gene or is natively present within such ***rep*** gene. For example:

- AAV1 **P5** promoter sequences natively control the transcription of the ***rep*** gene of AAV1 and AAV1 **P40** promoter sequences are natively found within the ***rep*** gene of AAV1. Thus, the AAV1 **P5** promoter sequences and the AAV1 **P40** promoter sequences are native AAV serotype promoter sequences of the ***rep*** gene of AAV1;
- AAV2 **P5** promoter sequences natively control the transcription of the ***rep*** gene of AAV2 and the AAV2 **P40** promoter sequences are natively found within the ***rep*** gene of AAV2. Thus, the AAV2 **P5** promoter sequences and the AAV2 **P40** promoter sequences are native AAV serotype promoter sequences of the ***rep*** gene of AAV2;
- AAV5 **P5** promoter sequences natively control the transcription of the ***rep*** gene of AAV5 and the AAV5 **P40** promoter sequences are natively found within the ***rep*** gene of AAV5. Thus, the AAV5 **P5** promoter sequences and the AAV5 **P40** promoter sequences are native AAV serotype promoter sequences of the ***rep*** gene of AAV5;
- AAV6 **P5** promoter sequences natively control the transcription of the ***rep*** gene of AAV6 and the AAV6 **P40** promoter sequences are natively found within the ***rep*** gene of AAV6. Thus, the AAV6 **P5** promoter sequences and the AAV6

P40 promoter sequences are native AAV serotype promoter sequences of the ***rep*** gene of AAV6; and

- AAV7 **P5** promoter sequences natively control the transcription of the ***rep*** gene of AAV7 and the AAV7 **P40** promoter sequences are natively found within the ***rep*** gene of AAV7. Thus, the AAV7 **P5** promoter sequences and the AAV7 **P40** promoter sequences are native AAV serotype promoter sequences of the ***rep*** gene of AAV7;

[0072] Native AAV **P5** and **P40** promoter sequences for AAV serotypes 1-8 are shown in **Table 1**. Such sequences, or subsequences thereof that are capable of mediating transcription, may be used in accordance with the methods of the present invention.

Table 1

SEQ ID NO	AAV Promoter	Native Serotype	Sequence
SEQ ID NO:9	**P5**	AAV1	ggtcctgtat tagctgtcac gtgagtgctt ttgcgacatt ttgcgacacc acgtggccat ttagggtata tatggccgag tgagcgagca ggatctccat tttgaccgcg aaatttgaac gagcagcagc c
SEQ ID NO:10	**P5**	AAV2	ggtcctgtat tagaggtcac gtgagtgttt tgcgacattt tgcgacacca tgtggtcacg ctgggtattt aagcccgagt gagcacgcag ggtctccatt ttgaagcggg aggtttgaac gcgcagccgc c
SEQ ID NO:11	**P5**	AAV3	ccagctgcgt cagcagtcag gtgacccttt tgcgacagtt tgcgacacca cgtggccgct gagggtatat attctcgagt gagcgaacca ggagctccat tttgaccgcg aaatttgaac gagcagcagc c
SEQ ID NO:12	**P5**	AAV4	ggtccctgta ttagcagtca cgtgagtgtc gtatttcgcg gagcgtagcg gagcgcatac caagctgcca cgtcacagcc acgtggtccg tttgcgacag tttgcgacac catgtggtca ggagggtata taaccgcgag tgagccagcg aggagctcca ttttgcccgc gaattttgaa cgagcagcag cc
SEQ ID NO:13	**P5**	AAV5	atgtgatgtg ttttatccaa taggaagaaa gcgcgcgtat gagttctcgc gagacttccg gggtataaaa gaccgagtga acgagcccgc cgccattctt tgctctggac tgctagagga ccctcgctgc c

Table 1			
SEQ ID NO	**AAV Promoter**	**Native Serotype**	**Sequence**
SEQ ID NO:14	**P5**	AAV6	ggtcctgtat tagaggtcac gtgagtgttt tgcgacattt tgcgacacca tgtggtcacg ctgggtattt aagcccgagt gagcacgcag ggtctccatt ttgaagcggg aggtttgaac gcgcagcgcc
SEQ ID NO:15	**P5**	AAV7	ggtcctgtat tagctgtcac gtgagtgctt ttgcgacatt ttgcgacacc acgtggccat ttgaggtata tatggccgag tgagcgagca ggatctccat tttgaccgcg aaatttgaac gagcagcagc c
SEQ ID NO:16	**P5**	AAV8	ggtcctgtat tagctgtcac gtgagtgctt ttgcggcatt ttgcgacacc acgtggccat ttgaggtata tatggccgag tgagcgagca ggatctccat tttgaccgcg aaatttgaac gagcagcagc c
SEQ ID NO:17	**P40**	AAV1	ggtgacaaag caggaagtca aagagttctt ccgctgggcg caggatcacg tgaccgaggt ggcgcatgag ttctacgtca gaaagggtgg agccaacaaa agacccgccc ccgatgacgc ggataaaagc gagcccaagc gggcctgccc ctcagtcgcg gatccatcga cgtcagacgc
SEQ ID NO:18	**P40**	AAV2	ggtcaccaag caggaagtca aagacttttt ccggtgggca aaggatcacg tggttgaggt ggagcatgaa ttctacgtca aaaagggtgg agccaagaaa agacccgccc ccagtgacgc agatataagt gagcccaaac gggtgcgcga gtcagttgcg cagccatcga cgtcagacgc
SEQ ID NO:19	**P40**	AAV3	ggtcaccaaa caggaagtaa aggacttttt ccggtgggct tccgatcacg tgactgacgt ggctcatgag ttctacgtca gaaagggtgg agctaagaaa cgccccgcct ccaatgacgc ggatgtaagc gagccaaaac gggagtgcac gtcacttgcg cagccgacaa cgtcagacgc
SEQ ID NO:20	**P40**	AAV4	ggtcaccaag caggaagtca aagacttttt ccggtgggcg tcagatcacg tgaccgaggt gactcacgag ttttacgtca gaaagggtgg agctagaaag aggcccgccc ccaatgacgc agatataagt gagcccaagc gggcctgtcc gtcagttgcg cagccatcga cgtcagacgc

