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(54) Title: RECOMBINANTLY-MODIFIED ADENO-ASSOCIATED VIRUS (RAAV) HAVING IMPROVED PACKAGING EFFICIENCY

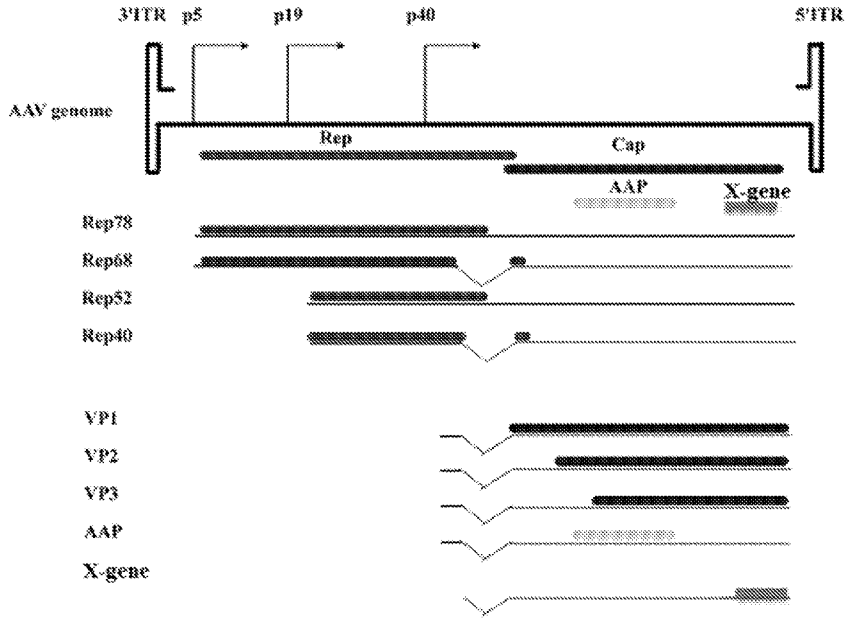

Figure 1

(57) Abstract: The present invention is directed to recombinantly-modified adeno-associated virus (rAAV) having improved packaging efficiency, pharmaceutical compositions comprising such rAAV, and methods for their production and use. The present invention is particularly directed to recombinantly-modified adeno-associated virus (rAAV) that have been further modified to comprise Cis-Elements, including replication origins, promoters and enhancers, that are capable of regulating the replication of an rAAV genome and that improve rAAV replication. Preferably, such Cis-Elements are provided within domains of the rAAV that precede and/or follow the 5' and/or 3' inverted terminal repeated sequences (ITR) of an rAAV. The invention particularly concerns the presence and the use of polynucleotide Cis-Elements that comprise actual or potential G-Quadruplex Sequences, polynucleotide Cis-Elements that comprise DNA sequences from wild-type AAV (wt AAV) and polynucleotide Cis- Elements that comprise DNA sequences from other viral

WO 2021/011034 A1

[Continued on next page]

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TITLE OF THE INVENTION:

Recombinantly-Modified Adeno-Associated Virus (rAAV) Having Improved Packaging Efficiency

FIELD OF THE INVENTION:

[0001] The present invention is directed to recombinantly-modified adeno-associated virus (rAAV) having improved packaging efficiency, pharmaceutical compositions comprising such rAAV, and methods for their production and use. The present invention is particularly directed to recombinantly-modified adeno-associated virus (rAAV) that have been further modified to comprise Cis-Elements, including replication origins, promoters and enhancers, that are capable of regulating the replication of an rAAV genome and that improve rAAV replication. Preferably, such Cis-Elements are provided within domains of the rAAV that precede and/or follow the 5' and/or 3' inverted terminal repeated sequences (ITR) of an rAAV. The invention particularly concerns the presence and the use of polynucleotide Cis-Elements that comprise actual or potential G-Quadruplex Sequences, polynucleotide Cis-Elements that comprise DNA sequences from wild-type AAV (wt AAV) and polynucleotide Cis-Elements that comprise DNA sequences from other viral genomes or from the human genome.

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: 2650-0003US_ST25.txt, created on July 15, 2019, and having a size of 63,309 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 4,700 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). 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 of Ad; 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).

[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] Typically, rAAV are 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 (*i.e.*, 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

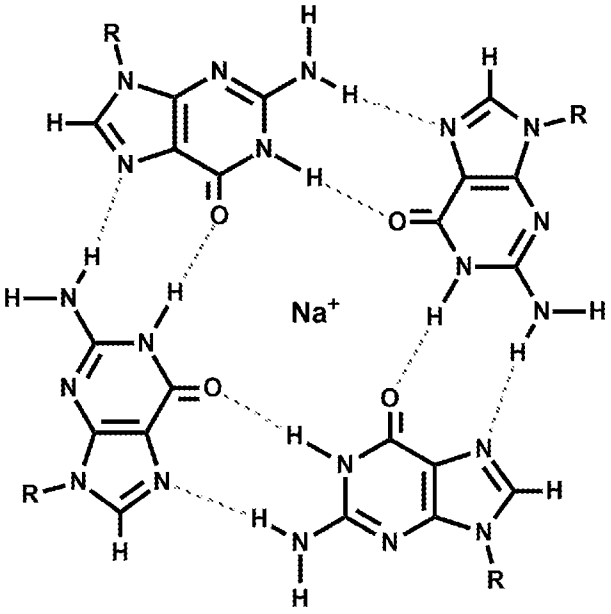
[0016] After production, rAAV are typically collected and purified by one or more overnight CsCl gradient centrifugations (Zolotukhin, S. *et al.* (1999) "*Recombinant Adeno-Associated Virus Purification Using Novel Methods Improves Infectious Titer And Yield*," Gene Ther. 6:973-985), followed by desalting 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.

V. G-Quadruplex Sequences and Structures

[0018] DNA can form several secondary structures besides the classic double helix; one that has received much attention in recent years is the **G-Quadruplex Structure**. G-Quadruplex Structures are formed from the stacking of three planar "**G-tetrad**" (also known as "**guanine quartet**") structures. Each G-tetrad is formed through Hoogsteen base pairing via hydrogen bond interactions involving four deoxyguanosine residues. The planar structure of the G-tetrad may be stabilized by cations (*e.g.*, Na^+). In the G-tetrad structure shown below, the guanines are attached to their respective polynucleotide chain(s) via "**R**".



G-Tetrad / Guanine Quartet

[0019] The stacking of G-tetrad structures to form a G-Quadruplex Structure is accomplished by the spontaneous interaction and/or looping of domains of one, two or four polynucleotide chains that together comprise a G-Quadruplex Sequence (**Figures 3A-3D**).

[0020] G-Quadruplex Structures are well known in the art (Bedrat, A. *et al.* (2016) "*Re-evaluation of G-Quadruplex propensity with G4Hunter*," Nucleic Acids Res. 44(4):1746-1759; Harris, L.M. *et al.* (2015) "*G-Quadruplexes In Pathogens: A Common Route To Virulence Control*?" PLoS Pathog. 11(2):e1004562 (pages 1-15); Siddiqui-Jain, A. *et al.* (2002) "*Direct Evidence For A G-Quadruplex In A Promoter Region And Its Targeting With A Small Molecule To Repress c-MYC Transcription*," Proc. Natl. Acad. Sci. (U.S.A.) 99:11593-11598; Wieland, M. *et al.* (2007) "*RNA Quadruplex-Based Modulation Of Gene Expression*," Chem. Biol., 14:757-763; Millevoi, S. *et al.* (2012) "*G-Quadruplexes In RNA Biology*," Wiley interdiscip. Rev. RNA 3:495-507; Lopes, J. *et al.* (2011) "*G-Quadruplex-Induced Instability During Leading-Strand Replication*," EMBO J. 30:4033-4046; Paeschke, K. *et al.* (2011) "*DNA Replication Through G-Quadruplex Motifs Is Promoted By The Saccharomyces Cerevisiae Pif1 DNA Helicase*," Cell 145:678-691; Besnard, E. *et al.* (2012) "*Unraveling Cell Type-Specific And Reprogrammable Human Replication Origin Signatures Associated With G-Quadruplex Consensus Motifs*," Nat. Struct. Mol. Biol.

19:837-844; and Valton, A.L. *et al.* (2014) "*G4 Motifs Affect Origin Positioning And Efficiency In Two Vertebrate Replicators*," EMBO J. 33:732-746).

[0021] Sequences capable of forming G-Quadruplex Structures have been recently identified within the genome of a number of viruses, *e.g.*, HIV, HSV, EBV influenza, papillomavirus and cauliflower mosaic virus (Piekna-Przybylska, D. *et al.* (2014) "*U3 Region In The HIV-1 Genome Adopts A G-Quadruplex Structure In Its RNA And DNA Sequence*," Biochemistry 53(16):2581-2593; Artusi, S. *et al.* (2015) "*The Herpes Simplex Virus-1 Genome Contains Multiple Clusters Of Repeated G-Quadruplex: Implications For The Antiviral Activity Of A G-Quadruplex Ligand*," Antivir. Res. 118:123-131; Tlučková, K. *et al.* (2013) "*Human Papillomavirus G-Quadruplexes*," Biochemistry 52(41):7207-7216; Métifiot, M. *et al.* (2014) "*G-Quadruplexes In Viruses: Function And Potential Therapeutic Applications*," Nucleic Acids Res. 42(20):12352-12366).

[0022] Viral G-Quadruplex Structures have been proposed to function as steric blocks to DNA replication and transcription (Satkunanathan, S. *et al.* (2017) "*The Function Of DNA Binding Protein Nucleophosmin In AAV Replication*," Virol. 510:46-54). For example, the presence of a G-Quadruplex Sequence in the wild-type Nuclease hypersensitive element III (NHE III_1) gene, a major regulator of c-MYC transcription, causes that gene to be expressed at a lower level than that of a mutated NHE III_1 gene (Siddiqui-Jain, A. *et al.* (2002) "*Direct Evidence For A G-Quadruplex In A Promoter Region And Its Targeting With A Small Molecule To Repress c-MYC Transcription*," Proc. Natl. Acad. Sci. (U.S.A.) 99:11593-11598; Harris, L.M. *et al.* (2015) "*G-Quadruplexes In Pathogens: A Common Route To Virulence Control*?" PLoS Pathog. 11(2):e1004562 (pages 1-15).

[0023] It has been proposed that limitations in high titer AAV production may be due to AAV's dependence on helper viruses and on an insufficient understanding of factors, viral or cellular, that contribute to AAV replication (Satkunanathan, S. *et al.* (2017) "*The Function Of DNA Binding Protein Nucleophosmin In AAV Replication*," Virol. 510:46-54). In this regard, investigations into the life cycle of AAV have revealed that AAV helper genes function to induce cellular factors that were either missing or inactivated in a normal cell cycle (Muzyczka, N. (1992) "*Use Of Adeno-Associated*

Virus As A General Transduction Vector For Mammalian Cells," Curr. Top. Microbiol. Immunol. 158:97-129; Ni, T.H. *et al.* (1998) "*Cellular Proteins Required For Adeno-Associated Virus DNA Replication In The Absence Of Adenovirus Coinfection*," J. Virol. 72(4):2777-2787). Additionally, cellular and viral DNA binding proteins have been found to play a vital role in AAV life cycle in unwinding AAV double-stranded DNA, nicking single-stranded DNA, facilitating single-stranded DNA association with nuclei and ultimately enhancing viral DNA production and protein expression (Weitzman, M.D. (2006) "*The Parvovirus Life Cycle: An Introduction To Molecular Interactions Important For Infection*," In: Kerr, J.R. *et al.* (Eds.) PARVOVIRUSES, Hodder Arnold, London, UK; Satkunanathan, S. *et al.* (2017) "*The Function Of DNA Binding Protein Nucleophosmin In AAV Replication*," Virol. 510:46-54).

[0024] For example, **Nucleophosmin (NPM1)** is a nucleolar protein that plays a role in many diverse functions, such as genome stability, DNA duplication and transcriptional regulation through its ability to bind to single-stranded nucleic acids. Nucleophosmin has been reported to enhance AAV infection by acting as a chaperone protein to mobilize AAV capsids into and out of the nucleolus (Nash, K. *et al.* (2009) "*Identification Of Cellular Proteins That Interact With The Adeno-Associated Virus Rep Protein*," J. Virol. 83(1):454-469; Ni, T.H. *et al.* (1998) "*Cellular Proteins Required For Adeno-Associated Virus DNA Replication In The Absence Of Adenovirus Coinfection*," J. Virol. 72(4):2777-2787; Nicolas, A. *et al.* (2012) *"Factors Influencing Helper-Independent Adeno-Associated Virus Replication*," Virology 432(1):1-9). Nucleophosmin has, however, also been found to negatively regulate DNA replication by binding to G-Quadruplex Sequences (Gallo, A. *et al.* (2012) "*Structure of Nucleophosmin DNA-binding Domain and Analysis of Its Complex with a G-Quadruplex Sequence from the c-MYC Promoter*," J. Biol. Chem. 287(32):26539-26548). The down-regulation of Nucleophosmin has been found to result in an increase in AAV2 and AAV8 vector production (Satkunanathan, S. *et al.* (2017) "*The Function Of DNA Binding Protein Nucleophosmin In AAV Replication*," Virol. 510:46-54), and the destruction or elimination of G-Quadruplex Sequences has been found to unblock G-Quadruplex Structure-mediated inhibition of viral DNA replication of HIV and HSV (Harris, L.M. *et al.* (2015) "*G-Quadruplexes In Pathogens: A Common Route To Virulence Control*?" PLoS Pathog. 11(2):e1004562 (pages 1-15)). Thus, the presence

of G-Quadruplex Sequences has been reported to inhibit AAV vector production (Satkunanathan, S. *et al.* (2017) "*The Function Of DNA Binding Protein Nucleophosmin In AAV Replication*," Virol. 510:46-54).

[0025] 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).

[0026] The present invention is directed to improved methods for increasing the efficiency of AAV and rAAV packaging through regulation of the replication of rAAV genomes.

SUMMARY OF THE INVENTION:

[0027] The present invention is directed to recombinantly-modified adeno-associated virus (rAAV) having improved packaging efficiency, pharmaceutical compositions comprising such rAAV, and methods for their production and use. The present invention is particularly directed to recombinantly-modified adeno-associated virus (rAAV) that have been further modified to comprise Cis-Elements, including replication origins, promoters and enhancers, that are capable of regulating the replication of an rAAV genome and that improve rAAV replication. Preferably, such Cis-Elements are provided within domains of the rAAV that precede and/or follow the 5' and/or 3' inverted terminal repeated sequences (ITR) of an rAAV. The invention particularly concerns the presence and the use of polynucleotide Cis-Elements that comprise actual or potential G-Quadruplex Sequences, polynucleotide Cis-Elements that comprise DNA sequences from wild-type AAV (wt AAV) and polynucleotide Cis-Elements that comprise DNA sequences from other viral genomes or from the human genome.

[0028] In detail, the invention provides a recombinantly-modified adeno-associated virus (rAAV) that comprises a Cis-Element in one or more of its **P1**, **P2**, **P3** or **P4 Domains**, wherein:

(1) the **P1 Domain** is 5' to a 5' ITR of the rAAV;

(2) the **P2 Domain** is 3' to the 5' ITR of the rAAV and 5' to a transgene cassette of the rAAV;

(3) the **P3 Domain** is 3' to the transgene cassette of the rAAV and 5' to a 3' ITR of the rAAV; and

(4) the **P4 Domain** is 3' to the 3' ITR of the rAAV; and

wherein the presence of the Cis-Element causes rAAV-producing cells to produce the rAAV at a higher production titer than would be attained with such rAAV if lacking the Cis-Element.

[0029] The invention also provides a pharmaceutical composition that comprises:

(A) a preparation of recombinantly-modified adeno-associated virus (rAAV) that comprise a Cis-Element in one or more of its **P1**, **P2**, **P3** or **P4 Domains**, wherein:

(1) the **P1 Domain** is 5' to a 5' ITR of the rAAV;

(2) the **P2 Domain** is 3' to the 5' ITR of the rAAV and 5' to a transgene cassette of the rAAV;

(3) the **P3 Domain** is 3' to the transgene cassette of the rAAV and 5' to a 3' ITR of the rAAV; and

(4) the **P4 Domain** is 3' to the 3' ITR of the rAAV; and

(B) a pharmaceutically acceptable carrier.

[0030] The invention also provides a method for increasing the production titer of recombinantly-modified adeno-associated virus (rAAV), wherein the method comprises:

(A) employing, as the rAAV for producing the production titer, an rAAV that has been modified to comprise an added Cis-Element in one or more of its **P1**, **P2**, **P3** or **P4 Domains**, wherein:

(1) the **P1 Domain** is 5' to a 5' ITR of the rAAV;

(2) the **P2 Domain** is 3' to the 5' ITR of the rAAV and 5' to a transgene cassette of the rAAV;

(3) the **P3 Domain** is 3' to the transgene cassette of the rAAV and 5' to a 3' ITR of the rAAV; and

(4) the **P4 Domain** is 3' to the 3' ITR of the rAAV; and

(B) culturing cells that have been transfected with the employed rAAV, wherein the cells additionally contain an AAV helper function-providing polynucleotide and a non-AAV helper function-providing polynucleotide, and wherein the culturing is conducted in a culture medium under conditions sufficient to permit the production of rAAV; wherein the presence of the Cis-Element in the employed rAAV causes the cells to produce the rAAV at an increased production titer relative to that which would be attained if the employed rAAV had lacked the Cis-Element.

[0031] The invention also provides the embodiment of such recombinantly-modified adeno-associated virus (rAAV), pharmaceutical composition, or method, wherein the employed rAAV has been modified to comprise an added Cis-Element in its **P1 Domain**.

[0032] The invention also provides the embodiment of such recombinantly-modified adeno-associated viruses (rAAV), pharmaceutical compositions, or methods, wherein the employed rAAV has been modified to comprise an added Cis-Element in its **P2 Domain**.

[0033] The invention also provides the embodiment of such recombinantly-modified adeno-associated viruses (rAAV), pharmaceutical compositions, or methods, wherein the employed rAAV has been modified to comprise an added Cis-Element in its **P3 Domain**.

[0034] The invention also provides the embodiment of such recombinantly-modified adeno-associated viruses (rAAV), pharmaceutical compositions, or methods, wherein the employed rAAV has been modified to comprise an added Cis-Element in its **P4 Domain**.

[0035] The invention also provides the embodiment of such recombinantly-modified adeno-associated viruses (rAAV), pharmaceutical compositions, or methods, wherein

the employed rAAV has been modified to comprise an added Cis-Element in its **P1 Domain** and in one or more of its **P2**, **P3** or **P4 Domain**.

[0036] The invention also provides the embodiment of such recombinantly-modified adeno-associated viruses (rAAV), pharmaceutical compositions, or methods, wherein the employed rAAV has been modified to comprise an added Cis-Element in its **P2 Domain** and in one or more of its **P3** or **P4 Domain**.

[0037] The invention also provides the embodiment of such recombinantly-modified adeno-associated viruses (rAAV), pharmaceutical compositions, or methods, wherein the employed rAAV has been modified to comprise an added Cis-Element in its **P3 Domain** and in its **P4 Domain**.

[0038] The invention also provides the embodiment of such recombinantly-modified adeno-associated viruses (rAAV), pharmaceutical compositions, or methods, wherein the added Cis-Element forms a G-Quadruplex Structure in the employed rAAV.

[0039] The invention also provides the embodiment of such recombinantly-modified adeno-associated viruses (rAAV), pharmaceutical compositions, or methods, wherein an added Cis-Element is selected from the group consisting of:

(1) a Potential G-Quadruplex Sequence of a wild type AAV genome or a Potential G-Quadruplex Sequence of a wild type AAV genome in a reversed orientation;

(2) an Actual G-Quadruplex Sequence of a wild type AAV genome or an Actual G-Quadruplex Sequence of a wild type AAV genome in a reversed orientation;

(3) a DNA sequence from wild-type AAV or a DNA sequence from wild-type AAV in a reversed orientation; and

(4) a DNA sequence from another viral genome or a DNA sequence from another viral genome in a reversed orientation.

[0040] The invention also provides the embodiment of such recombinantly-modified adeno-associated viruses (rAAV), pharmaceutical compositions, or 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.

[0041] The invention also provides the embodiment of such recombinantly-modified adeno-associated viruses (rAAV), pharmaceutical compositions, or methods, wherein the employed rAAV belongs to the rAAV1, rAAV2, rAAV5, rAAV6, rAAV7, rAAV8, rAAV9 or rAAV10 serotype, or to a hybrid of the serotypes.

[0042] The invention also provides the embodiment of such recombinantly-modified adeno-associated viruses (rAAV), pharmaceutical compositions, or methods, wherein the employed rAAV belongs to the rAAV2, rAAV5, or rAAV9 serotype, or to a hybrid of the serotypes.

[0043] The invention also provides the embodiment of such recombinantly-modified adeno-associated viruses (rAAV), pharmaceutical compositions, or methods, wherein the cells are human embryonic kidney cells, baby hamster kidney cells or sf9 insect cells.

[0044] The invention also provides the embodiment of such recombinantly-modified adeno-associated viruses (rAAV), pharmaceutical compositions, or methods, wherein the cells are HEK293 human embryonic kidney cells.

[0045] The invention also provides the embodiment of such recombinantly-modified adeno-associated viruses (rAAV), pharmaceutical compositions, or methods, wherein the cells are BHK21 baby hamster kidney cells.

[0046] The invention also provides such recombinantly-modified adeno-associated viruses (rAAV) and pharmaceutical compositions, wherein the transgene cassette encodes a protein, or comprises a transcribed nucleic acid, that is therapeutic for a genetic or heritable disease or condition, for use in the treatment of the genetic or heritable disease or condition.

BRIEF DESCRIPTION OF THE DRAWINGS:

[0047] **Figure 1** provides a schematic genetic map of the wild-type (**Wt**) AAV genome.

[0048] **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.

[0049] **Figures 3A-3D** show illustrative **G-Quadruplex Structures** formed by the stacking of multiple G-tetrads. **Figures 3A-3D** depict such G-tetrad as gray planar rectangles whose vertices are connected to the polynucleotide backbone. The G-Quadruplex Structures may be formed from a single polynucleotide chain (**Figures 3A-3B**), that spontaneously loops back upon itself, from two polynucleotide strands (**Figure 3C**) that loop back upon each other, or from four polynucleotide chains (**Figure 3D**) that loop back upon each other. The ability of such polynucleotide chains to form such G-tetrads and to form such loops depends upon their presence of **G-Quadruplex Sequences**; such one, two or four polypeptide chains may form loops in either an anti-parallel manner (*e.g.*, **Figure 3A**) or in a parallel manner (*e.g.*, **Figure 3B**) (see, Harris, L.M. *et al.* (2015) "*G-Quadruplexes In Pathogens: A Common Route To Virulence Control*?" PLoS Pathog. 11(2):e1004562 (pages 1-15).

[0050] **Figure 4** shows a map of the AAV helper plasmid vector **pAAV-RC2**.

[0051] **Figure 5** shows a map of the non-AAV helper plasmid vector **pHelper-Kan**.

[0052] **Figure 6** shows a map of the rAAV plasmid vector **pAV-CMV-EGFP**.

[0053] **Figure 7** shows a map of the rAAV plasmid vector **pAV-TBG-EGFP**.

[0054] **Figure 8** shows the overall structure and approach followed for the development of the exemplary rAAV constructs described herein (**ITR**: adeno-

associated virus (AAV)-specific palindromic inverted terminal repeated sequences; **Pro**: promoter. **Gene**: transgene; **PolyA**: polynucleotide comprising poly-deoxyadenosine sequence; **CisE**: polynucleotide comprising a Cis-Element).

[0055] **Figures 9A-9B** show the increase in rAAV production titers obtained by introducing a Cis-Element within the **P2 Domain** of the rAAV plasmid vector **pAV-TBG-EGFP**. **Figure 9A** shows the **P2 Domain** of the rAAV plasmid vector **pAV-TBG-EGFP**. **Figure 9B** shows the production titers of rAAV obtained using rAAV plasmid vectors containing any of Cis-Elements CisE1-CisE27 (**Table 1**), relative to that obtained using the parental rAAV plasmid vector, **pAV-TBG-EGFP**. The production titers of rAAV were obtained using the parental or derivative rAAV plasmids in a triple plasmid transfection system with a helper plasmid providing the AAV ***rep*** and ***cap*** functions and an Ad helper plasmid that provided the required adenoviral functions.

[0056] **Figures 10A-10B** show the increase in rAAV production titers obtained by introducing a Cis-Element within the **P1 Domain** of the rAAV plasmid vector **pAV-TBG-EGFP** (**Figure 10A**). **Figure 10B** shows the production titers of rAAV obtained using rAAV plasmid vectors containing any of Cis-Elements CisE1, CisE20, CisE21, CisE27, CisE28, CisE29, or CisE30 (**Table 1**), relative to that obtained using the parental rAAV plasmid vector, **pAV-TBG-EGFP**. The production titers of rAAV were obtained using the parental or derivative rAAV plasmid vectors in a triple plasmid transfection system with an AAV helper plasmid providing the AAV ***rep*** and ***cap*** functions and an Ad helper plasmid that provided the required adenoviral functions.

[0057] **Figures 11A-11B** show the increase in rAAV production titers obtained by introducing the same Cis-Element within the **P1 Domain** or **P2 Domain** of the rAAV plasmid vector **pAV-TBG-EGFP** (**Figure 11A**). **Figure 11B** shows the production titers of rAAV obtained using rAAV plasmids containing any of Cis-Elements CisE1, CisE20, or CisE21 (**Table 1**), relative to that obtained using the parental rAAV plasmid, **pAV-TBG-EGFP**. The production titers of rAAV were obtained using the parental or derivative rAAV plasmids in a triple plasmid transfection system with an AAV helper plasmid providing the AAV ***rep*** and ***cap*** functions and an Ad helper plasmid that provided the required adenoviral functions.

[0058] **Figures 12A-12B** show the effect of Cis-Element orientation on the ability of a Cis-Element, inserted within the **P1 Domain** the rAAV plasmid vector **pAV-TBG-EGFP**, to cause an increase in rAAV production titer (**Figure 12A**). **Figure 12B** shows the production titers of rAAV obtained using rAAV plasmid vectors containing Cis-Element CisE21 (**Table 1**) in the forward orientation (**SEQ ID NO:41**) ("CisE21-For-P1") or in the reverse orientation (**SEQ ID NO:42**) ("CisE21-Rev-P1"), relative to that obtained using the parental rAAV plasmid vector, **pAV-TBG-EGFP**. The production titers of rAAV were obtained using the parental or derivative rAAV plasmids in a triple plasmid transfection system with an AAV helper plasmid providing the AAV ***rep*** and ***cap*** functions and an Ad helper plasmid that provided the required adenoviral functions.

[0059] **Figures 13A-13D** show the effect of Cis-Element orientation on the ability of a Cis-Element, inserted within both the **P1 Domain** the rAAV plasmid vector **pAV-TBG-EGFP** and the **P4 Domain** of such plasmid vector to cause an increase in rAAV production titer (**Figure 13A**). **Figure 13B** shows the production titers of rAAV obtained using rAAV plasmid vectors containing different Cis-Elements (Cis-Element CisE21, CisE22, CisE23, CisE24, CisE25, CisE26, CisE28, CisE29, CisE31, CisE32, CisE33, CisE34, or CisE35) within the **P1 Domain**, while maintaining the same Cis-Element (CisE30-Rev) within the **P4 Domain** of the rAAV plasmid vector. **Figure 13C** shows the production titers of rAAV obtained using rAAV plasmid vectors containing different Cis-Elements (Cis-Element CisE21, CisE22, CisE23, CisE24, CisE25, CisE27, CisE28, CisE32, CisE33, or CisE34) within its **P1 Domain** and Cis-Element CisE35-Rev within its **P4 Domain**. **Figure 13D** shows the production titers of rAAV obtained using rAAV plasmid vectors containing different Cis-Elements Cis-Element CisE22-Rev, CisE27-Rev, CisE29-Rev, or CisE35-Rev) within the **P4 Domain**, while maintaining the same Cis-Element (CisE28) within the **P1 Domain** of the rAAV plasmid vector. The production titers of rAAV were obtained using the parental or derivative rAAV plasmids in a triple plasmid transfection system with an AAV helper plasmid providing the AAV ***rep*** and ***cap*** functions and an Ad helper plasmid that provided the required adenoviral functions.

DETAILED DESCRIPTION OF THE INVENTION:

I. The Methods of the Present Invention

[0060] The present invention is directed to recombinantly-modified adeno-associated virus (rAAV) having improved packaging efficiency, pharmaceutical compositions comprising such rAAV, and methods for their production and use. The present invention is particularly directed to recombinantly-modified adeno-associated virus (rAAV) that have been further modified to comprise Cis-Elements, including replication origins, promoters and enhancers, that are capable of regulating the replication of an rAAV genome and that improve rAAV replication. Preferably, such Cis-Elements are provided within domains of the rAAV that precede and/or follow the 5' and/or 3' inverted terminal repeated sequences (ITR) of an rAAV. The invention particularly concerns the presence and the use of polynucleotide Cis-Elements that comprise actual or potential G-Quadruplex Sequences, polynucleotide Cis-Elements that comprise DNA sequences from wild-type AAV (wt AAV) and polynucleotide Cis-Elements that comprise DNA sequences from other viral genomes or from the human genome.

[0061] The present invention is based in part on the recognition that high levels of DNA replication increase both the amount of rAAV genomes particles and, consequently, the efficiency of rAAV packaging, and thus result in high production titers of rAAV stocks. Such desired high levels of DNA replication can be attained by modifying rAAV or rAAV plasmid vectors to contain additional polynucleotides that comprise replication origins, promoters, enhancers, *etc*. Because such polynucleotides act to increase the replication of rAAV vectors on which they are present, they are referred to herein as "**Cis-Elements**." The invention encompasses recombinant AAV vectors and rAAV plasmid vectors that carry such Cis-Elements and their use in the production of novel stable cell lines capable of generating high titer rAAV preparations. The Cis-Elements of the present invention are preferably introduced into an rAAV plasmid vector. Such introduction is preferably accomplished using well-known methods of recombinant DNA technology.

[0062] 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.

[0063] 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**, such as pAAV-RC (Agilent; Addgene; Cell Biolabs), pAAV-RC2 (Cell Biolabs), *etc.*, are commercially available. Plasmid **pAAV-RC2 (SEQ ID NO:1; Figure 4)** is an AAV helper plasmid that may be used in accordance with the present invention to provide **AAV helper functions**.

Coding Strand of Plasmid pAAV-RC2 (SEQ ID NO:1):

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
atcagaatcc caattctgat gcgccggtga tcagatcaaa aacttcagcc
aggtacatgg agctggtcgg gtggctcgtg gacaagggga ttacctcgga
gaagcagtgg atccaggagg accaggcctc atacatctcc ttcaatgcgg
cctccaactc gcggtcccaa atcaaggctg ccttggacaa tgcgggaaag
attatgagcc tgactaaaac cgcccccgac tacctggtgg gccagcagcc
cgtggaggac atttccagca atcggattta taaaattttg gaactaaacg
ggtacgatcc ccaatatgcg gcttccgtct ttctgggatg ggccacgaaa
aagttcggca agaggaacac catctggctg tttgggcctg caactaccgg
gaagaccaac atcgcggagg ccatagccca cactgtgccc ttctacgggt
gcgtaaactg gaccaatgag aactttccct tcaacgactg tgtcgacaag

atggtgatct ggtgggagga ggggaagatg accgccaagg tcgtggagtc
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[0064] In **SEQ ID NO:1**, residues 85-1950 of **pAAV-RC2** encode the **Rep** protein, **Rep78** (with residues 484-663 corresponding to the **P19** promoter, residues 1464-1643 corresponding to the **P40** promoter and residues 1668-1676 being a donor site); residues 1967-4174 encode the capsid protein, **VP1**; residues 1992-2016 encodes a portion of the **Rep68** protein; residues 4175-4256 encode a polyA sequence; 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**).

[0065] 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 (commercially available from Cell Biolabs, Inc., Invitrogen and Stratagene) 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). Plasmid **pHelper-Kan (SEQ ID NO:2; Figure 5)** 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:2):

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ggaacaagag tccactatta aagaacgtgg actccaacgt caaagggcga
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ggagcccccg atttagagct tgacggggaa agccggcgaa cgtggcgaga
aaggaaggga agaaagcgaa aggagcgggc gctagggcgc tggcaagtgt
agcggtcacg ctgcgcgtaa ccaccacacc cgccgcgctt aatgcgccgc
tacagggcgc gatggatcc

[0066] In **SEQ ID NO:2**, 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 5**).

[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:3**; **Figure 6**) 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:3):

```
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gagggcctat ttcccatgat tccttcatat ttgcatatac gatacaaggc
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tacaaaatac gtgacgtaga aagtaataat ttcttgggta gtttgcagtt
```

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actggaccag gcagcagcgt cagaagactt ttttggaaaa gcttgactag
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tggccttttg ctggcctttt gctcacatgt

[0068] In **SEQ ID NO:3**, 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 6**).

[0069] A second illustrative rAAV plasmid vector that may be used in accordance with the present invention is **pAV-TBG-EGFP** (**SEQ ID NO:4**; **Figure 7**) 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:4):

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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
atgtcatgat aataatggtt tcttagacgt caggtggcac ttttcgggga
aatgtgcgcg gaacccctat ttgtttattt ttctaaatac attcaaatat
gtatccgctc atgagacaat aaccctgata aatgcttcaa taatattgaa
aaaggaagag tatgagtatt caacatttcc gtgtcgccct tattcccttt
tttgcggcat tttgccttcc tgtttttgct cacccagaaa cgctggtgaa
agtaaaagat gctgaagatc agttgggtgc acgagtgggt tacatcgaac
tggatctcaa cagcggtaag atccttgaga gttttcgccc cgaagaacgt
tttccaatga tgagcacttt taaagttctg ctatgtggcg cggtattatc
ccgtattgac gccgggcaag agcaactcgg tcgccgcata cactattctc
agaatgactt ggttgagtac tcaccagtca cagaaaagca tcttacggat
ggcatgacag taagagaatt atgcagtgct gccataacca tgagtgataa
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 catttttaat 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
atgctcgtca gggggggcgga gcctatggaa aaacgccagc aacgcggcct
ttttacggtt cctggccttt tgctggcctt ttgctcacat gt

[0070] In **SEQ ID NO:4**, 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 7**).

[0071] In particular, the present invention provides a recombinantly-modified adeno-associated virus (rAAV), such as **pAV-CMV-EGFP** or **pAV-TBG-EGFP**, that comprises a Cis-Element in one or more of its **P1**, **P2**, **P3** or **P4 Domains**, wherein:

(1) the **P1 Domain** is 5' to a 5' ITR of the rAAV;

(2) the **P2 Domain** is 3' to the 5' ITR of the rAAV and 5' to a transgene cassette of the rAAV;

(3) the **P3 Domain** is 3' to the transgene cassette of the rAAV and 5' to a 3' ITR of the rAAV; and

(4) the **P4 Domain** is 3' to the 3' ITR of the rAAV; and

wherein the presence of the Cis-Element causes rAAV-producing cells to produce the rAAV at a higher production titer than would be attained with such rAAV if lacking the Cis-Element.

[0072] In one embodiment, the Cis-Element will preferably comprise an introduced nucleotide sequence that was not previously present in rAAV vector. In other embodiments, the introduced nucleotide sequence was previously present in such rAAV plasmid vector, and has been positioned in the recombinantly-produced rAAV plasmid vector, adjacent to, or immediately adjacent to, such previously present nucleotide sequence. Alternatively, such introduced nucleotide sequence may be positioned at a site that is not adjacent to such previously present nucleotide sequence.