Table 1

SEQ ID NO	AAV Promoter	Native Serotype	Sequence
SEQ ID NO:21	**P40**	AAV5	gattactaag caggaagtca aggacttttt tgcttgggca aaggtcaatc aggtgccggt gactcacgag tttaaagttc ccagggaatt ggcgggaact aaaggggcgg agaaatctct aaaacgccca ctgggtgacg tcaccaatac tagctataaa agtctggaga agcgggcctg gagcatgagg ctctcatttg ttcccgagac gcctcgcagt tcagacg
SEQ ID NO:22	**P40**	AAV6	ggtgacaaag caggaagtca aagagttctt ccgctgggcg caggatcacg tgaccgaggt ggcgcatgag ttctacgtca gaaagggtgg agccaacaag agacccgccc ccgatgacgc ggataaaagc gagcccaagc gggcctgccc ctcagtcgcg gatccatcga cgtcagacgc
SEQ ID NO:23	**P40**	AAV7	ggtgacgaag caggaagtca aagagttctt ccgctgggcc agtgatcacg tgaccgaggt ggcgcatgag ttctacgtca gaaagggcgg agccagcaaa agacccgccc ccgatgacgc ggatataagc gagcccaagc gggcctgccc ctcagtcgcg gatccatcga cgtcagacgc
SEQ ID NO:24	**P40**	AAV8	ggtgacaaag caggaagtca aagagttctt ccgctgggcc agtgatcacg tgaccgaggt ggcgcatgag ttttacgtca gaaagggcgg agccagcaaa agacccgccc ccgatgacgc ggataaaagc gagcccaagc gggcctgccc ctcagtcgcg gatccatcga cgtcagacgc

[0073] In contrast, the term "**non-native AAV serotype promoter sequence**" is intended to denote a promoter sequence that does not natively control a ***rep*** gene of an AAV and is not natively found within such ***rep*** gene. Illustrative, non-limiting examples of non-native AAV serotype promoter sequences include: the AAV1 **P5** promoter when used to direct the expression of an AAV2, AAV5, AAV6, or AAV7 ***rep*** gene; the AAV2 **P5** promoter when used to direct the expression of an AAV1, AAV5, AAV6, or AAV7 ***rep*** gene; the AAV5 **P5** promoter when used to direct the expression of an AAV1, AAV2, AAV6, or AAV7 ***rep*** gene; the AAV6 **P5** promoter when used to direct the expression of an AAV1, AAV2, AAV5, or AAV7 ***rep*** gene; the AAV7 **P5** promoter when used to direct the expression of an AAV1, AAV2, AAV5, or AAV6 ***rep*** gene; the AAV1 **P40** promoter, when present within an AAV2, AAV5, AAV6, or AAV7 ***rep*** gene; the AAV2 **P40** promoter, when present within an AAV1, AAV5, AAV6, or AAV7 ***rep*** gene; the AAV5 **P40** promoter, when present within an AAV1,

AAV2, AAV6, or AAV7 ***rep*** gene; the AAV6 **P40** promoter, when present within an AAV1, AAV2, AAV5, or AAV7 ***rep*** gene; the AAV7 **P40** promoter, when present within an AAV1, AAV2, AAV5, or AAV6 ***rep*** gene, *etc.*

[0074] In one embodiment, one or more of such AAV serotype promoter sequences can be genetically engineered into recombinant AAV helper plasmids that are designed to provide the **Rep** and **Cap** proteins to replace or augment the existing **P5** or **P40** promoters of such plasmids. Such modification is preferably accomplished using well-known methods of recombinant DNA technology.

[0075] The identity of the serotype of promoter sequences is indicated herein by denoting the involved promoter (*e.g.*, **P5**, **P40**, *etc.*), the serotype of the ***rep*** gene with which it is natively associated, and the name of the vector. Thus, for example, a **pAAV-RC2** plasmid that comprises a **P5** promoter sequence that is natively associated with AAV2 is denoted as **P5(2)-RC2**; a **pAAV-RC2** plasmid that comprises a **P5** promoter sequence that is natively associated with AAV3 is denoted as **P5(3)-RC2**; a **pAAV-RC5** plasmid that comprises a **P40** promoter sequence that is natively associated with AAV7 is denoted as **P40(7)-RC5**; a **pAAV-RC2** plasmid that comprises a **P5** promoter sequence that is natively associated with AAV3 and a **P40** promoter sequence that is natively associated with AAV8 is denoted as **P5(3)/P40(8)-RC2**; *etc.*

[0076] In one embodiment, the introduced AAV serotype promoter sequence will replace an initially present AAV serotype promoter sequence. In other embodiments, the introduced AAV serotype promoter sequence will be present in addition to such initially present AAV serotype promoter sequence, and will be positioned 5' to, or 3' to, such initially present AAV serotype promoter sequence. The introduced nucleotide sequence may be positioned adjacent to, or apart from, such initially present AAV serotype promoter sequence.

[0077] The substitution or addition of one or more of such AAV serotype promoter sequences invention increases rAAV production titers. As used herein, the term "**production titer**" is intended to denote the amount of concentration of infectious rAAV in a preparation. Such amounts or concentrations are preferably determined by titering the AAV or rAAV in such preparation. The production titers of the rAAV

preparations of the present invention are preferably titered after subjecting producing cells (*e.g.*, HEK293 transformed with an rAAV plasmid vector, an AAV helper vector providing **Rep** and **Cap** proteins, and an Ad helper vector providing required adenovirus transcription and translation factors) to three rounds of freeze / thawing, followed by sonication to release the rAAV particles. The preparation is then centrifuged. The employed AAV helper vector is localized to the supernatant. An aliquot of the preparation is treated with proteinase K, and the number of AAV genomes is determined. An aliquot of the preparation is infected into HeLa-32C2 cells (which express AAV2 **Rep** and **Cap** proteins), and infectious titer is measured using the infectious center assay (**ICA**) (François, A. *et al.* (2018) "*Accurate Titration of Infectious AAV Particles Requires Measurement of Biologically Active Vector Genomes and Suitable Controls*," Molec. Ther. Meth. Clin. Develop. 10:223-236) or more preferably, as the median tissue culture infective dose (**TCID50**) (Zen, Z. *et al.* (2004) "*Infectious Titer Assay For Adeno-Associated Virus Vectors With Sensitivity Sufficient To Detect Single Infectious Events*," Hum. Gene Ther. 15:709-715).

[0078] As used herein, an rAAV production titer is said to be "**increased**" by the methods of the present invention if the production titer obtained from the use of the methods of the present invention is at least 10% greater, more preferably at least 20% greater, still more preferably at least 30% greater, still more preferably at least 40% greater, still more preferably at least 50% greater, still more preferably at least 60% greater, still more preferably at least 70% greater, still more preferably at least 80% greater, still more preferably at least 90% greater, still more preferably at least 2-fold greater, still more preferably at least 110% greater, still more preferably at least 120% greater, still more preferably at least 130% greater, still more preferably at least 140% greater, still more preferably at least 2.5-fold greater, still more preferably at least 160% greater, still more preferably at least 170% greater, still more preferably at least 180% greater, still more preferably at least 190% greater, and still more preferably at least 3-fold greater than the titer obtained from a similarly conducted production in which the additionally provided ions were not provided.