[0073] As shown in **Figure 8**, an rAAV or an rAAV plasmid vector of the present invention may be envisioned as having, in the 5' to 3' direction:

(1) the 5' terminus of the rAAV;
(2) a polynucleotide region (the "**P1 Domain**")
(3) a "**5' ITR**," which is an ITR that is located at or near the 5' end of the polynucleotide chain that comprises the coding strand of the transgene cassette of the rAAV;
(4) a polynucleotide region (the "**P2 Domain**")
(5) a transgene cassette (including a preceding (*i.e.*, 5'-positioned) promoter ("**Pro**"), the transgene encoding sequence ("**Gene**"), and following (*i.e.*, 3'-positioned) poly-A sequence ("**PolyA**");
(6) a polynucleotide region (the "**P3 Domain**")
(7) a "**3' ITR**," which is an ITR that is located at or near the 3' end of the polynucleotide chain that comprises the coding strand of the transgene cassette of the rAAV;
(8) a polynucleotide region (the "**P4 Domain**"); and
(9) the 3' terminus of the rAAV."

[0074] Such **P1**, **P2**, **P3**, and **P4 Domains** need not all be present in any particular rAAV or rAAV plasmid vector, and an rAAV or rAAV plasmid vector may lack any 1, 2, or 3 of these Domains, or may lack all 4 of such Domains. The boundaries of such Domains are defined by the other domains of the rAAV or rAAV plasmid vector. Thus,

the **P1 Domain** extends from the 5' terminus of the rAAV or rAAV plasmid vector to the 5' terminus of the 5' ITR. The **PciI** site of **pAV-CMV-EGFP** or **pAV-TBG-EGFP** is an example of a suitable site within the **P1 Domain** of an rAAV or rAAVplasmid vector for insertion of a Cis-Element. The **P2 Domain** extends from the 3' terminus of the 5' ITR to the 5' terminus of the transgene cassette. The **P3 Domain** extends from the 3' terminus of the transgene cassette to the 5' terminus of the 3' ITR. The **EcoR1** site of **pAV-CMV-EGFP** or the **SpeI** site of **pAV-TBG-EGFP** are examples of suitable sites within the **P2 Domain** of an rAAV or rAAVplasmid vector for insertion of a Cis-Element. The **P3 Domain** extends from the 3' terminus of the poly(A) sequence to the 3' ITR of the rAAV or rAAV plasmid vector. The **PmlI** site of **pAV-CMV-EGFP** or **pAV-TBG-EGFP** is an example of a suitable site within the **P3 Domain** of an rAAV or rAAVplasmid vector for insertion of a Cis-Element. The **P4 Domain** extends from the 3' terminus of the 3' ITR to the 3' terminus of the rAAV or rAAV plasmid vector. The **KasI** site of **pAV-CMV-EGFP** or **pAV-TBG-EGFP** is an example of a suitable site within the **P4 Domain** of an rAAV or rAAVplasmid vector for insertion of a Cis-Element. The precise location of a Cis-Element of the present invention within a particular **P1**, **P2**, **P3**, or **P4 Domain** is not material to the ability of such positioned Cis-Element to mediate an increase in rAAV production titers. Insertions of Cis-Element(s) may be made by ligating a Cis-Element into a suitable restriction site or by employing primers to install such Cis-Elements.

[0075] The present invention employs the nomenclature of CisE1, CisE2, *etc*. to identify particular Cis-Elements. Such designation is followed by "For" or "Rev" in some cases to indicate that the Cis-Element is being (respectively) inserted into the rAAV in its forward orientation or in its reverse orientation. When "For" or "Rev" are not indicated, the Cis-Element is being inserted into the rAAV in its forward orientation. Lastly, the present invention employs the nomenclature **P1**, **P2**, **P3**, or **P4 Domain** to indicate the domain within which the Cis-Element has been inserted. Thus, for example, an rAAV or rAAV plasmid vector containing Cis-Element CisE1 of the present invention within its **P1 Domain** in its forward orientation is referred to herein by the designation "**CisE1-For-P1**" or "**CisE1-P1**;" an rAAV or rAAV plasmid vector containing Cis-Element CisE21 of the present invention within its **P2 Domain** in its forward orientation is referred to herein by the designation "**CisE21-For-P2**" or

"**CisE1-P2**;" an rAAV or rAAV plasmid vector containing Cis-Element CisE30 of the present invention within its **P4 Domain** in its reverse orientation is referred to herein by the designation "**CisE30-Rev-P4**." Thus, with reference to the constructs shown schematically in **Figure 8**, constructs CisE-P1, CisE-Rev-P1, CisE-P1&P4, CisE-P1~P4 all contain Cis-Elements within the **P1 Domain** (the orientation of the Cis-Element may be reversed, as in CisE-Rev-P1); the construct CisE-P1&P4 contains Cis-Elements within the **P1 Domain** and within the **P4 Domain**; the construct CisE-P1~P4 contains Cis-Elements within the **P1**, **P2**, **P3** and **P4 Domain**s.

[0076] In one embodiment, such Cis-Elements are actual or potential "**G-Quadruplex Sequences**" capable of forming a **G-Quadruplex Structure**. The G-Quadruplex Structures of particular relevance to the present invention comprise only a single polynucleotide chain, and have the general formula of four series, each composed of 3 or more deoxyguanosine residues, wherein the first, second and third such series is separated from the fourth such series by from 1 to 7 of any other nucleotide residue.

[0077] In some cases, a particular polynucleotide will comprise a sequence that is known to comprise a G-Quadruplex Sequence (*i.e.*, an "**Actual G-Quadruplex Sequence**"). In other cases, a particular sequence will be predicted to comprise a G-Quadruplex Sequence that can form a G-Quadruplex Structure (*i.e.*, a "**Potential G-Quadruplex Sequence**"). Predictive algorithms for determining whether any particular polynucleotide is a potential G-Quadruplex Sequence are well known, and thus the recognition of whether a particular polynucleotide is a potential G-Quadruplex Sequence may be readily accomplished. Examples of such predictive algorithms include **G4P Calculator** (Eddy, J. *et al.* (2006) "*Gene Function Correlates With Potential For G4 DNA Formation In The Human Genome*," Nucleic Acids Res. 34:3887-3896), **QuadParser** (Huppert, J.L. *et al.* (2005) "*Prevalence Of Quadruplexes In The Human Genome*," Nucleic Acids Res. 33:2908-29168) and **GHunter** (Bedrat, A. *et al.* (2016) "*Re-evaluation of G-Quadruplex propensity with G4Hunter*," Nucleic Acids Res. 44(4):1746-1759), have been developed to identify potential G-Quadruplex Sequences (Huppert, J.L. *et al.* (2007) "*G-Quadruplexes In Promoters Throughout The Human Genome*," Nucleic Acids Res. 35:406-413; Verma, A. *et al.* (2008) "*Genome-Wide Computational And Expression Analyses Reveal G-Quadruplex DNA Motifs As*

Conserved Cis-Regulatory Elements In Human And Related Species," J. Med. Chem. 51:5641-5649).

[0078] The invention further encompasses compositions such as plasmids that are genetically engineered to replicate high levels of recombinant viral genomes. The replication of viral genomes may be regulated through the use of Cis-Elements, including replication origins, promoters and enhancers. Such Cis-Elements can be genetically engineered into recombinant plasmids that are designed to pack AAV vectors. Further, the invention encompasses the Cis-Elements can be located before or after ITRs.

[0079] Cis-elements of the present invention that increase AAV production particularly include:

(1) Potential G-Quadruplex Sequences of wild type AAV genomes (*e.g.*, CisE1 – CisE16 (**SEQ ID NOs:5**, 7, **9**, **11**, **13**, **15**, **17**, **19**, **21**, **23**, **25**, **27**, **29**, **31**, **33** and **35**)), or Potential G-Quadruplex Sequences in a reversed orientation (*e.g.*, CisE1-Rev – CisE16-Rev (**SEQ ID NOs:6**, **8**, **10**, **12**, **14**, **16**, **18**, **20**, **22**, **24**, **26**, **28**, **30**, **32**, **34** and **36**));

(2) Actual G-Quadruplex Sequences (*e.g.*, CisE17 – CisE20 (**SEQ ID NOs:37**, **39**, **41** and **43**)) or Actual G-Quadruplex Sequences in a reversed orientation (*e.g.*, CisE17-Rev – CisE20-Rev (**SEQ ID NOs:38**, **40**, **42** and **44**);

(3) Other DNA sequences from wild-type AAV (*e.g.*, CisE21 – CisE26 and CisE31 – CisE35 (**SEQ ID NOs:45**, **47**, **49**, **51**, **53**, **55**, **65**, **67**, **69**, **71** and **73**)) or other DNA sequences from wild-type AAV in a reversed orientation (*e.g.*, CisE21-Rev – CisE26-Rev and CisE31-Rev – CisE35-Rev (**SEQ ID NOs: 46**, **48**, **50**, **52**, **54**, **56**, **66**, **68**, **72** and **74**); and

(4) DNA sequences from other viral genomes (*e.g.*, CisE27 – CisE30 (**SEQ ID NOs:57**, **59**, **61** and **63**)) or such DNA sequences from other viral sources in a reversed orientation (*e.g.*, CisE27-Rev – CisE30-Rev (**SEQ ID NOs: 58**, **60**, **62** and **64**) and human genomes.

[0080] **Table 1** provides the sequences, sequence designations, and origins of such preferred exemplary Cis-Elements of the present invention.

SEQ ID NO	Sequence Designation	Source or Origin	Sequence
5	CisE1	GQ4	gggtggccga gaaggaatgg
6	CisE1-Rev	GQ4-Rev	ggtaaggaag agccggtggg
7	CisE2	GQ5	ggaatggcgc cgtgtgagta aggccccgg
8	CisE2-Rev	GQ5-Rev	ggccccggaa tgagtgtgcc gcggtaagg
9	CisE3	GQ6	ggaaaccacc ggggtgaaat ccatgg
10	CisE3-Rev	GQ6-Rev	ggtacctaaa gtggggccac caaagg
11	CisE4	GQ7	ggaggcggga acaaggtggt gg
12	CisE4-Rev	GQ7-Rev	ggtggtggaa caagggcgga gg
13	CisE5	GQ10	ggatccagga ggaccagg
14	CisE5-Rev	GQ10-Rev	ggaccaggag gacctagg
15	CisE6	GQ12	ggcaagagga acaccatctg gctgtttggg
16	CisE6-Rev	GQ12-Rev	gggtttgtcg gtctaccaca aggagaacgg
17	CisE7	GQ14	ggaggaagca aggtgcgcgt gg
18	CisE7-Rev	GQ14-Rev	ggtgcgcgtg gaacgaagga gg
19	CisE8	GQ15	ggtgggcaaa ggatcacgtg gttgaggtgg
20	CisE8-Rev	GQ15-Rev	ggtggagttg gtgcactagg aaacgggtgg
21	CisE9	GQ16	ggcataagga cgacagcagg gg
22	CisE9-Rev	GQ16-Rev	ggggacgaca gcaggaatac gg
23	CisE10	GQ17	ggggcaacct cggacgagca gtcttccagg
24	CisE10-Rev	GQ17-Rev	ggaccttctg acgagcaggc tccaacgggg
25	CisE11	GQ18	ggttcttgaa cctctgggcc tggttgagg
26	CisE11-Rev	GQ18-Rev	ggagttggtc cgggtctcca agttcttgg
27	CisE12	GQ19	ggctccggga aaaaagaggc cgg
28	CisE12-Rev	GQ19-Rev	ggccggagaa aaaagggcct cgg

SEQ ID NO	Sequence Designation	Source or Origin	Sequence
29	CisE13	GQ20	ggaaccggaa aggcggg
30	CisE13-Rev	GQ20-Rev	gggcggaaag gccaagg
31	CisE14	GQ22	gggcgccgac ggagtgggta attcctcgg
32	CisE14-Rev	GQ22-Rev	ggctccttaa tgggtgaggc agccgcggg
33	CisE15	GQ23	ggaccagtct aggaactggc ttcctgg
34	CisE15-Rev	GQ23-Rev	ggtccttcgg tcaaggatct gaccagg
35	CisE16	GQ24	ggtgaatccg ggcccggcca tgg
36	CisE16-Rev	GQ24-Rev	ggtaccggcc cgggcctaag tgg
37	CisE17	c-Myc	atggggaggg tggggagggt ggggaaggtg ggga
38	CisE17-Rev	c-Myc-Rev	aggggtggaa ggggtgggag gggtgggagg ggta
39	CisE18	Chicken β-actin	gggggggggg gggcggg
40	CisE18-Rev	Chicken β-actin-Rev	gggcgggggg ggggggg
41	CisE19	VEGF	ggggcgggcc gggggcgggg tcccggggcg g
42	CisE19-Rev	VEGF-Rev	ggcggggccc tggggcgggg gccgggcggg g
43	CisE20	BCL-2	aggggcgggc gcgggaggaa gggggcggga gcggggctg
44	CisE20-Rev	BCL-2-Rev	gtcggggcga gggcgggga aggagggcgc gggcggga

SEQ ID NO	Sequence Designation	Source or Origin	Sequence
45	CisE21	P5	ggtcctgtat tagaggtcac gtgagtgttt tgcgacattt tgcgacacca tgtggtcacg ctgggtattt aagcccgagt gagcacgcag ggtctccatt ttgaagcggg aggtttgaac gcgcagccgc catgccgggg ttttacgaga ttgtgattaa ggtccccagc gaccttgacg agcatctgcc cggcatttct gacagctttg tgaactgggt ggccgagaag gaatgggagt tgccgccaga ttctgacatg gatctgaatc tgattgagca ggcacccctg accgtggccg agaagctgca gcgcgacttt ctgacggaat ggcgccgtgt gagtaaggcc ccggaggccc ttttctttgt gcaatttgag aagggagaga gctacttcca catgcacgtg ctcgtggaaa ccaccggggt ga
46	CisE21-Rev	P5-Rev	agtggggcca ccaaaggtgc tcgtgcacgt acaccttcat cgagagaggg aagagtttaa cgtgtttctt ttcccggagg ccccggaatg agtgtgccgc ggtaaggcag tctttcagcg cgacgtcgaa gagccggtgc cagtccccac ggacgagtta gtctaagtct aggtacagtc ttagaccgcc gttgagggta aggaagagcc ggtgggtcaa gtgtttcgac agtctttacg gcccgtctac gagcagttcc agcgacccct ggaattagtg ttagagcatt ttggggccgt accgccgacg cgcaagtttg gagggcgaag ttttacctct gggacgcacg agtgagcccg aatttatggg tcgcactggt gtaccacagc gttttacagc gttttgtgag tgcactggag attatgtcct gg
47	CisE22	P19	gtcacaaaga ccagaaatgg cgccggaggc gggaacaagg tggtggatga gtgctacatc cccaattact tgctccccaa aacccagcct gagctccagt gggcgtggac taatatggaa cagtatttaa gcgcctgttt gaatctcacg gagcgtaaac ggttggtggc gcagcatctg
48	CisE22-Rev	P19-Rev	gtctacgacg cggtggttgg caaatgcgag gcactctaag tttgtccgcg aatttatgac aaggtataat caggtgcggg tgacctcgag tccgacccaa aacccctcgt tcattaaccc ctacatcgtg agtaggtggt ggaacaaggg cggaggccgc ggtaaagacc agaaacactg

SEQ ID NO	Sequence Designation	Source or Origin	Sequence
49	CisE23	P40	gtcacaaaga ccagaaatgg cgccggaggt caccaagcag gaagtcaaag actttttccg gtgggcaaag gatcacgtgg ttgaggtgga gcatgaattc tacgtcaaaa agggtggagc caagaaaaga cccgccccca gtgacgcaga tataagtgag cccaaacggg tgcgcgagtc agttgcgcag ccatcgacgt cagacgcggc gggaacaagg tggtggatga gtgctacatc cccaattact tgctccccaa aacccagcct gagctccagt gggcgtggac taatatggaa cagtatttaa gcgcctgttt gaatctcacg gagcgtaaac ggttggtggc gcagcatctg
50	CisE23-Rev	P40-Rev	gtctacgacg cggtggttgg caaatgcgag gcactctaag tttgtccgcg aatttatgac aaggtataat caggtgcggg tgacctcgag tccgacccaa aacccctcgt tcattaaccc ctacatcgtg agtaggtggt ggaacaaggg cggcgcagac tgcagctacc gacgcgttga ctgagcgcgt gggcaaaccc gagtgaatat agacgcagtg acccccgccc agaaaagaac cgaggtggga aaaactgcat cttaagtacg aggtggagtt ggtgcactag gaaacgggtg gccttttca gaaactgaag gacgaaccac tggaggccgc ggtaaagacc agaaacactg
51	CisE24	P5(209-331)	cgtgagtgtt ttgcgacatt ttgcgacacc atgtggtcac gctgggtatt taagcccgag tgagcacgca gggtctccat tttgaagcgg gaggtttgaa cgcgcagccg ccatgccggg gtt
52	CisE24-Rev	P5(209-331)-Rev	ttggggccgt accgccgacg cgcaagtttg gagggcgaag ttttacctct gggacgcacg agtgagcccg aatttatggg tcgcactggt gtaccacagc gttttacagc gttttgtgag tgc
53	CisE25	P5(317-431)	cgccatgccg gggttttacg agattgtgat taaggtcccc agcgaccttg acgagcatct gcccggcatt tctgacagct ttgtgaactg ggtggccgag aaggaatggg agttg

SEQ ID NO	Sequence Designation	Source or Origin	Sequence
54	CisE25-Rev	P5(317-431)-Rev	gttgagggta aggaagagcc ggtgggtcaa gtgtttcgac agtctttacg gcccgtctac gagcagttcc agcgaccccct ggaattagtg ttagagcatt ttggggccgt accgc
55	CisE26	P5(406-543)	gggtggccga gaaggaatgg gagttgccgc cagattctga catggatctg aatctgattg agcaggcacc cctgaccgtg gccgagaagc tgcagcgcga ctttctgacg gaatggcgcc gtgtgagtaa ggccccgg
56	CisE26-Rev	P5(406-543)-Rev	ggccccggaa tgagtgtgcc gcggtaaggc agtctttcag cgcgacgtcg aagagccggt gccagtcccc acggacgagt tagtctaagt ctaggtacag tcttagaccg ccgttgaggg taaggaagag ccggtggg
57	CisE27	P143	cgttgaaaac caaattgact ccggtcacta cgttttccaa ttttctaaag aatcctttac acacaatgtc aggcggcaag tttagcgcca tcacattctc gtacgtgtac gcccacaatt catcgtgatc caaaatttcg tttttagccg actgagtcaa atatatcatg tagtgtatgc caaaataata gcccaacgat acgcacaatt tggtatcgtc aaagtcaaac caatgattgc aggccctatt aaacactatt ttctcttgtt ttttgtaagg ctcacatcgc ttcaaagctt cattcaaagc ttctttgtcg caggcaaata atgattcaca caaaagttcc aaaaacagtt tgatgtcg
58	CisE27-Rev	P143-Rev	gctgtagttt gacaaaaacc ttgaaaacac acttagtaat aaacggacgc tgtttcttcg aaacttactt cgaaacttcg ctacactcgg aatgtttttt gttctctttt atcacaaatt atcccggacg ttagtaacca aactgaaact gctatggttt aacacgcata gcaacccgat aataaaaccg tatgtgatgt actatataaa ctgagtcagc cgatttttgc tttaaaacct agtgctactt aacacccgca tgtgcatgct cttacactac cgcgatttga acggcggact gtaacacaca tttcctaaga aatcttttaa ccttttgcat cactggcctc agttaaacca aaagttgc

SEQ ID NO	Sequence Designation	Source or Origin	Sequence
59	CisE28	CMV	gacattgatt attgactagt tattaatagt aatcaattac ggggtcatta gttcatagcc catatatgga gttccgcgtt acataactta cggtaaatgg cccgcctggc tgaccgccca acgacccccg cccattgacg tcaataatga cgtatgttcc catagtaacg ccaataggga ctttccattg acgtcaatgg gtggagtatt tacggtaaac tgcccacttg gcagtacatc aagtgtatca tatgccaagt acgccccta ttgacgtcaa tgacggtaaa tggcccgcct ggcattatgc ccagtacatg accttatggg actttcctac ttggcagtac atctacgtat tagtcatcgc tattaccatg gtgatgcggt tttggcagta catcaatggg cgtggatagc ggtttgactc acggggattt ccaagtctcc accccattga cgtcaatggg agtttgtttt ggcaccaaaa tcaacgggac tttccaaaat gtcgtaacaa ctccgcccca ttgacgcaaa tgggcggtag gcgtgtacgg tgggaggtct atataagcag
60	CisE28-Rev	CMV-Rev	gacgaatata tctggagggt ggcatgtgcg gatggcgggt aaacgcagtt accccgcctc aacaatgctg taaaaccttt cagggcaact aaaaccacgg ttttgtttga gggtaactgc agttacccca cctctgaacc tttaggggca ctcagtttgg cgataggtgc gggtaactac atgacggttt tggcgtagtg gtaccattat cgctactgat tatgcatcta catgacggtt catcctttca gggtattcca gtacatgacc cgtattacgg tccgcccggt aaatggcagt aactgcagtt atcccccgca tgaaccgtat actatgtgaa ctacatgacg gttcacccgt caaatggcat ttatgaggtg ggtaactgca gttacctttc agggataacc gcaatgatac ccttgtatgc agtaataact gcagttaccc gcccccagca acccgccagt cggtccgccc ggtaaatggc attcaataca ttgcgccttg aggtatatac ccgatacttg attactgggg cattaactaa tgataattat tgatcagtta ttagttacag

SEQ ID NO	Sequence Designation	Source or Origin	Sequence
61	CisE29	SV40	gtgtgtcagt tagggtgtgg aaagtcccca ggctccccag caggcagaag tatgcaaagc atgcatctca attagtcagc aaccaggtgt ggaaagtccc caggctcccc agcaggcaga agtatgcaaa gcatgcatct caattagtca gcaaccatag tcccgccccc aactccgccc atcccgcccc taactccgcc cagttccgcc cattctccgc cccatggctg actaattttt tttatttatg cagaggccga ggccgcctcg gcctctgagc tattccagaa gtagtgagga ggctttttg gaggcctagg cttttgcaaa
62	CisE29-Rev	SV40-Rev	aaacgttttc ggatccggag gttttttcgg aggagtgatg aagaccttat cgagtctccg gctccgccgg agccggagac gtatttattt tttttaatca gtcggtaccc cgcctcttac ccgccttgac ccgcctcaat ccccgcccta cccgcctcaa tccccgcccc gataccaacg actgattaac tctacgtacg aaacgtatga agacggacga cccctcggac ccctgaaagg tgtggaccaa cgactgatta actctacgta cgaaacgtat gaagacggac gacccctcgg acccctgaaa ggtgtgggat tgactgtgtg
63	CisE30	RLTR	gcatcaggcg ccgtgcggta tttcacaccg catatggatc catgcatgtt cgaatttaaa tttaattaac atcatcaata atatacctta ttttggattg aagccaatat gataatgagg gggtggagtt tgtgacgtgg cgcggggcgt gggaacgggg cgggtgacgt aggttttagg gcggagtaac ttgtatgtgt tgggaattgt agttttctta aaatgggaag tgacgtaacg tgggaatccg gaggcgcccc tgc
64	CisE30-Rev	RLTR-Rev	cgtccccgcg gaggcctaag ggtgcaatgc agtgaagggt aaaattcttt tgatgttaag ggttgtgtat gttcaatgag gcgggatttt ggatgcagtg ggcggggcaa gggtgcgggg cgcggtgcag tgtttgaggt gggggagtaa tagtataacc gaagttaggt tttattccat ataataacta ctacaattaa tttaaattta agcttgtacg tacctaggta tacgccacac tttatggcgt gccgcggact acg

SEQ ID NO	Sequence Designation	Source or Origin	Sequence
65	CisE31	GQ4-7	gggtggccga gaaggaatgg gagttgccgc cagattctga catggatctg aatctgattg agcaggcacc cctgaccgtg gccgagaagc tgcagcgcga ctttctgacg gaatggcgcc gtgtgagtaa ggccccggag gccctttct ttgtgcaatt tgagaaggga gagagctact tccacatgca cgtgctcgtg gaaaccaccg gggtgaaatc catggttttg ggacgtttcc tgagtcagat tcgcgaaaaa ctgattcaga gaatttaccg cgggatcgag ccgactttgc caaactggtt cgcggtcaca aagaccagaa atggcgccgg aggcgggaac aaggtggtgg
66	CisE31-Rev	GQ4-7-Rev	ggtggtggaa caagggcgga ggccgcggta aagaccagaa acactggcgc ttggtcaaac cgtttcagcc gagctagggc gccatttaag agacttagtc aaaaagcgct tagactgagt cctttgcagg gttttggtac ctaaagtggg gccaccaaag gtgctcgtgc acgtacacct tcatcgagag agggaagagt ttaacgtgtt tcttttcccg gaggccccgg aatgagtgtg ccgcggtaag gcagtctttc agcgcgacgt cgaagagccg gtgccagtcc ccacggacga gttagtctaa gtctaggtac agtcttagac cgccgttgag ggtaaggaag agccggtggg
67	CisE32	GQ9-12	ggtcgggtgg ctcgtggaca aggggattac ctcggagaag cagtggatcc aggaggacca ggcctcatac atctccttca atgcggcctc caactcgcgg tcccaaatca aggctgcctt ggacaatgcg ggaaagatta tgagcctgac taaaaccgcc cccgactacc tggtgggcca gcagcccgtg gaggacattt ccagcaatcg gatttataaa attttggaac taaacgggta cgatccccaa tatgcggctt ccgtctttct gggatgggcc acgaaaaagt tcggcaagag gaacaccatc tggctgtttg gg

SEQ ID NO	Sequence Designation	Source or Origin	Sequence
68	CisE32-Rev	GQ9-12-Rev	gggtttgtcg gtctaccaca aggagaacgg cttgaaaaag caccgggtag ggtctttctg ccttcggcgt ataaccccta gcatgggcaa atcaaggttt taaaatattt aggctaacga cctttacagg aggtgcccga cgaccgggtg gtccatcagc ccccgccaaa atcagtccga gtattagaaa gggcgtaaca ggttccgtcg gaactaaacc ctggcgctca acctccggcg taacttcctc tacatactcc ggaccaggag gacctaggtg acgaagaggc tccattaggg gaacaggtgc tcggtgggct gg
69	CisE33	GQ14-15	ggaggaagca aggtgcgcgt ggaccagaaa tgcaagtcct cggcccagat agacccgact cccgtgatcg tcacctccaa caccaacatg tgcgccgtga ttgacgggaa ctcaacgacc ttcgaacacc agcagccgtt gcaagaccgg atgttcaaat ttgaactcac ccgccgtctg gatcatgact ttgggaaggt caccaagcag gaagtcaaag acttttccg gtgggcaaag gatcacgtgg ttgaggtgg
70	CisE33-Rev	GQ14-15-Rev	ggtggagttg gtgcactagg aaacgggtgg ccttttcag aaactgaagg acgaaccact ggaagggttt cagtactagg tctgccgccc actcaagttt aaacttgtag gccagaacgt tgccgacgac cacaagcttc cagcaactca agggcagtta gtgccgcgtg tacaaccaca acctccactg ctagtgccct cagcccagat agacccggct cctgaacgta aagaccaggt gcgcgtggaa cgaaggagg

SEQ ID NO	Sequence Designation	Source or Origin	Sequence
71	CisE34	GQ16-22	ggcataagga cgacagcagg ggtcttgtgc ttcctgggta caagtacctc ggacccttca acggactcga caagggagag ccggtcaacg aggcagacgc cgcggccctc gagcacgaca aagcctacga ccggcagctc gacagcggag acaacccgta cctcaagtac aaccacgccg acgcggagtt tcaggagcgc cttaaagaag atacgtcttt tgggggcaac ctcggacgag cagtcttcca ggcgaaaaag agggttcttg aacctctggg cctggttgag gaacctgtta agacggctcc gggaaaaaag aggccggtag agcactctcc tgtggagcca gactcctcct cgggaaccgg aaaggcgggc cagcagcctg caagaaaaag attgaatttt ggtcagactg gagacgcaga ctcagtacct gacccccagc ctctcggaca gccaccagca gccccctctg gtctgggaac taatacgatg gctacaggca gtggcgcacc aatggcagac aataacgagg gcgccgacgg agtgggtaat tcctcgg
72	CisE34-Rev	GQ16-22-Rev	ggctccttaa tgggtgaggc agccgcggga gcaataacag acggtaacca cgcggtgacg gacatcggta gcataatcaa gggtctggtc tcccccgacg accaccgaca ggctctccga cccccagtcc atgactcaga cgcagaggtc agactggttt taagttagaa aaagaacgtc cgacgaccgg gcggaaaggc caagggctcc tcctcagacc gaggtgtcct ctcacgagat ggccggagaa aaaagggcct cggcagaatt gtccaaggag ttggtccggg tctccaagtt cttgggagaa aaagcggacc ttctgacgag caggctccaa cgggggtttt ctgcatagaa gaaattccgc gaggactttg aggcgcagcc gcaccaacat gaactccatg cccaacagag gcgacagctc gacggccagc atccgaaaca gcacgagctc ccggcgccgc agacggagca actggccgag agggaacagc tcaggcaact tcccaggctc catgaacatg ggtccttcgt gttctgggga cgacagcagg aatacgg

SEQ ID NO	Sequence Designation	Source or Origin	Sequence
73	CisE35	GQ23-25	ggaccagtct aggaactggc ttcctggacc ctgttaccgc cagcagcgag tatcaaagac atctgcggat aacaacaaca gtgaatactc gtggactgga gctaccaagt accacctcaa tggcagagac tctctggtga atccgggccc ggccatggca agccacaagg acgatgaaga aaagtttttt cctcagagcg gggttctcat ctttgggaag caagg
74	CisE35-Rev	GQ23-25-Rev	ggaacgaagg gtttctactc ttggggcgag actccttttt tgaaaagaag tagcaggaac accgaacggt accggcccgg gcctaagtgg tctctcagag acggtaactc caccatgaac catcgaggtc aggtgctcat aagtgacaac aacaataggc gtctacagaa actatgagcg acgaccgcca ttgtcccagg tccttcggtc aaggatctga ccagg

[0081] The inclusion of one or more of the Cis-Elements of the present 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).

[0082] 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.

[0083] 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.

[0084] 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.

[0085] 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 transfected with a desired rAAV or rAAV plasmid vector, and with such one or more viruses and/or helper plasmids that can provide proteins or RNA molecules that are not 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, in the case of rAAV, ***cap*** genes that encode VP capsid proteins required to form infectious particles. Such proteins or RNA molecules also include the viral transcription and translation factors (E1a, E1b, E2a, VA and E4) required for AAV proliferation. In one embodiment for producing the rAAV of the present invention, all of these 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 required ***rep*** and ***cap*** genes are provided by one plasmid, and the genes that encode the viral transcription and translation factors are provided on a second plasmid, so that such plasmids, in concert with the rAAV, comprise a triple plasmid transfection system.

[0086] 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 the rAAV1, rAAV2, rAAV5, rAAV6, rAAV7, rAAV8, rAAV9, and rAAV10 serotypes, and including hybrid serotypes (*e.g.*, AAV2/5 and rAAV2/5, which is a hybrid of serotypes 2 and 5 and thus has the trophism of both such serotypes).

[0087] 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.

[0088] 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.

[0089] 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).

[0090] 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.

[0091] In particular, the present invention thus provides a method for increasing the production titer of a recombinantly-modified AAV (rAAV) wherein such method comprises the steps:

(A) employing, as the rAAV for producing the production titer, an rAAV that has been modified to comprise an added Cis-Element in one or more of its **P1**, **P2**, **P3** or **P4 Domains**, wherein:

(1) the **P1 Domain** is 5' to a 5' ITR of the rAAV;

(2) the **P2 Domain** is 3' to the 5' ITR of the rAAV and 5' to a transgene cassette of the rAAV;

(3) the **P3 Domain** is 3' to the transgene cassette of the rAAV and 5' to a 3' ITR of the rAAV; and

(4) the **P4 Domain** is 3' to the 3' ITR of the rAAV; and

(B) culturing cells that have been transfected with the employed rAAV, wherein the cells additionally contain an AAV helper function-providing polynucleotide and a non-AAV helper function-providing polynucleotide, and wherein the culturing is conducted in a culture medium under conditions sufficient to permit the production of rAAV;

wherein the presence of the Cis-Element in the employed rAAV causes the cells to produce the rAAV at an increased production titer relative to that which would be attained if the employed rAAV had lacked the Cis-Element.

II. Pharmaceutical Compositions of the Present Invention

[0092] The present invention provides a pharmaceutical composition that comprises:

(A) a preparation of recombinantly-modified adeno-associated virus (rAAV) that comprise a Cis-Element in one or more of its **P1**, **P2**, **P3** or **P4 Domains**, wherein:

(1) the **P1 Domain** is 5' to a 5' ITR of the rAAV;

(2) the **P2 Domain** is 3' to the 5' ITR of the rAAV and 5' to a transgene cassette of the rAAV;

(3) the **P3 Domain** is 3' to the transgene cassette of the rAAV and 5' to a 3' ITR of the rAAV; and

(4) the **P4 Domain** is 3' to the 3' ITR of the rAAV; and

(B) a pharmaceutically acceptable carrier.

[0093] 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**")

[0094] 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.

[0095] 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.

[0096] 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.

[0097] 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

[0098] 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

[0099] 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-E22**:

E1. A recombinantly-modified adeno-associated virus (rAAV) that comprises a Cis-Element in one or more of its **P1**, **P2**, **P3** or **P4 Domain**s, wherein:

(1) the **P1 Domain** is 5' to a 5' ITR of the rAAV;

(2) the **P2 Domain** is 3' to the 5' ITR of the rAAV and 5' to a transgene cassette of the rAAV;

(3) the **P3 Domain** is 3' to the transgene cassette of the rAAV and 5' to a 3' ITR of the rAAV; and

(4) the **P4 Domain** is 3' to the 3' ITR of the rAAV; and

wherein the presence of the Cis-Element causes rAAV-producing cells to produce the rAAV at a higher production titer than would be attained with such rAAV if lacking the Cis-Element.

E2. A pharmaceutical composition that comprises:

(A) a preparation of recombinantly-modified adeno-associated virus (rAAV) that comprise a Cis-Element in one or more of its **P1**, **P2**, **P3** or **P4 Domain**s, wherein:

(1) the **P1 Domain** is 5' to a 5' ITR of the rAAV;

(2) the **P2 Domain** is 3' to the 5' ITR of the rAAV and 5' to a transgene cassette of the rAAV;

(3) the **P3 Domain** is 3' to the transgene cassette of the rAAV and 5' to a 3' ITR of the rAAV; and

(4) the **P4 Domain** is 3' to the 3' ITR of the rAAV; and

(B) a pharmaceutically acceptable carrier.

E3. A method for increasing the production titer of recombinantly-modified adeno-associated virus (rAAV), wherein the method comprises:

(A) employing, as the rAAV for producing the production titer, an rAAV that has been modified to comprise an added Cis-Element in one or more of its **P1**, **P2**, **P3** or **P4 Domain**s, wherein:

(1) the **P1 Domain** is 5' to a 5' ITR of the rAAV;

(2) the **P2 Domain** is 3' to the 5' ITR of the rAAV and 5' to a transgene cassette of the rAAV;

(3) the **P3 Domain** is 3' to the transgene cassette of the rAAV and 5' to a 3' ITR of the rAAV; and

(4) the **P4 Domain** is 3' to the 3' ITR of the rAAV; and

(B) culturing cells that have been transfected with the employed rAAV, wherein the cells additionally contain an AAV helper function-providing polynucleotide and a non-AAV helper function-providing

polynucleotide, and wherein the culturing is conducted in a culture medium under conditions sufficient to permit the production of rAAV; wherein the presence of the Cis-Element in the employed rAAV causes the cells to produce the rAAV at an increased production titer relative to that which would be attained if the employed rAAV had lacked the Cis-Element.