[0079] The rAAV whose production titer may be increased using the methods of the present invention may comprise any transgene cassette that permits the rAAV to be packaged into an rAAV plasmid vector that may be encapsidated within an AAV capsid

particle. Without limitation, such transgene cassette(s) may be of human, primate (including chimpanzee, gibbon, gorilla, orangutan, *etc.*), cercopithecine (including baboon, cynomolgus monkey, velvet monkey, *etc.*), canine, glirine (including rat, mouse, hamster, guinea pig, *etc.*) , feline, ovine, caprine, or equine origin.

[0080] In preferred embodiments, such an rAAV or rAAV plasmid vector will encode a protein (*e.g.*, an enzyme, hormone, antibody, receptor, ligand, *etc.*), or comprise a transcribed nucleic acid, that is relevant to a genetic or heritable disease or condition, such that it may be used in gene therapy to treat such disease or condition.

[0081] The methods of the present invention may be used to increase the production titer of rAAV and rAAV plasmid vectors in cells that have been additionally transfected with:

(1) an AAD helper vector possessing a non-native AAV serotype promoter sequence and capable of expressing proteins or RNA molecules that are not natively provided by such rAAV or rAAV plasmid vectors, but are required for their production. As discussed above, such proteins or RNA molecules include the genes encoding the **Rep52** and **Rep78** proteins that are required for vector transcription control and replication, and for the packaging of viral genomes into the viral capsule, and ***cap*** genes that encode VP capsid proteins required to form infectious particles; and

(2) an Ad helper vector that can provide the non-AAV helper proteins (*e.g.*, E1a, E1b, E2a, VA and E4) or RNA molecules that are not provided by such rAAV or rAAV plasmid vectors, but are required for their production.

[0082] In one embodiment for producing the rAAV of the present invention, all of such genes and RNA molecules are provided on the same helper virus (or more preferably, helper vector) so as to comprise, in concert with an rAAV, a double plasmid transfection system. More preferably, however, for producing the rAAV of the present invention, the AAV helper function-providing polynucleotide that provides the required ***rep*** and ***cap*** genes and such non-native AAV serotype promoter sequences are provided on a vector that is separate from the vector that comprises the non-AAV helper function-providing polynucleotide, so that such vectors or plasmids, in concert with the rAAV, comprise a triple plasmid transfection system.

[0083] The invention thus derives in part from the recognition that the production of rAAV may be increased by causing the expression of Rep and Cap proteins to be directed by promoter sequences that are not native promoter sequences. Thus, by modifying a particular rAAV to replace its native **P5** and/or **P40** AAV serotype promoter sequence(s) with a non-native **P5** and/or **P40** AAV serotype promoter sequence (or by incorporating a non-native **P5** and/or **P40** AAV serotype promoter sequence into such rAAV), the methods of the present invention may be employed to increase the production titer of rAAV belonging to any serotype, including the AAV1, AAV2, AAV5, AAV6, AAV7, AAV8, AAV9 and AAV10 serotypes, and including hybrid serotypes (*e.g.*, AAV2/5 and rAAV2/5, which is a hybrid of AAV serotypes 2 and 5 and thus has the trophism of both such serotypes).

[0084] The methods of the present invention may be employed to increase the production titers of rAAV that are to be produced using "helper" RNA or proteins provided by an adenovirus, a herpes simplex virus, a cytomegalovirus, a vaccinia virus or a papillomavirus.

[0085] The methods of the present invention may be employed to increase the production titers of rAAV produced by cells in adherent monolayer culture or in suspension culture, and may be used with any method capable of producing rAAV. Preferably, however, rAAV is produced by transfecting baby hamster kidney (BHK) cells, or more preferably, human embryonic kidney (HEK) cells grown in tissue culture with the plasmid vectors described above. The BHK cell line BHK-21 (ATCC CCL-10), which lacks endogenous retroviruses is a preferred BHK cell line. The HEK cell line HEK293 (ATCC CRL-1573) and its derivatives, such as HEK293T (ATCC CRL-3216, which is a highly transfectable derivative of the HEK293 cell line into which the temperature-sensitive gene for SV40 T-antigen was inserted) or HEK293T/17 (ATCC® CRL-11268, which was selected for its ease of transfection) are particularly preferred. The HEK293T/17 SF cell line (ATCC ACS-4500) is a derivative of the 293T/17 cell line (ATCC CRL-11268), adapted to serum-free medium and suspension, and may be employed if desired.

[0086] The preferred base medium of the present invention for culturing such cells is Eagle's Minimum Essential Medium (ATCC Catalog No. 30-2003) or Dulbecco's

Modified Eagle's Medium (DMEM; Mediatech, Manassas, VA). Fetal bovine serum (*e.g.*, FBS; HyClone Laboratories, South Logan, UT) is added to a final concentration of 10% in order to make the complete growth medium. Eagle's Minimum Essential Medium and Dulbecco's Modified Eagle's Medium are complex media that contain amino acids, vitamins, and optionally glucose, in addition to various inorganic salts. The media differ in that Dulbecco's modified Eagle's medium contains approximately four times as much of the vitamins and amino acids present in the original formula of Eagle's Minimum Essential Medium, and two to four times as much glucose. Additionally, it contains iron in the form of ferric sulfate and phenol red for pH indication (Yao, T *et al.* (2017) "*Animal-Cell Culture Media: History, Characteristics, And Current Issues*," Reproduc. Med. Biol. 16(2): 99-117).

[0087] Cells to be used for such transfection are preferably passaged twice weekly to maintain them in exponential growth phase. For small-scale transfections, an aliquot of, for example, 1 x 10^6 HEK293 or BHK cells per well on a multi-well plate, or 1.5 x 10^7 HEK293 cells per 15-cm dish, may be employed. For large-scale production HEK293 or BHK cells may be collected from multiple confluent 15-cm plates, and split into two 10-layer cell stacks (Corning, Corning, NY) containing 1 liter of complete culturing medium. In one embodiment, such cells are grown for 4 days in such medium before transfection. The day before transfection, the two cell stacks may be trypsinized and the cells (*e.g.*, approximately 6 x 10^8 cells) may be resuspended in 200 ml of medium. Preferably, the cells are allowed to attach for 24 hours before transfection. Confluency of the cell stacks may be monitored using a Diaphot inverted microscope (Nikon, Melville, NY) from which the phase-contrast hardware had been removed in order to accommodate the cell stack on the microscope stage.