E4. The recombinantly-modified adeno-associated virus (rAAV) of **E1**, the pharmaceutical composition of **E2**, or the method of **E3**, wherein the employed rAAV has been modified to comprise an added Cis-Element in its **P1 Domain**.

E5. The recombinantly-modified adeno-associated virus (rAAV) of **E1**, the pharmaceutical composition of **E2**, or the method of **E3**, wherein the employed rAAV has been modified to comprise an added Cis-Element in its **P2 Domain**.

E6. The recombinantly-modified adeno-associated virus (rAAV) of **E1**, the pharmaceutical composition of **E2**, or the method of **E3**, wherein the employed rAAV has been modified to comprise an added Cis-Element in its **P3 Domain**.

E7. The recombinantly-modified adeno-associated virus (rAAV) of **E1**, the pharmaceutical composition of **E2**, or the method of **E3**, wherein the employed rAAV has been modified to comprise an added Cis-Element in its **P4 Domain**.

E8. The recombinantly-modified adeno-associated virus (rAAV) of **E1**, the pharmaceutical composition of **E2**, or the method of **E3**, wherein the employed rAAV has been modified to comprise an added Cis-Element in its **P1 Domain** and in one or more of its **P2**, **P3** or **P4 Domain**.

E9. The recombinantly-modified adeno-associated virus (rAAV) of **E1**, the pharmaceutical composition of **E2**, or the method of **E3**, wherein the employed rAAV has been modified to comprise an added Cis-Element in its **P2 Domain** and in one or more of its **P3** or **P4 Domain**.

E10. The recombinantly-modified adeno-associated virus (rAAV) of **E1**, the pharmaceutical composition of **E2**, or the method of **E3**, wherein the employed rAAV has been modified to comprise an added Cis-Element in its **P3 Domain** and in its **P4 Domain**.

E11. The recombinantly-modified adeno-associated virus (rAAV) of any one of **E1** or **E4-E10**, the pharmaceutical composition of any one of **E2** or **E4-E10**, or the

method of any one of **E3-E10**, wherein the added Cis-Element forms a G-Quadruplex Structure in the employed rAAV.

E12. The recombinantly-modified adeno-associated virus (rAAV) of any one of **E1** or **E4-E11**, the pharmaceutical composition of any one of **E2** or **E4-E11**, or the method of any one of **E3-E11**, wherein an added Cis-Element is selected from the group consisting of:

(1) a Potential G-Quadruplex Sequence of a wild type AAV genome or a Potential G-Quadruplex Sequence of a wild type AAV genome in a reversed orientation;

(2) an Actual G-Quadruplex Sequence of a wild type AAV genome or an Actual G-Quadruplex Sequence of a wild type AAV genome in a reversed orientation;

(3) a DNA sequence from wild-type AAV or a DNA sequence from wild-type AAV in a reversed orientation; and

(4) a DNA sequence from another viral genome or a DNA sequence from another viral genome in a reversed orientation.

E13. The recombinantly-modified adeno-associated virus (rAAV) of any one of **E1** or **E4-E12**, the pharmaceutical composition of any one of **E2** or **E4-E12**, or the method of any one of **E3-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. The recombinantly-modified adeno-associated virus (rAAV) of any one of **E1** or **E4-E13**, the pharmaceutical composition of any one of **E2** or **E4-E13**, or the method of any one of **E3-E13**, wherein the employed rAAV belongs to the rAAV1, rAAV2, rAAV5, rAAV6, rAAV7, rAAV8, rAAV9 or rAAV10 serotype, or to a hybrid of the serotypes.

E15. The recombinantly-modified adeno-associated virus, the pharmaceutical composition, or the method of **E14**, wherein the employed rAAV belongs to the rAAV2, rAAV5, or rAAV9 serotype, or to a hybrid of the serotypes.

E16. The recombinantly-modified adeno-associated virus (rAAV) of any one of **E1** or **E4-E15**, or the method of any one of **E3-E15**, wherein the cells are human embryonic kidney cells.

E17. The recombinantly-modified adeno-associated virus (rAAV) of **E16**, or the method of **E16**, wherein the cells are human embryonic kidney cells.

E18. The recombinantly-modified adeno-associated virus (rAAV) of **E17**, or the method of **E17**, wherein the are HEK293 cells.

E19. The recombinantly-modified adeno-associated virus (rAAV) of **E16**, or the method of **E16**, wherein the cells are baby hamster kidney cells.

E20. The recombinantly-modified adeno-associated virus (rAAV) of **E19**, or the method of **E19**, wherein the are BHK21 cells.

E21. The recombinantly-modified adeno-associated virus (rAAV) of **E16**, or the method of **E16**, wherein the cells are sf9 insect cells.

E22. The preparation of recombinantly-modified adeno-associated virus (rAAV) of **E14**, or the pharmaceutical composition of **E15**, wherein the transgene cassette encodes a protein, or comprises a transcribed nucleic acid, that is therapeutic for a genetic or heritable disease or condition, for use in the treatment of the genetic or heritable disease or condition.

EXAMPLES

[00100] 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 rAAV Plasmid Vectors Having a Cis-Element within the rAAV Plasmid Vector's P2 Domain

[00101] In order to demonstrate the ability of the Cis-Elements of the present invention to increase rAAV production titers, the parent rAAV plasmid vector **pAV-TBG-EGFP** was modified to contain a cis element within its **P2 Domain**.

[00102] A series of 27 derivatives of plasmid **pAV-TBG-EGFP** were constructed by inserting one of Cis-Element CisE1-CisE27 (**Table 1**) into the **SpeI** site of the plasmid that is located within the plasmid's **P2 Domain** (**Figure** 7; **Figure 9A**). The production titers of rAAV obtained using the derivative plasmids in a triple plasmid transfection system (**Figure 2**) with the AAV helper plasmid **pRC2** providing the AAV ***rep*** and ***cap***

functions and the Ad helper plasmid **pHelper** that provided the required adenoviral functions were measured and compared to those obtained with the parental **pAV-TBG-EGFP** plasmid.

[00103] The results of the investigation are shown in **Figure 9B**, and indicate that all of the Cis-Elements introduced within the **P2 Domain** of the rAAV plasmid vector **pAV-TBG-EGFP** resulted in an increase in rAAV production titer. The insertion of CisE19 into the **P2 Domain** of the rAAV plasmid vector was found to mediate a 2.5-fold increase in production titer. The insertion of CisE26 into the **P2 Domain** of the rAAV plasmid vector was found to mediate a 4-fold increase in production titer.

Example 2
Comparison of rAAV Production Titers by Cells Transfected with rAAV Plasmid Vectors Having a Cis-Element within the rAAV Plasmid Vector's P1 Domain

[00104] In order to further demonstrate the ability of the Cis-Elements of the present invention to increase rAAV production titers, the rAAV plasmid vector, **pAV-TBG-EGFP** was modified to contain a Cis-Element within the plasmid's **P1 Domain**. The effect of that modification on rAAV titer was then assessed as described above.

[00105] More specifically, a series of 7 derivatives of rAAV plasmid vector **pAV-TBG-EGFP** were constructed by inserting one of Cis-Element CisE1, CisE20, CisE21, CisE27, CisE28, CisE29, or CisE30 (**Table 1**) into the **PciI** site of the plasmid that is located within the plasmid's **P1 Domain** (**Figure 7**; **Figure 10A**). The production titers of rAAV were obtained essentially as described in **Example 1** using a triple plasmid transfection system (**Figure 2**) with an AAV helper plasmid providing the AAV ***rep*** and ***cap*** functions (**pHelper-Kan**) and an Ad helper plasmid that provided the required adenoviral functions (**pRC2**). The production titers were compared with those obtained with the parental **pAV-TBG-EGFP** plasmid. The results of the investigation are shown in **Figure 10B**, and indicate that all of the Cis-Elements introduced within the **P1 Domain** of **pAV-TBG-EGFP** resulted in an increase in rAAV production titer. The insertion of CisE30 within the **P1 Domain** of the rAAV plasmid vector was found to mediate a 3.0-fold increase in production titer.

Example 3
Comparison of rAAV Production Titers by Cells Transfected with rAAV Plasmid Vectors Having a Cis-Element within Either the rAAV Plasmid Vector's P1 Domain or P2 Domain

[00106] The effect on rAAV production titers of inserting the same Cis-Element either within the **P1 Domain** of an rAAV plasmid vector or within the **P2 Domain** of such rAAV plasmid vector was investigated by introducing Cis-Element CisE1, CisE20 or CisE21 into either the **P1 Domain** of the rAAV plasmid vector **pAV-TBG-EGFP** or within the **P2 Domain** of plasmid vector **pAV-TBG-EGFP** (**Figure 11A**), essentially as described above.

[00107] The production titers of rAAV were obtained essentially as described in **Example 1** using a triple plasmid transfection system (**Figure 2**) with an AAV helper plasmid providing the AAV ***rep*** and ***cap*** functions (**pHelper**) and an Ad helper plasmid that provided the required adenoviral functions (**pRC2**). The results of the investigation are shown in **Figure 11B**, and indicate that positioning a Cis-Element within either the **P1** or **P2 Domain** of an rAAV plasmid vector resulted in an increase in rAAV production titer.

Example 4
Effect of Orientation of Cis-Elements in an rAAV Plasmid Vector on rAAV Yield Produced by Transfected Cells

[00108] The effect of the orientation of a Cis-Element in an rAAV plasmid vector on rAAV production titers was investigated by inserting the Cis-Element CisE21 (**Table 1**) within the **P1 Domain** of the rAAV plasmid vector **pAV-TBG-EGFP** in either the "forward" orientation (**SEQ ID NO:45**) or in the "reverse" orientation (**SEQ ID NO:46**) (**Figure 12A**), essentially as described above.

[00109] The production titers of rAAV were obtained essentially as described in **Example 1** using a triple plasmid transfection system (**Figure 2**) with an AAV helper plasmid (**pRC2**) providing the AAV ***rep*** and ***cap*** functions and an Ad helper plasmid (**pHelper**) that provided the required adenoviral functions. The results of the investigation are shown in **Figure 12B**, and indicate that an increased rAAV production titer was obtained using rAAV plasmid vectors having either orientation of the inserted Cis-Element.

Example 5

Comparison of rAAV Production Titers by Cells Transfected with rAAV Plasmid Vectors Having a Cis-Element within the rAAV Plasmid Vector's P1 Domain and Its P4 Domain

[00110] The effect on rAAV production titers of inserting different Cis-Elements within the **P1 Domain** of an rAAV plasmid vector, while maintaining the same Cis-Element within the **P4 Domain** of such rAAV plasmid vector (**Figure 13A**), was investigated by introducing different Cis-Elements within the **P1 Domain** of an rAAV plasmid vector that contained the same Cis-Element in the **KasI** site that is located within the **P4 Domain** of such rAAV plasmid vector. The production titers of rAAV were obtained essentially as described in **Example 1** using a triple plasmid transfection system (**Figure 2**) with an AAV helper plasmid providing the AAV ***rep*** and ***cap*** functions and an Ad helper plasmid that provided the required adenoviral functions.

[00111] The results of the investigation are shown in **Figure 13B** and **Figure 13C**. **Figure 13B** shows the production titers of rAAV that were obtained using plasmid vector **pAV-TBG-EGFP** that contained Cis-Element CisE21, CisE22, CisE23, CisE24, CisE25, CisE26, CisE28, CisE29, CisE31, CisE32, CisE33, CisE34, or CisE35 within its **P1 Domain** and Cis-Element CisE30-Rev within its **P4 Domain**. **Figure 13C** shows the production titers of rAAV that were obtained using plasmid vector **pAV-TBG-EGFP** that contained Cis-Element CisE21, CisE22, CisE23, CisE24, CisE25, CisE27, CisE28, CisE32, CisE33, or CisE34 within its **P1 Domain** and Cis-Element CisE35-Rev within its **P4 Domain**. The results of the investigation indicate that the presence of two Cis-Elements in the rAAV plasmid vectors synergistically enhanced the increased production titer, relative to that obtained using only a single Cis-Element.

[00112] The effect on rAAV production titers of inserting different Cis-Elements within the **P4 Domain** of an rAAV plasmid vector, while maintaining the same Cis-Element within the **P1 Domain** of such rAAV plasmid vector, was investigated by introducing Cis-Element CisE28 into the **P1 Domain** of the rAAV plasmid vector **pAV-TBG-EGFP**. The plasmid vectors were then further modified to contain Cis-Element CisE22-Rev, CisE27-Rev, CisE29-Rev, or CisE35-Rev within its **P4 Domain** (**Figure 13D**), essentially as described above. An rAAV having a Cis28 Cis-Element

within its **P1 Domain** and a CisE35-Rev Cis-Element within its **P4 Domain** exhibited a 2-3 fold increased production titer relative to that of the parent rAAV.

[00113] 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.

What Is Claimed Is:

Claim 1. A recombinantly-modified adeno-associated virus (rAAV) that comprises a Cis-Element in one or more of its **P1**, **P2**, **P3** or **P4 Domain**s, wherein:

(1) said **P1 Domain** is 5' to a 5' ITR of said rAAV;

(2) said **P2 Domain** is 3' to said 5' ITR of said rAAV and 5' to a transgene cassette of said rAAV;

(3) said **P3 Domain** is 3' to said transgene cassette of said rAAV and 5' to a 3' ITR of said rAAV; and

(4) said **P4 Domain** is 3' to said 3' ITR of said rAAV; and

wherein the presence of said Cis-Element causes rAAV-producing cells to produce said rAAV at a higher production titer than would be attained with such rAAV if lacking said Cis-Element.

Claim 2. The recombinantly-modified adeno-associated virus (rAAV) of claim 1, wherein said employed rAAV has been modified to comprise an added Cis-Element in its **P1 Domain**.

Claim 3. The recombinantly-modified adeno-associated virus (rAAV) of claim 1, wherein said employed rAAV has been modified to comprise an added Cis-Element in its **P2 Domain**.

Claim 4. The recombinantly-modified adeno-associated virus (rAAV) of claim 1, wherein said employed rAAV has been modified to comprise an added Cis-Element in its **P3 Domain**.

Claim 5. The recombinantly-modified adeno-associated virus (rAAV) of claim 1, wherein said employed rAAV has been modified to comprise an added Cis-Element in its **P4 Domain**.

Claim 6. The recombinantly-modified adeno-associated virus (rAAV) of claim 1, wherein said employed rAAV has been modified to comprise an added Cis-Element in its **P1 Domain** and in in one or more of its **P2**, **P3** or **P4 Domain**.

Claim 7. The recombinantly-modified adeno-associated virus (rAAV) of claim 1, wherein said employed rAAV has been modified to comprise an added Cis-Element in its **P2 Domain** and in one or more of its **P3** or **P4 Domain**.

Claim 8. The recombinantly-modified adeno-associated virus (rAAV) of claim 1, wherein said employed rAAV has been modified to comprise an added Cis-Element in its **P3 Domain** and its **P4 Domain**.

Claim 9. The recombinantly-modified adeno-associated virus (rAAV) of claim 1, wherein said added Cis-Element forms a G-Quadruplex Structure in said employed rAAV.

Claim 10. The recombinantly-modified adeno-associated virus (rAAV) of claim 1, wherein an added Cis-Element is selected from the group consisting of:

(1) a Potential G-Quadruplex Sequence of a wild type AAV genome or a Potential G-Quadruplex Sequence of a wild type AAV genome in a reversed orientation;

(2) an Actual G-Quadruplex Sequence of a wild type AAV genome or an Actual G-Quadruplex Sequence of a wild type AAV genome in a reversed orientation;

(3) a DNA sequence from wild-type AAV or a DNA sequence from wild-type AAV in a reversed orientation; and

(4) a DNA sequence from another viral genome or a DNA sequence from another viral genome in a reversed orientation.

Claim 11. The recombinantly-modified adeno-associated virus (rAAV) of claim 1, wherein said transgene cassette encodes a protein, or comprises a transcribed nucleic acid, that is therapeutic for a genetic or heritable disease or condition.

Claim 12. The recombinantly-modified adeno-associated virus (rAAV) of claim 1, wherein said employed rAAV belongs to the rAAV1, rAAV2, rAAV5, rAAV6, rAAV7, rAAV8, rAAV9 or rAAV10 serotype, or to a hybrid of said serotypes.

Claim 13. A pharmaceutical composition that comprises:

(A) the recombinantly-modified adeno-associated virus (rAAV) of claim 1; and

(B) a pharmaceutically acceptable carrier.

Claim 14. The pharmaceutical composition of claim 13, wherein said employed rAAV belongs to the rAAV1, rAAV2, rAAV5, rAAV6, rAAV7, rAAV8, rAAV9 or rAAV10 serotype, or to a hybrid of said serotypes.

Claim 15. A method for increasing the production titer of recombinantly-modified adeno-associated virus (rAAV), wherein said method comprises:

(A) employing, as said rAAV for producing said production titer, an rAAV that has been modified to comprise an added Cis-Element in one or more of its **P1**, **P2**, **P3** or **P4 Domain**s, wherein:

(1) said **P1 Domain** is 5' to a 5' ITR of said rAAV;

(2) said **P2 Domain** is 3' to said 5' ITR of said rAAV and 5' to a transgene cassette of said rAAV;

(3) said **P3 Domain** is 3' to said transgene cassette of said rAAV and 5' to a 3' ITR of said rAAV; and

(4) said **P4 Domain** is 3' to said 3' ITR of said rAAV; and

(B) culturing cells that have been transfected with said employed rAAV, wherein said cells additionally contain an AAV helper function-providing polynucleotide and a non-AAV helper function-providing polynucleotide, and wherein said culturing is conducted in a culture medium under conditions sufficient to permit the production of rAAV;

wherein the presence of said Cis-Element in said employed rAAV causes said cells to produce said rAAV at an increased production titer relative to that which would be attained if said employed rAAV had lacked said Cis-Element.