[0088] In particular, the present invention thus provides a method for increasing the production titer of a recombinantly-modified adeno-associated virus (rAAV) that comprises a transgene cassette, wherein the method comprises culturing cells that have been transfected with:

(1) the rAAV;

(2) a recombinantly-modified adeno-associated virus (AAV) helper vector that comprises an AAV helper function-providing polynucleotide, wherein such polynucleotide comprises a non-native AAV serotype **P5**

or **P40** promoter sequence in replacement of, or in addition to, a native AAV serotype promoter sequence; and

(3) a vector that comprises a non-AAV helper function-providing polynucleotide;

wherein the culturing is conducted in a culture medium under conditions sufficient to permit the production of the rAAV, and wherein the presence of the non-native AAV serotype **P5** or **P40** promoter sequence causes the cells to produce the rAAV at an increased production titer relative to that which would be attained if the AAV helper function-providing polynucleotide contained native serotype **P5** and **P40** promoters.

[0089] The present invention further provides a method for increasing the production titer of a recombinantly-modified adeno-associated virus (rAAV) that comprises a transgene cassette, wherein the method comprises culturing cells that have been transfected with:

(1) the rAAV; and

(2) a recombinantly-modified adeno-associated virus (AAV) helper vector that comprises:

(a) an AAV helper function-providing polynucleotide, wherein such polynucleotide comprises a non-native AAV serotype **P5** or **P40** promoter sequence in replacement of, or in addition to, a native AAV serotype promoter sequence; and

(b) a non-AAV helper function-providing polynucleotide;

wherein the culturing is conducted in a culture medium under conditions sufficient to permit the production of the rAAV, and wherein the presence of the non-native AAV serotype **P5** or **P40** promoter sequence causes the cells to produce the rAAV at an increased production titer relative to that which would be attained if the AAV helper function-providing polynucleotide contained native serotype **P5** and **P40** promoters.

[0090] In preferred embodiments, the transgene cassette of such rAAV encodes a protein, or comprises a transcribed nucleic acid, that is therapeutic for a genetic or heritable disease or condition.

II. Pharmaceutical Compositions of the Present Invention

[0091] The invention additionally includes pharmaceutical compositions that comprise a pharmaceutically acceptable preparation of rAAV produced in accordance with the methods of the present invention, and a pharmaceutically acceptable carrier. The rAAV of such pharmaceutical compositions comprises a transgene cassette that encodes a protein, or comprises a transcribed nucleic acid, that is therapeutic for a genetic or heritable disease or condition, and is present in such pharmaceutical composition in an amount effective to ("**effective amount**")

[0092] The term "**pharmaceutically acceptable**" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "**carrier**" refers to a diluent, adjuvant (*e.g.*, Freund's adjuvant complete and incomplete), excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. Suitable pharmaceutical excipients are described in US Patents No. 8,852,607; 8,192,975; 6,764,845; 6,759,050; and 7,598,070.

[0093] Generally, the ingredients of compositions of the invention are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free concentrate, or as an aqueous solution in a hermetically sealed container such as a vial, an ampoule or sachette indicating the quantity of active agent.

Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline, or other diluent can be provided so that the ingredients may be mixed prior to administration.

[0094] The invention also provides a pharmaceutical pack or kit comprising one or more containers such pharmaceutical composition. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

[0095] The rAAV of such pharmaceutical compositions is preferably packaged in a hermetically sealed container, such as a vial, an ampoule or sachette indicating the quantity of the molecule, and optionally including instructions for use. In one embodiment, the rAAV of such kit is supplied as a dry sterilized lyophilized powder or water-free concentrate in a hermetically sealed container and can be reconstituted, *e.g.*, with water, saline, or other diluent to the appropriate concentration for administration to a subject. The lyophilized material should be stored at between 2°C and 8°C in their original container and the material should be administered within 12 hours, preferably within 6 hours, within 5 hours, within 3 hours, or within 1 hour after being reconstituted. In another embodiment, the rAAV of such kit is supplied as an aqueous solution in a hermetically sealed container and can be diluted, *e.g.*, with water, saline, or other diluent, to the appropriate concentration for administration to a subject. The kit can further comprise one or more other prophylactic and/or therapeutic agents useful for the treatment of the disease or condition, in one or more containers; and/or the kit can further comprise one or more cytotoxic antibodies that bind one or more cancer antigens associated with cancer. In certain embodiments, the other prophylactic or therapeutic agent is a chemotherapeutic. In other embodiments, the prophylactic or therapeutic agent is a biological or hormonal therapeutic.

III. Uses of the Invention

[0096] The methods of the present invention may be used to facilitate the production of rAAV, and may particularly be used to facilitate the production of rAAV that

comprise transgene cassettes that encode a protein (*e.g.*, an enzyme, hormone, antibody, receptor, ligand, *etc.*), or of rAAV that comprise a transcribed nucleic acid, that is relevant to a genetic or heritable disease or condition, such that it may be used in gene therapy to treat such disease or condition. Examples of such diseases and conditions include: achromatopsia (**ACHM**); alpha-1 antitrypsin (**AAT**) deficiency; Alzheimer's Disease; aromatic L-amino acid decarboxylase (**AADC**) deficiency; choroideremia (**CHM**); cancer; Duchenne muscular dystrophy; dysferlin deficiency; follistatin gene deficiency (**BMDSIBM**); hemophilia A; hemophilia B; hepatitis A; hepatitis B; hepatitis C; Huntington's disease; idiopathic Parkinson's disease; late-infantile neuronal ceroid lipofuscinosis (**LINCL**, an infantile form of Batten disease); Leber congenital amaurosis (**LCA**); Leber's hereditary optic neuropathy (**LHON**); limb girdle muscular dystrophy 1B (**LGMD1B**); limb girdle muscular dystrophy 1C (**LGMD1C**); limb girdle muscular dystrophy 2A (**LGMD2A**); limb girdle muscular dystrophy 2B (**LGMD2B**); limb girdle muscular dystrophy 2I (**LGMD2I**); limb girdle muscular dystrophy 2L (**LGMD2L**); lipoprotein lipase (**LPL**) deficiency; metachromatic leukodystrophy; neurological disability; neuromotor deficit; neuroskeletal impairment; Parkinson's disease; rheumatoid arthritis; Sanfilippo A syndrome; spinal muscular atrophy (**SMA**); X-linked retinoschisis (**XLRS**); α-sarcoglycan deficiency (**LGMD2D**); β-sarcoglycan deficiency (**LGMD2E**); γ-sarcoglycan deficiency (**LGMD2C**) and δ-sarcoglycan deficiency (**LGMD2F**).

IV. Embodiments of the Invention

[0097] The invention concerns a recombinantly-modified adeno-associated virus (AAV) helper vector that comprises an AAV helper function-providing polynucleotide, and uses and compositions thereof. It is particularly directed to the following embodiments **E1-E16**:

E1. A recombinantly-modified adeno-associated virus (AAV) helper vector that comprises an AAV helper function-providing polynucleotide, wherein the polynucleotide comprises a non-native AAV serotype **P5** or **P40** promoter sequence.

E2. The recombinantly-modified adeno-associated virus (AAV) helper vector of **E1**, wherein the AAV helper function-providing polynucleotide vector comprises a non-native AAV serotype **P5** promoter sequence.

E3. The recombinantly-modified adeno-associated virus (AAV) helper vector of any one of **E1** or **E2**, wherein the AAV helper function-providing polynucleotide vector comprises a non-native AAV serotype **P40** promoter sequence.

E4. The recombinantly-modified adeno-associated virus (AAV) helper vector of any one of **E1-E3**, wherein the vector is a plasmid vector.

E5. The recombinantly-modified adeno-associated virus (AAV) helper vector of **E1**, wherein the non-native AAV serotype **P5** or **P40** promoter sequence replaces a native AAV serotype promoter sequence.

E6. The recombinantly-modified adeno-associated virus (AAV) helper vector of any one of **E1-E5**, wherein the vector additionally comprises a non-AAV helper function-providing polynucleotide.

E7. A method for increasing the production titer of a recombinantly-modified adeno-associated virus (rAAV) that comprises a transgene cassette, wherein the method comprises culturing cells that have been transfected with:

(1) the rAAV;

(2) the recombinantly-modified adeno-associated virus (AAV) helper vector of **E6**;

wherein the culturing is conducted in a culture medium under conditions sufficient to permit the production of the rAAV and wherein the presence of the non-native AAV serotype **P5** or **P40** promoter sequence causes the cells to produce the rAAV at an increased production titer relative to that which would be attained if the AAV helper function-providing polynucleotide contained native serotype **P5** and **P40** promoters.

E8. A method for increasing the production titer of a recombinantly-modified adeno-associated virus (rAAV) that comprises a transgene cassette, wherein the method comprises culturing cells that have been transfected with:

(1) the rAAV;

(2) the recombinantly-modified adeno-associated virus (AAV) helper vector of any one of **E1-E6**; and

(3) an additional vector, especially a plasmid vector, that comprises a non-AAV helper function-providing polynucleotide;

wherein the culturing is conducted in a culture medium under conditions sufficient to permit the production of the rAAV, and wherein the presence of the non-native AAV serotype **P5** or **P40** promoter sequence causes the cells to produce the rAAV at an increased production titer relative to that which would be attained if the AAV helper function-providing polynucleotide contained native serotype **P5** and **P40** promoters.

E9. The method of any one of **E7**-**E8**, wherein:

(A) the AAV helper function-providing polynucleotide of the vector encodes an AAV1 **Cap** protein, and the non-native AAV serotype promoter sequence is a promoter sequence of an AAV of serotype AAV3, AAV4, AAV5, AAV6, AAV7 or AAV8, or a hybrid of one or more of the serotypes;

(B) the AAV helper function-providing polynucleotide of the vector encodes an AAV2 **Cap** protein, and the non-native AAV serotype promoter sequence is a promoter sequence of an AAV of serotype AAV1, AAV3, AAV4, AAV5, AAV6, AAV7 or AAV8, or a hybrid of one or more of the serotypes;

(C) the AAV helper function-providing polynucleotide of the vector encodes an AAV3 **Cap** protein, and the non-native AAV serotype promoter sequence is a promoter sequence of an AAV of serotype AAV1, AAV4, AAV5, AAV6, AAV7 or AAV8, or a hybrid of one or more of the serotypes;

(D) the AAV helper function-providing polynucleotide of the vector encodes an AAV4 **Cap** protein, and the non-native AAV serotype promoter sequence is a promoter sequence of an AAV of serotype AAV1, AAV3, AAV5, AAV6, AAV7 or AAV8, or a hybrid of one or more of the serotypes;

(E) the AAV helper function-providing polynucleotide of the vector encodes an AAV5 **Cap** protein, and the non-native AAV serotype promoter sequence is a promoter sequence of an AAV of serotype AAV1, AAV3, AAV4, AAV6, AAV7 or AAV8, or a hybrid of one or more of the serotypes;

(F) the AAV helper function-providing polynucleotide of the vector encodes an AAV6 **Cap** protein, and the non-native AAV serotype promoter sequence is a promoter sequence of an AAV of serotype AAV1, AAV3, AAV4, AAV5, AAV7 or AAV8, or a hybrid of one or more of the serotypes;

(G) the AAV helper function-providing polynucleotide of the vector encodes an AAV7 **Cap** protein, and the non-native AAV serotype promoter sequence is a promoter sequence of an AAV of serotype AAV1, AAV3, AAV4, AAV5, AAV6 or AAV8, or a hybrid of one or more of the serotypes; or

(H) the AAV helper function-providing polynucleotide of the vector encodes an AAV8 **Cap** protein, and the non-native AAV serotype promoter sequence is a promoter sequence of an AAV of serotype AAV1, AAV3, AAV4, AAV5, AAV6 or AAV7, or a hybrid of one or more of the serotypes.

E10. The method of any one of **E7-E9**, wherein the cells are human embryonic kidney cells, baby hamster kidney cells or sf9 insect cells.

E11. The method of **E10**, wherein the cells are HEK293 human embryonic kidney cells.

E12. The method of **E10**, wherein the cells are BHK21 baby hamster kidney cells.

E13. The method of any one of **E7-E12**, wherein the transgene cassette encodes a protein, or comprises a transcribed nucleic acid, that is therapeutic for a genetic or heritable disease or condition.

E14. A preparation of the recombinantly-modified adeno-associated virus (rAAV) produced by the method of **E13**.

E15. A pharmaceutical composition that comprises the recombinantly-modified adeno-associated virus (rAAV) produced by the method of **E13**, and a pharmaceutically acceptable carrier.

E16. The preparation of recombinantly-modified adeno-associated virus (rAAV) of **E14**, or the pharmaceutical composition of **E15**, for use in the treatment of the genetic or heritable disease or condition.

EXAMPLES

[0098] Having now generally described the invention, the same will be more readily understood through reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention unless specified.

Example 1
Comparison Of rAAV Production Titers By Cells Transfected With AAV RC2 Helper Plasmid Vectors Having A Non-Native AAV Serotype P5 Promoter Sequence

[0099] In order to demonstrate the ability of non-native AAV serotype promoter sequences to affect the production titer of rAAV, derivatives of AAV helper plasmid AAV RC2 (having an AAV2 ***rep*** gene and a ***cap*** gene that encodes **Cap** protein of the AAV2 serotype) were constructed that comprised a non-native AAV serotype promoter sequence (**Figure 11**) in lieu of the native AAV2 serotype **P5** promoter of such plasmid (**Figure 12A**; downward striped rectangle). The **P19** and **P40** promoters of the constructs were not changed, and thus were both native AAV2 serotype promoter sequences (**Figure 12A**; solid black rectangles).