Claim 16. The method of claim 15, wherein said cells are human embryonic kidney cells or baby hamster kidney cells.

Claim 17. The method of claim 16, wherein said cells are HEK293 human embryonic kidney cells.

Claim 18. The method of claim 16, wherein said cells are BHK21 baby hamster kidney cells.

Claim 19. The method of claim 15, wherein said cells are sf9 insect cells.

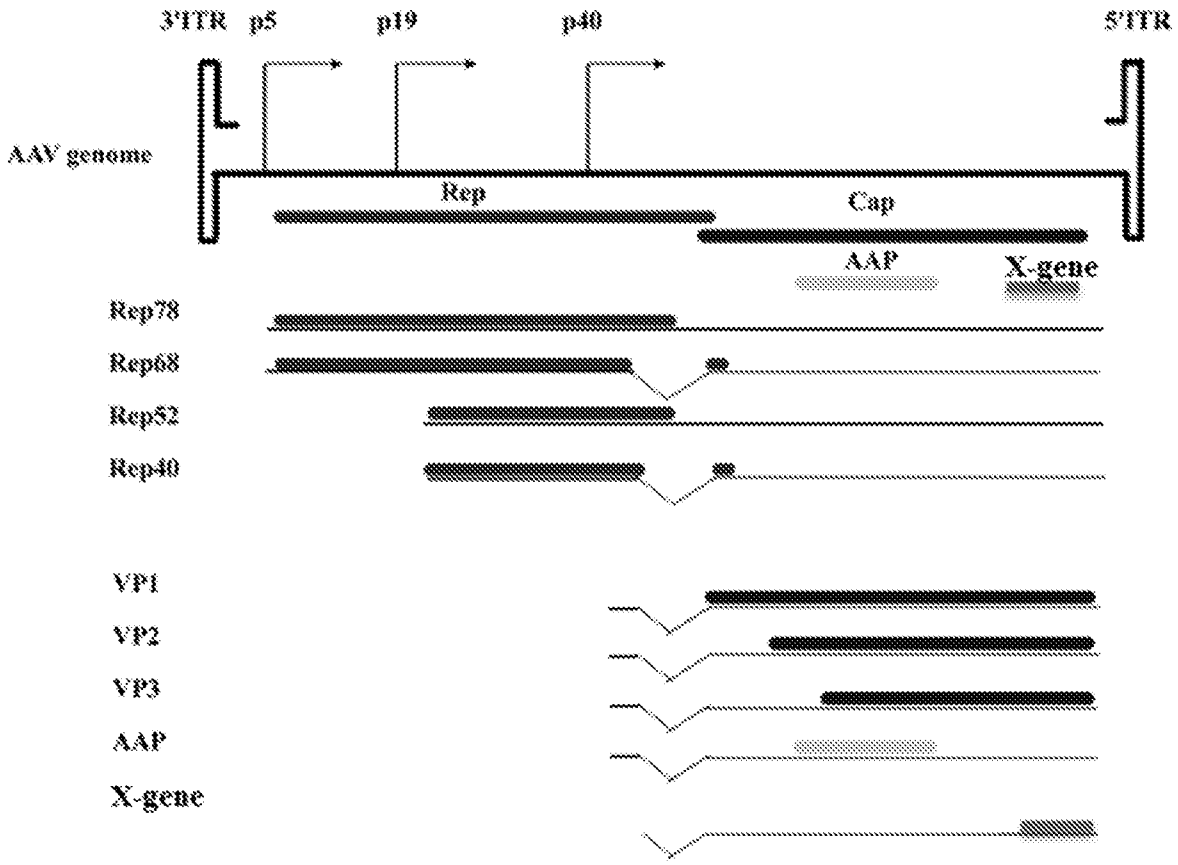


Figure 1

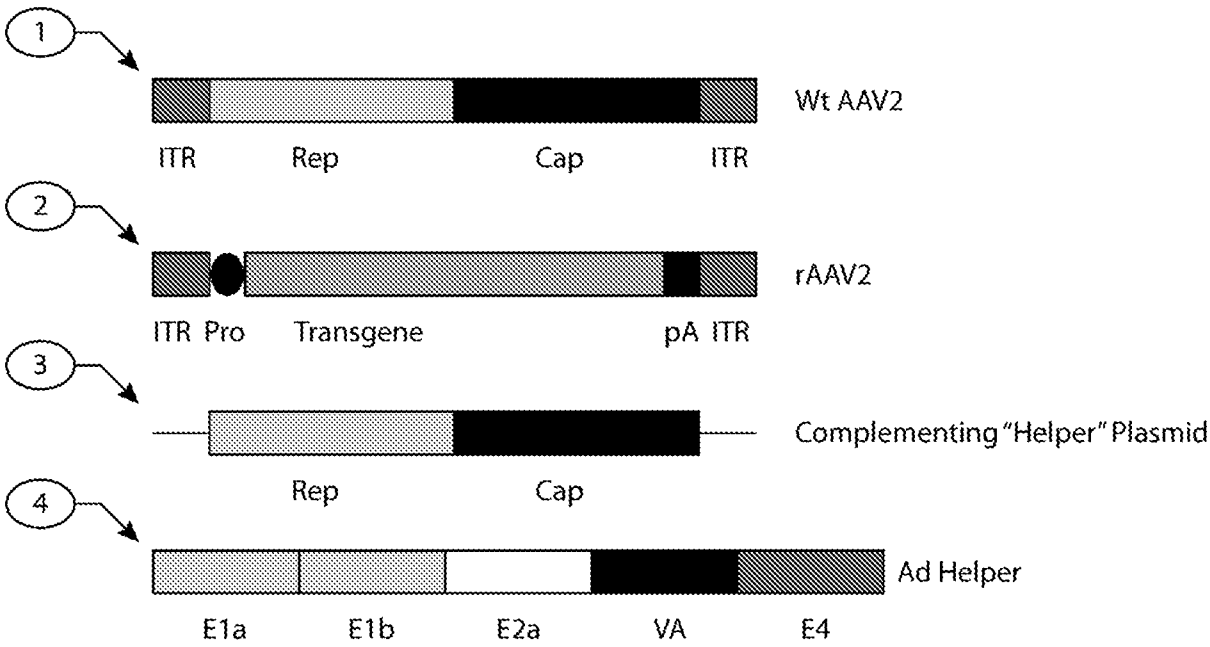


Figure 2

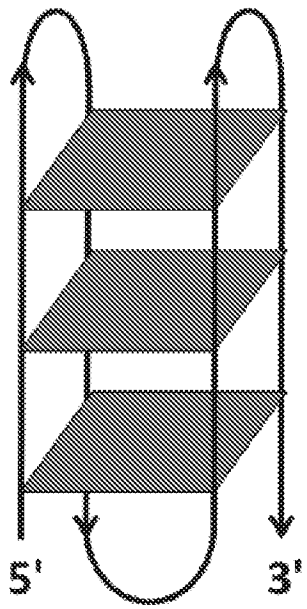


Figure 3A

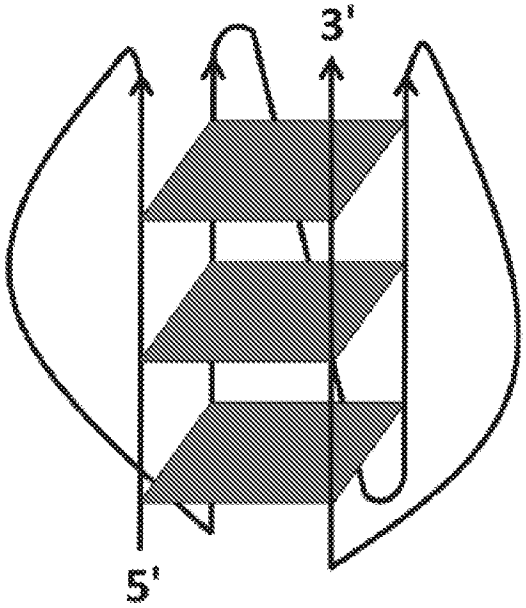


Figure 3B

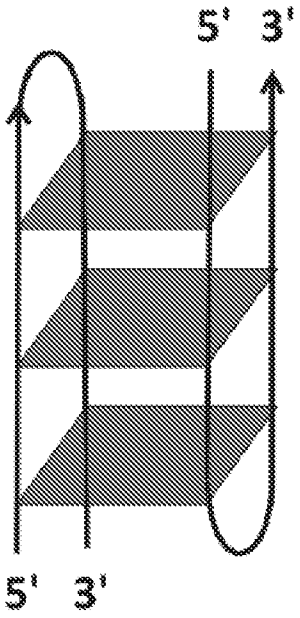


Figure 3C

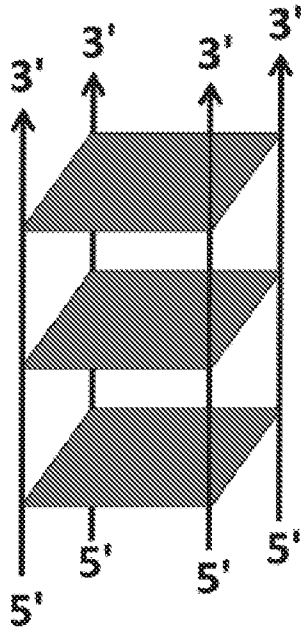
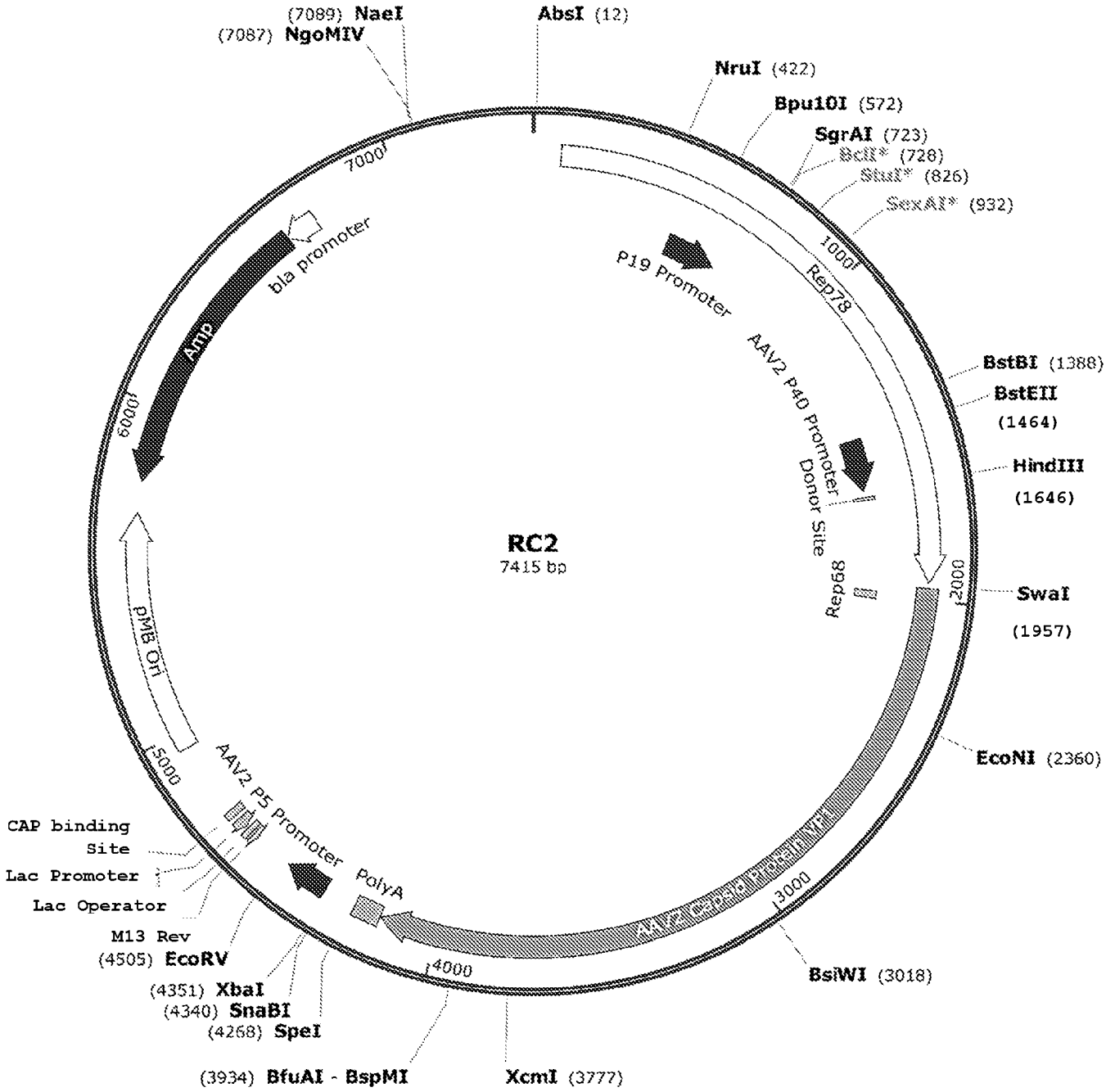


Figure 3D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(1957)
EcoNI (2360)
BsiWI (3018)
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M13 Rev
Lac Operator
Lac Promoter
CAP binding
Site
AAV2 P5 Promoter
PolyA
pMB Ori
bla promoter
Amp
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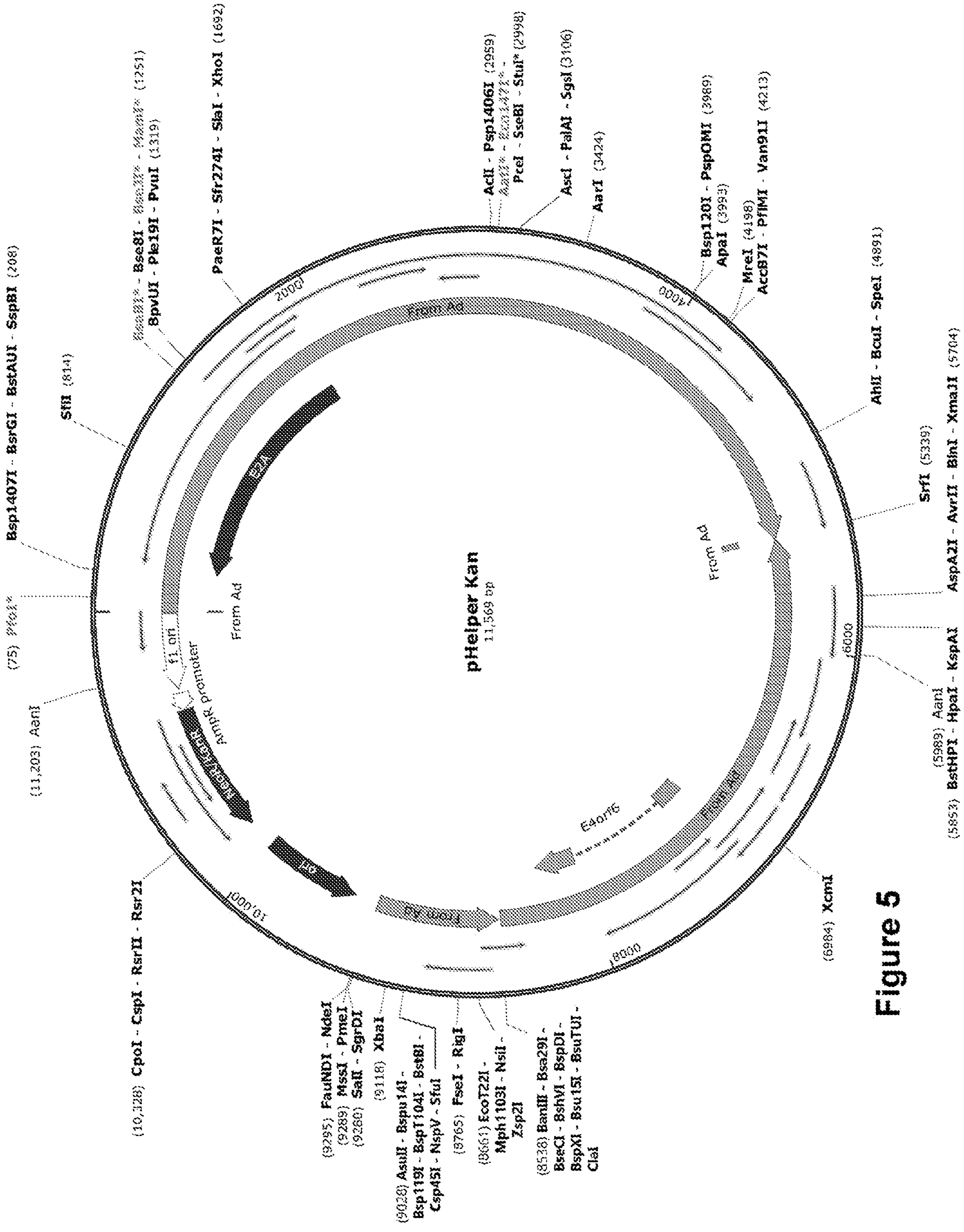
Figure 4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PceI - SseBI - StuI* (2998)
AscI - PalAI - SgsI (3106)
AarI (3424)
Bsp120I - PspOMI (3989)
ApaI (3993)
MreI (4198)
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SrfI (5339)
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(75) PfoI*
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Figure 5