[00100] The following constructs were employed; the sequences of the promoter regions are shown in **Table 1**:

(1) **Parent-RC2** – **pAAV-RC2** (**SEQ ID NO:2**), which contains the AAV2 ***rep*** gene and a partial portion of the full AAV2 serotype **P5** promoter sequence (**SEQ ID NO:10**), and the AAV2 ***cap*** gene, whose expression is controlled by a native AAV2 **P40** promoter sequence (**SEQ ID NO:18**);

(2) **P5(1)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV serotype **P5** promoter sequences had been replaced with the **P5** promoter sequences of AAV serotype 1 (**SEQ ID NO:9**);

(3) **P5(2)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which the partial AAV2 serotype **P5** promoter sequences of **Parent-RC2** had been replaced with the full-length **P5** promoter sequences of AAV serotype 2 (**SEQ ID NO:10**);

(4) **P5(3)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV2 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequences of AAV serotype 3 (**SEQ ID NO:11**);

(5) **P5(4)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV2 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequences of AAV serotype 4 (**SEQ ID NO:12**);

(6) **P5(5)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV2 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequences of AAV serotype 5 (**SEQ ID NO:13**);

(7) **P5(6)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV2 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequences of AAV serotype 6 (**SEQ ID NO:14**);

(8) **P5(7)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV2 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequences of AAV serotype 7 (**SEQ ID NO:15**); and

(9) **P5(8)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV2 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequences of AAV serotype 8 (**SEQ ID NO:16**).

[00101] Figure 12B shows the production titers of rAAV obtained using such AAV helper plasmid vectors. The production titers of rAAV were obtained using a triple plasmid transfection system with an rAAV (pGOI; BBa_K404119), and an Ad helper plasmid (pHelper) that provided the required adenoviral functions. Plasmid pGOI is an rAAV plasmid vector that comprises, in the 5' to 3' direction, a 5' ITR, a CMV promoter, a β-globin intron, a polynucleotide encoding the yellow fluorescent protein mVenus (Nagai, T. *et al.* (2002) "*A Variant Of Yellow Fluorescent Protein With Fast And Efficient Maturation For Cell-Biological Applications*," Nat. Biotechnol. 20(1):87-90), the polyA domain of human growth hormone and a 3' ITR. **Figure 12B** reveals that the serotype of the **P5** promoter affects rAAV production titers, and indicates that replacing the native AAV2 **P5** promoter of the plasmid vector **pAAV-RC2** with an AAV5 serotype **P5** promoter greatly decreased rAAV production titer, whereas replacing the native AAV2 **P5** promoter of the plasmid vector **pAAV-RC2** with a **P5** promoter of AAV serotype 1, 3, 5, 7 or 8 greatly increased rAAV production titer.

Example 2
Comparison Of rAAV Production Titers By Cells Transfected With AAV RC2 Helper Plasmid Vectors Having A Non-Native AAV Serotype P40 Promoter Sequence

[00102] In order to further demonstrate the ability of non-native AAV serotype promoter sequences to affect the production titer of rAAV, derivatives of AAV helper plasmid AAV RC2 (having an AAV2 ***rep*** gene and a ***cap*** gene that encodes **Cap** protein of the AAV2 serotype) were constructed that comprised a non-native AAV serotype promoter sequence (**Figure 11**) in lieu of the native serotype **P40** promoter of such plasmid (**Figure 13A**; upward striped rectangle). The **P5** and **P19** promoters of the constructs were not changed, and thus were both native AAV2 serotype promoter sequences (**Figure 13A**; solid black rectangles).

[00103] The following constructs were employed; the sequences of the promoter regions are shown in **Table 1**:

(1) **Parent-RC2** – **pAAV-RC2** (**SEQ ID NO:2**), which contains the AAV2 ***rep*** gene and a partial portion of the full AAV2 serotype **P5** promoter sequence (**SEQ ID NO:10**), and the AAV2 ***cap*** gene, whose expression is controlled by a native AAV2 **P40** promoter sequence (**SEQ ID NO:18**);

(2) **P40(1)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV2 serotype **P40** promoter sequences had been replaced with the **P40** promoter sequences of AAV serotype 1 (**SEQ ID NO:17**);

(3) **P40(2)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which the AAV2 serotype **P40** promoter sequences of **Parent-RC2** had been replaced with the **P40** promoter sequences of AAV serotype 2 (**SEQ ID NO:18**);

(4) **P40(3)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV2 serotype **P40** promoter sequences had been replaced with the **P40** promoter sequences of AAV serotype 3 (**SEQ ID NO:19**);

(5) **P40(4)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV2 serotype **P40** promoter sequences had been replaced with the **P40** promoter sequences of AAV serotype 4 (**SEQ ID NO:20**);

(6) **P40(5)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV2 serotype **P40** promoter sequences had been replaced with the **P40** promoter sequences of AAV serotype 5 (**SEQ ID NO:21**);

(7) **P40(6)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV2 serotype **P40** promoter sequences had been replaced with the **P40** promoter sequences of AAV serotype 6 (**SEQ ID NO:22**);

(8) **P40(7)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV2 serotype **P40** promoter sequences had been replaced with the **P40** promoter sequences of AAV serotype 7 (**SEQ ID NO:23**); and

(9) **P40(8)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV2 serotype **P40** promoter sequences had been replaced with the **P40** promoter sequences of AAV serotype 8 (**SEQ ID NO:24**).

[00104] **Figure 13B** shows the production titers of rAAV obtained using such AAV helper plasmid vectors. Production titers of rAAV were obtained essentially as described in **Example 1**. The results of the investigation reveal that the serotype of the **P40** promoter also affects rAAV production titers, and indicate that replacing the native AAV2 **P40** promoter of the plasmid vector **pAAV-RC2** with an AAV5 serotype **P40** promoter greatly decreased rAAV production titer, whereas replacing the native AAV2 **P40** promoter of the plasmid vector **pAAV-RC2** with an AAV1 serotype **P40** promoter or with an AAV8 serotype **P40** promoter greatly increased rAAV production titer.

Example 3
Comparison Of rAAV Production Titers By Cells Transfected With AAV RC2 Helper Plasmid Vectors Having Non-Native AAV Serotype P5 and P40 Promoter Sequences

[00105] In order to further demonstrate the ability of non-native AAV serotype promoter sequences to affect the production titer of rAAV, derivatives of AAV helper plasmid AAV RC2 (having an AAV2 ***rep*** gene and a ***cap*** gene that encodes **Cap** protein of the AAV2 serotype) were constructed that comprised non-native AAV serotype promoter sequences (**Figure 11**) in lieu of the native AAV2 serotype **P5** (**Figure 14A**; downward striped rectangle) and **P40** (**Figure 14A**; upward striped rectangle) promoters of such plasmid. The AAV2 **P19** promoter of the constructs were not changed, and thus was the native AAV2 serotype promoter sequence (**Figure 14A**; solid black rectangle).

[00106] The following constructs were employed; the sequences of the promoter regions are shown in **Table 1**:

(1) **Parent-RC2** – **pAAV-RC2** (**SEQ ID NO:2**), which contains the AAV2 ***rep*** gene and a partial portion of the full AAV2 serotype **P5** promoter sequence (**SEQ ID NO:10**), and the AAV2 ***cap*** gene, whose expression is controlled by a native AAV2 **P40** promoter sequence (**SEQ ID NO:18**);

(2) **P5(2)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which the partial AAV2 serotype **P5** promoter sequences of **Parent-RC2** had been replaced with the full-length **P5** promoter sequences of AAV serotype 2 (**SEQ ID NO:10**);

(3) **P5(3)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV2 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequence of AAV3 (**SEQ ID NO:11**);

(4) **P5(5)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV2 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequence of AAV5 (**SEQ ID NO:13**);

(5) **P40(1)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV2 serotype **P40** promoter sequences had been replaced with the **P40** promoter sequence of AAV1 (**SEQ ID NO:17**);

(6) **P5(2)/P40(1)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV2 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequence of AAV2 (**SEQ ID NO:10)** and in which native **P40** promoter sequences had been replaced with the **P40** promoter sequence of AAV1 (**SEQ ID NO:17**);

(7) **P5(3)/P40(1)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV2 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequence of AAV3 (**SEQ ID NO:11**) and in which native **P40** promoter sequences had been replaced with the **P40** promoter sequence of AAV1 (**SEQ ID NO:17**); and

(8) **P5(5)/P40(1)-RC2** – a derivative of plasmid vector **pAAV-RC2** in which native AAV2 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequence of AAV5 (**SEQ ID NO:13**) and in which native **P40** promoter sequences had been replaced with the **P40** promoter sequence of AAV1 (**SEQ ID NO:17**).

[00107] Production titers of rAAV were obtained essentially as described in **Example 1**. **Figure 14B** shows the production titers of rAAV obtained using such AAV helper plasmid vectors. As shown in **Figure 14B**, the replacement of the native **P5** and **P40** promoters of **pAAV-RC2** with the **P5** promoter sequence of AAV3 or AAV5 and the **P40** promoter sequence of AAV1 synergistically increased rAAV production titers.

Example 4
Comparison Of rAAV Production Titers By Cells Transfected With AAV RC6 Helper Plasmid Vectors Having Non-Native AAV Serotype P5 and P40 Promoter Sequences

[00108] In order to further demonstrate the ability of non-native AAV serotype promoter sequences to affect the production titer of rAAV, derivatives of AAV helper plasmid AAV RC6 (having an AAV2 ***rep*** gene and a ***cap*** gene that encodes **Cap** protein of the AAV6 serotype) were constructed that comprised non-native AAV serotype promoter sequences (**Figure 11**) in lieu of the native AAV2 serotype **P5** (**Figure 15A**; downward striped rectangle) and **P40** (**Figure 15A**; downward striped rectangle) promoters of such plasmid. The AAV2 **P19** promoter of the constructs were not changed, and thus was the native AAV2 serotype promoter sequence (**Figure 15A**; solid black rectangle).

[00109] The following constructions were employed; the sequences of the promoter regions are shown in **Table 1**:

(1) **Parent-RC6 – pAAV-RC6** (**SEQ ID NO:4**), which contains the AAV2 ***rep*** gene and its native AAV2 serotype **P5** promoter sequence (**SEQ ID NO:10**), and the AAV6 ***cap*** gene, whose expression is controlled by a native AAV2 **P40** promoter sequence (**SEQ ID NO:18**);

(2) **P5(1)-RC6** – a derivative of plasmid vector **pAAV-RC6** in which native AAV2 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequence of AAV1 (**SEQ ID NO:9**);

(3) **P5(2)-RC6** – a derivative of plasmid vector **pAAV-RC6** in which native AAV2 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequence of AAV2 (**SEQ ID NO:10**);

(4) **P5(3)-RC6** – a derivative of plasmid vector **pAAV-RC6** in which native AAV2 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequence of AAV3 (**SEQ ID NO:11**);

(5) **P5(7)-RC6** – a derivative of plasmid vector **pAAV-RC6** in which native AAV2 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequence of AAV7 (**SEQ ID NO:15**); and

(6) **P5(8)-RC6** – a derivative of plasmid vector **pAAV-RC6** in which native AAV2 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequence of AAV8 (**SEQ ID NO:16**).

[00110] **Figure 15B** shows the production titers of rAAV obtained using such AAV helper plasmid vectors. Production titers of rAAV were obtained essentially as described in **Example 1**.

[00111] The results of the investigation are shown in **Figures 15B** and **15C**, and reveal that the production titers of rAAV obtained using such AAV helper plasmid vectors. As shown in such Figures, the replacement of the native **P5** and **P40** promoters of **pAAV-RC6** with the **P5** promoter sequence of AAV serotype 1, 2, 3, 7 or 8 increased rAAV production titers.

Example 5
Comparison Of rAAV Production Titers By Cells Transfected With AAV RC1, AAV RC5 or AAV RC7 Helper Plasmid Vectors Having Non-Native AAV Serotype P5 and P40 Promoter Sequences

[00112] In order to further demonstrate the ability of non-native AAV serotype promoter sequences to affect the production titer of rAAV, derivatives of AAV helper plasmid AAV RC1 (having an AAV2 ***rep*** gene and a ***cap*** gene that encodes **Cap** protein of the AAV1 serotype), derivatives of AAV helper plasmid AAV RC5 (having an AAV2 ***rep*** gene and a ***cap*** gene that encodes **Cap** protein of the AAV5 serotype) and derivatives of AAV helper plasmid AAV RC7 (having an AAV2 ***rep*** gene and a ***cap*** gene that encodes **Cap** protein of the AAV7 serotype) were constructed that comprised non-native AAV serotype promoter sequences (**Figure 11**) in lieu of the native AAV2 serotype **P5** (**Figure 16A**; downward striped rectangle) and/or **P40** (**Figure 16A**; upward striped rectangle) promoter sequences of such plasmids.