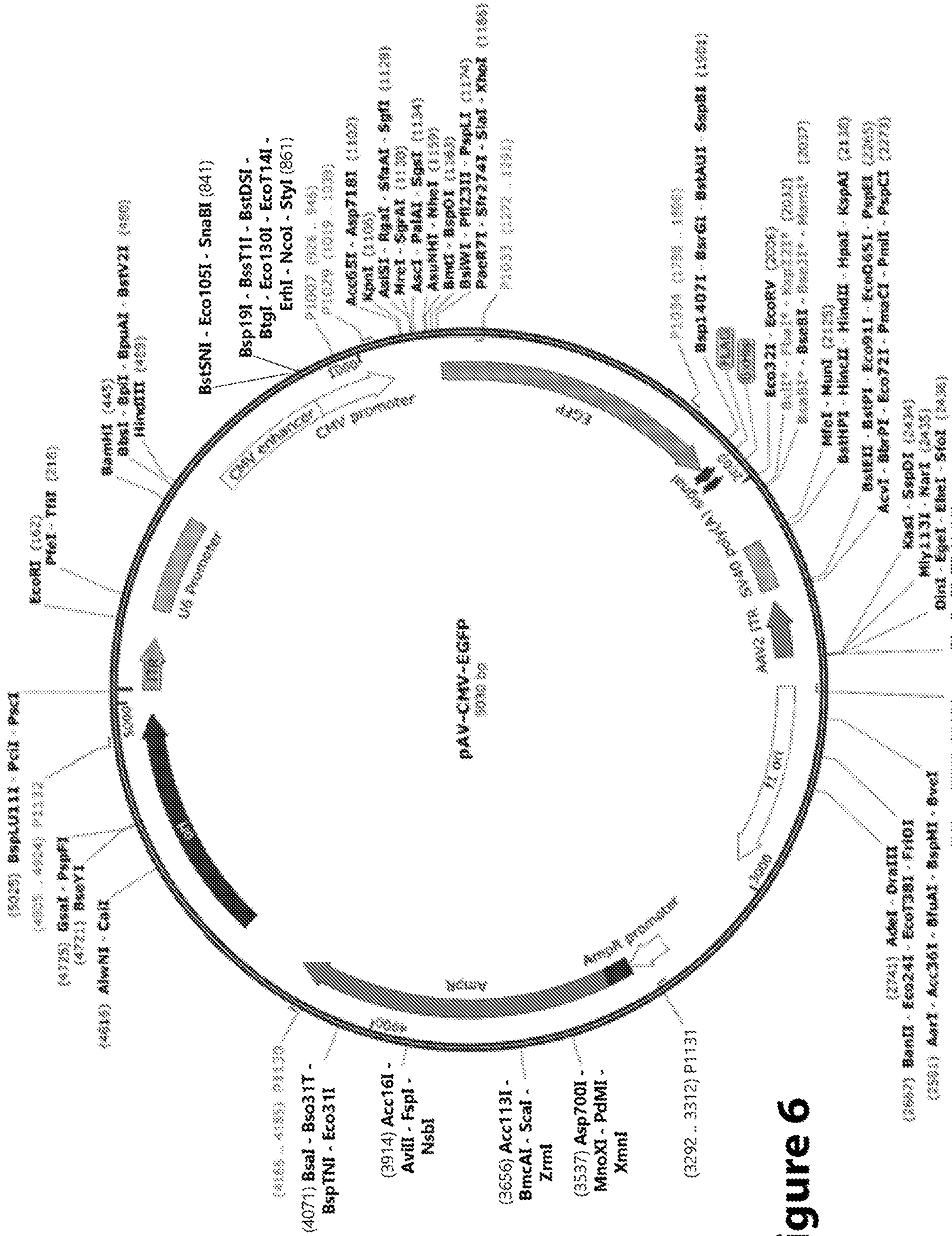
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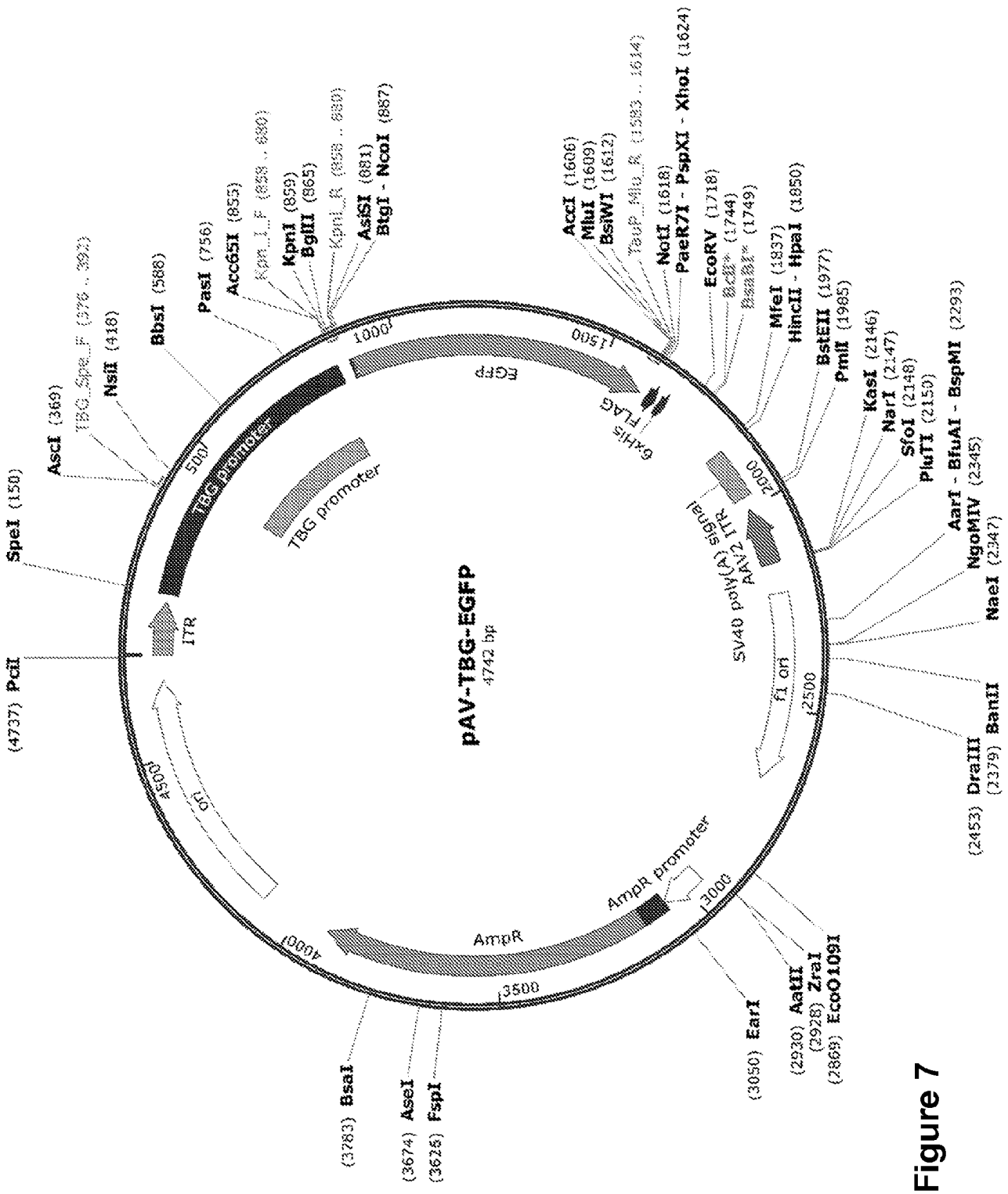
Figure 6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BsiWI (1612)
NotI (1618)
PaeR7I - PspXI - XhoI (1624)
EcoRV (1718)
BsaBI* (1744)
BsaAI* (1749)
MfeI (1837)
HincII - HpaI (1850)
BstEII (1977)
PmlI (1985)
KasI (2146)
NarI (2147)
SfoI (2148)
PluTI (2159)
AarI - BfuAI - BspMI (2293)
NgoMIV (2345)
NaeI (2347)
(2379) BanII
(2453) DraIII
(2869) EcoO109I
(2928) ZraI
(2930) AatII
(3050) EarI
(3626) FspI
(3674) AseI
(3783) BsaI

Figure 7

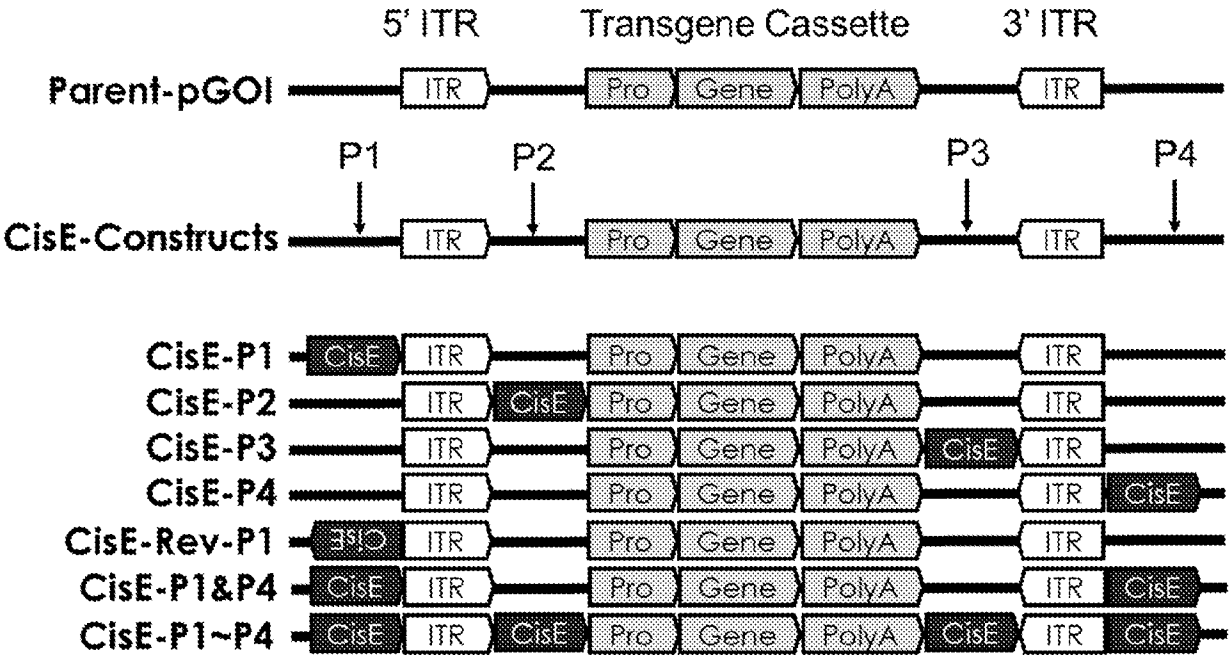

Figure 8

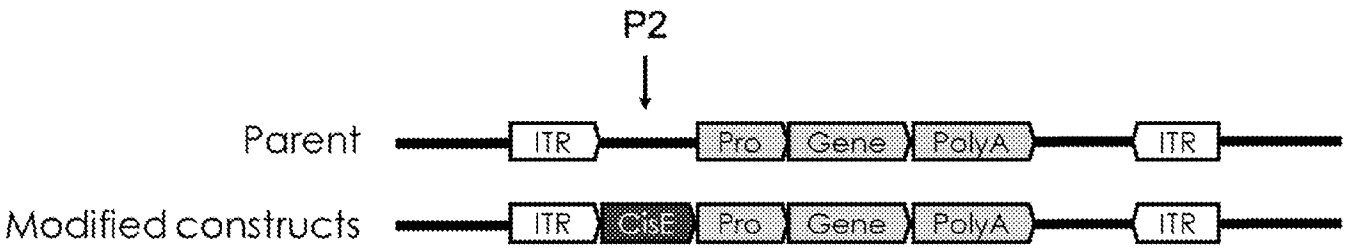

Figure 9A

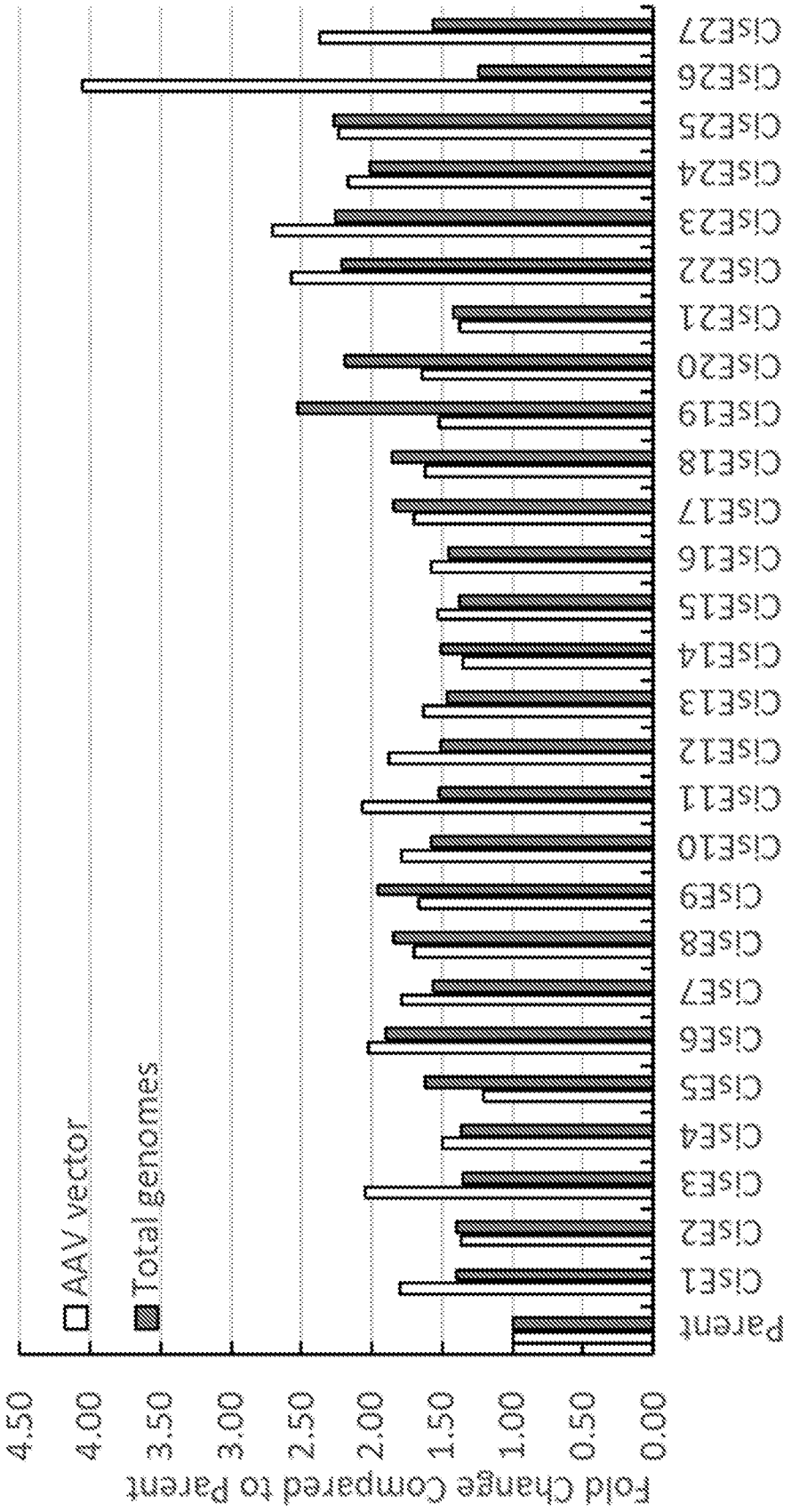
Fold Change Compared to Parent
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
AAV vector
Total genomes
Parent
CisE1
CisE2
CisE3
CisE4
CisE5
CisE6
CisE7
CisE8
CisE9
CisE10
CisE11
CisE12
CisE13
CisE14
CisE15
CisE16
CisE17
CisE18
CisE19
CisE20
CisE21
CisE22
CisE23
CisE24
CisE25
CisE26
CisE27