[00113] The following constructions were employed; the sequences of the promoter regions are shown in **Table 1**:

(1) **Parent-RC1** – **pAAV-RC1** (**SEQ ID NO:1**), which contains the AAV2 ***rep*** gene and its native AAV2 serotype **P5** promoter sequence (**SEQ ID NO:10**), and the AAV1 ***cap*** gene, whose expression is controlled by a native AAV2 **P40** promoter sequence (**SEQ ID NO:18**);

(2) **Parent-RC5** – **pAAV-RC5** (**SEQ ID NO:3**), which contains the AAV2 ***rep*** gene and its native AAV2 serotype **P5** promoter sequence (**SEQ ID NO:10**), and the AAV5 ***cap*** gene, whose expression is controlled by a native AAV2 **P40** promoter sequence (**SEQ ID NO:18**);

(3) **Parent-RC7** – **pAAV-RC7** (**SEQ ID NO:5**), which contains the AAV2 ***rep*** gene and its native AAV2 serotype **P5** promoter sequence (**SEQ ID NO:10**), and the AAV7 ***cap*** gene, whose expression is controlled by a native AAV2 **P40** promoter sequence (**SEQ ID NO:18**);

(4) **P5(2)-RC1** – a derivative of plasmid vector **pAAV-RC1** in which native AAV1 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequence of AAV2 (**SEQ ID NO:10**);

(5) **P5(7)-RC1** – a derivative of plasmid vector **pAAV-RC1** in which native AAV1 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequence of AAV7 (**SEQ ID NO:15**);

(6) **P5(8)-RC1** – a derivative of plasmid vector **pAAV-RC1** in which native AAV1 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequence of AAV8 (**SEQ ID NO:16**);

(7) **P5(7)-RC5** – a derivative of plasmid vector **pAAV-RC5** in which native AAV5 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequence of AAV7 (**SEQ ID NO:15**);

(8) **P5(2)-RC7** – a derivative of plasmid vector **pAAV-RC7** in which native AAV7 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequence of AAV2 (**SEQ ID NO:10**).

(9) **P5(7)-RC7** – a derivative of plasmid vector **pAAV-RC7** in which native AAV7 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequence of AAV7 (**SEQ ID NO:15**); and

(10) **P5(8)-RC7** – a derivative of plasmid vector **pAAV-RC7** in which native AAV7 serotype **P5** promoter sequences had been replaced with the **P5** promoter sequence of AAV8 (**SEQ ID NO:16**).

[00114] Production titers of rAAV were obtained essentially as described in **Example 1**. The results of the investigation are shown in **Figure 16B**, and reveals that the replacement of the native **P5** promoter sequences of **pAAV-RC1**, **pAAV-RC5**, and **pAAV-RC7** with **P5** promoter sequence of AAV serotype 2, 7 or 8 increased rAAV production titers.

[00115] All publications and patents mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference in its entirety. While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth.

Claims:

Claim 1. A recombinantly-modified adeno-associated virus (AAV) helper vector that comprises an AAV helper function-providing polynucleotide, wherein said polynucleotide comprises an AAV serotype P40 promoter sequence that is not native to a rep gene of the AAV.

Claim 2. The recombinantly-modified adeno-associated virus (AAV) helper vector of claim 1, wherein said AAV helper function-providing polynucleotide vector further comprises an AAV serotype P5 promoter sequence that is not native to the rep gene of the AAV.

Claim 3. The recombinantly-modified adeno-associated virus (AAV) helper vector of claim 1, wherein said vector is a plasmid vector.

Claim 4. The recombinantly-modified adeno-associated virus (AAV) helper vector of claim 1, wherein said AAV serotype P40 promoter sequence replaces an AAV serotype promoter sequence that is native to the rep gene of the AAV.

Claim 5. The recombinantly-modified adeno-associated virus (AAV) helper vector of claim 1, wherein said vector additionally comprises a non-AAV helper function-providing polynucleotide.

Claim 6. A method for increasing the production titer of a recombinantly-modified adeno-associated virus (rAAV) that comprises a transgene cassette, wherein said method comprises culturing cells that have been transfected with:

(1) said rAAV; and

(2) the recombinantly-modified adeno-associated virus (AAV) helper vector of claim 5;

wherein said culturing is conducted in a culture medium under conditions sufficient to permit the production of said rAAV, and wherein the presence of said the AAV serotype P40 promoter sequence that is not native to the rep gene of the AAV causes said cells to produce said rAAV at an increased production titer

relative to that which would be attained if said AAV helper function-providing polynucleotide contained an AAV serotype P40 promoter sequence that is native to the rep gene of the AAV.

Claim 7. The method of claim 6, wherein said transgene cassette is capable of being used in gene therapy.

Claim 8. The method of claim 6, wherein said transgene cassette comprises a promoter, a polyadenylation site, or combination thereof.

Claim 9. The method of claim 6, wherein said transgene cassette encodes a protein, or wherein said transgene cassette comprises a polynucleotide that is transcribed into an RNA molecule, wherein said protein or said RNA molecule is therapeutic for a genetic or heritable disease or condition.

Claim 10. The method of claim 9, wherein said protein or said polynucleotide of said transgene cassette is capable of being used in therapy.

Claim 11. The method of claim 6, wherein said cells are human embryonic kidney cells, baby hamster kidney cells or sf9 insect cells.

Claim 12. The method of claim 11, wherein said cells are HEK293 human embryonic kidney cells.

Claim 13. The method of claim 11, wherein said cells are BHK21 baby hamster kidney cells.

Claim 14. A method for increasing the production titer of a recombinantly-modified adeno-associated virus (rAAV) that comprises a transgene cassette, wherein said method comprises culturing cells that have been transfected with:

(1) said rAAV;

(2) the recombinantly-modified adeno-associated virus (AAV) helper vector of claim 1; and

(3) an additional vector that comprises a non-AAV helper function-providing polynucleotide;

wherein said culturing is conducted in a culture medium under conditions sufficient to permit the production of said rAAV, and wherein the presence of said AAV serotype P40 promoter sequence that is not native to the rep gene of the AAV causes said cells to produce said rAAV at an increased production titer relative to that which would be attained if said AAV helper function-providing polynucleotide contained an AAV serotype P40 promoter sequence that is native to the rep gene of the AAV.

Claim 15. The method of claim 14, wherein said transgene cassette is capable of being used in gene therapy.

Claim 16. The method of claim 14, wherein said transgene cassette comprises a promoter, a polyadenylation site, or combination thereof.

Claim 17. The method of claim 14, wherein said transgene cassette encodes a protein, or wherein said transgene cassette comprises a polynucleotide that is transcribed into an RNA molecule, wherein said protein or said RNA molecule is therapeutic for a genetic or heritable disease or condition.

Claim 18. The method of claim 17, wherein said polynucleotide of said transgene cassette is capable of being used in gene therapy.

Claim 19. The method of claim 14, wherein said cells are human embryonic kidney cells, baby hamster kidney cells or sf9 insect cells.

Claim 20. The method of claim 19, wherein said cells are HEK293 human embryonic kidney cells.

Claim 21. The method of claim 19, wherein said cells are BHK21 baby hamster kidney cells.