Figure 9B

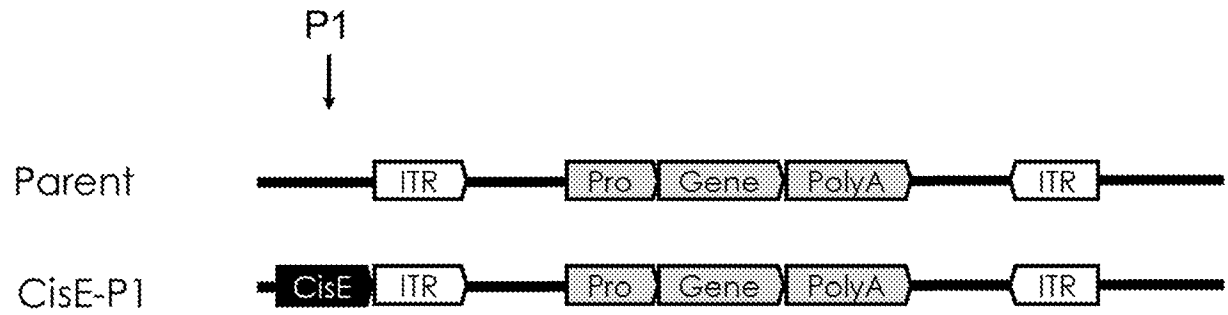

Figure 10A

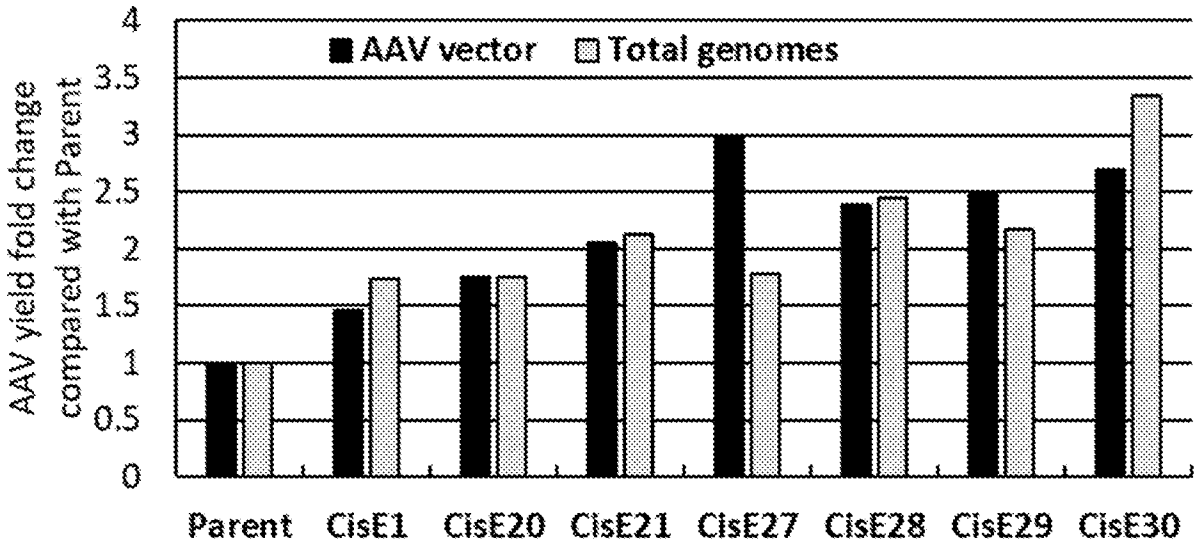

Figure 10B

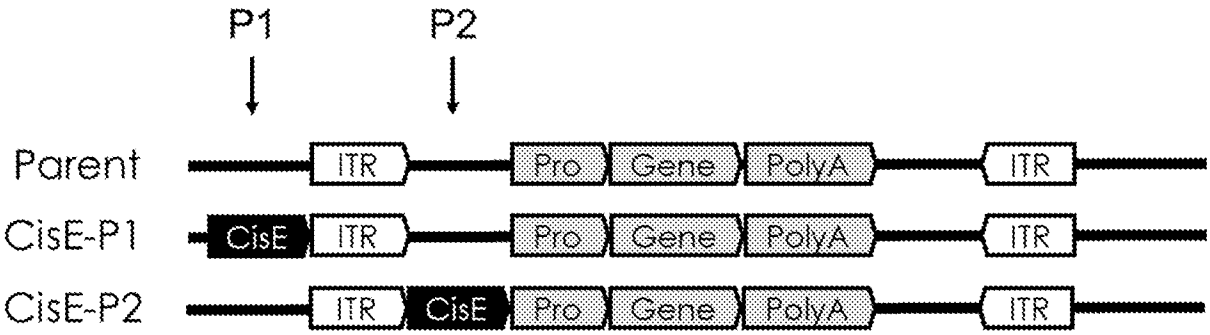

Figure 11A

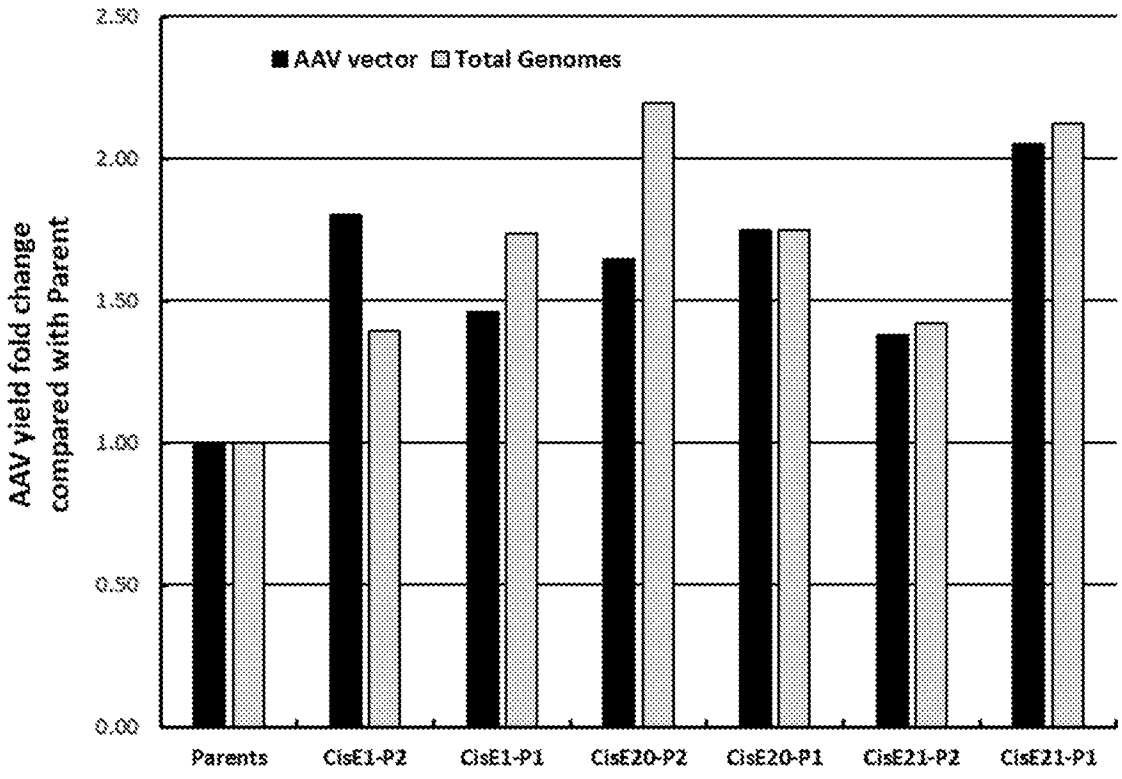

Figure 11B

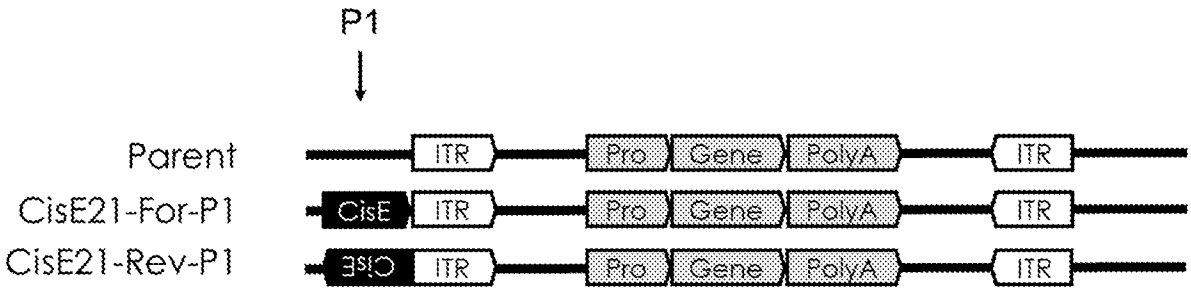

Figure 12A

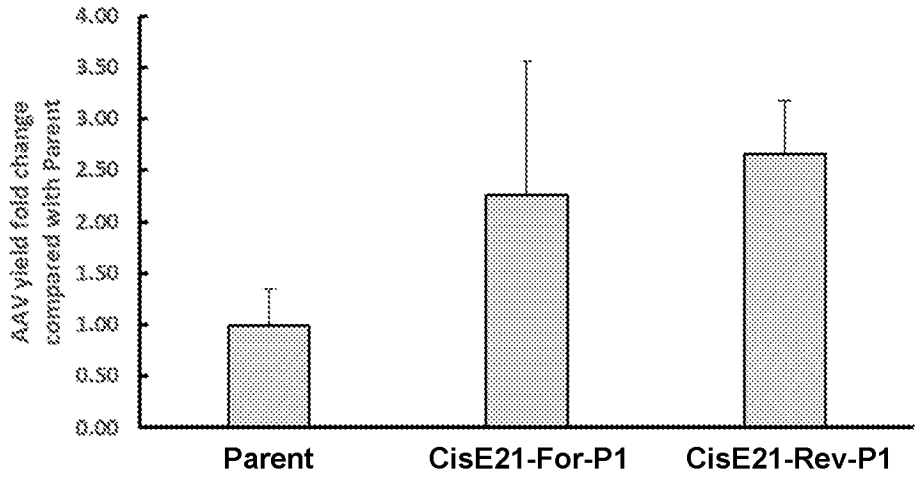

Figure 12B

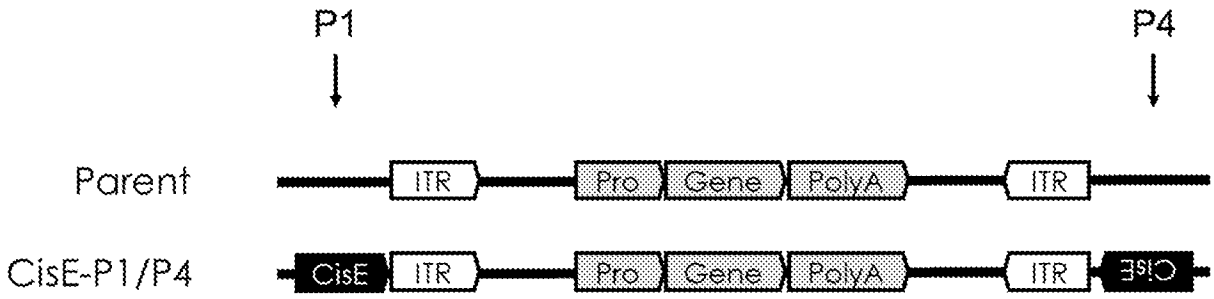

Figure 13A

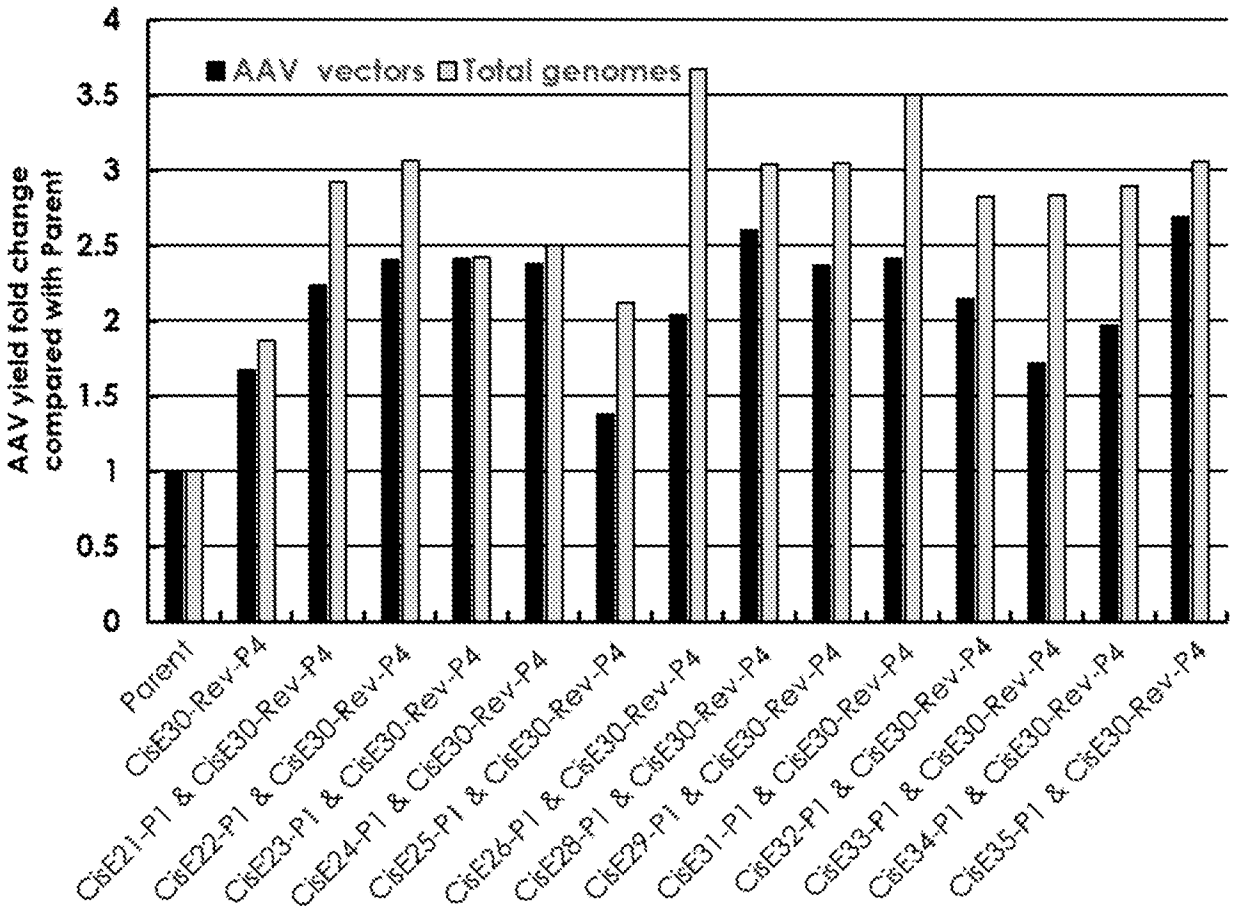

Figure 13B

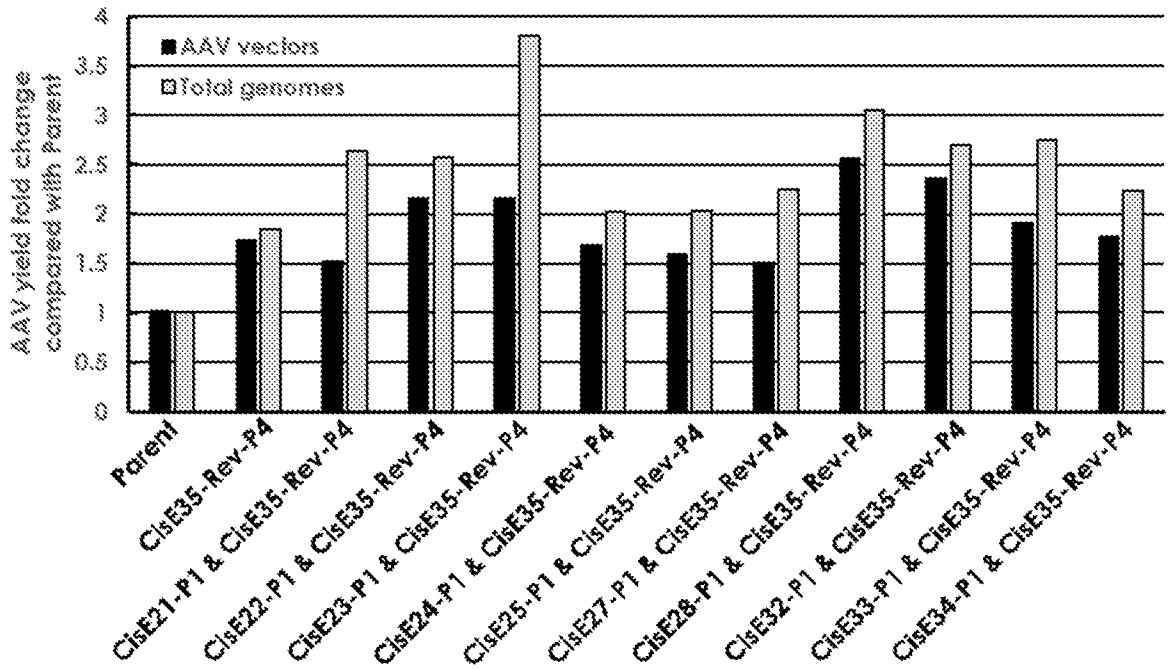

Figure 13C

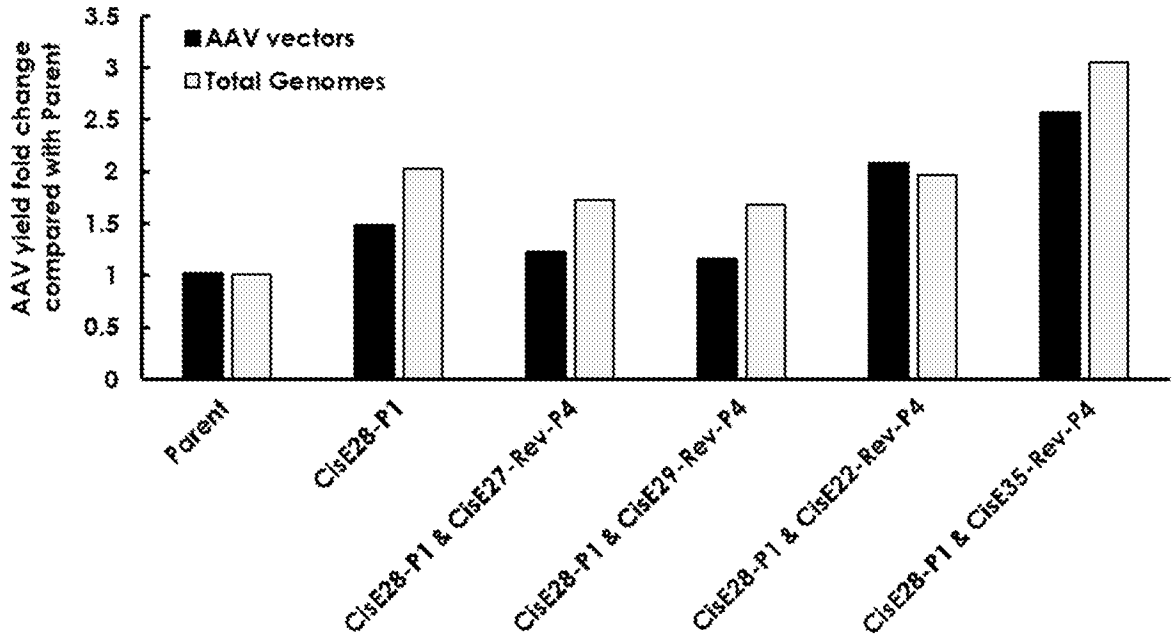

Figure 13D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US20/22326

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12N 15/861; A61K 35/761; C07K 14/075; A61K 48/00; C12N 15/69 (2020.01)

CPC - C12N 15/861; A61K 35/761; C07K 14/075; A61K 48/0058; C12N 15/69

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2006/0205079 A1 (LYNCH, CM et al.) 14 September 2006; paragraphs [0037], [0039], [0047], [0048], [0049], [0110], [0111]	1-19
A	US 2011/0151434 A1 (GAO, G et al.) 23 June 2011; paragraphs [0017], [0042], [0072], [0075], [0111], [0172], [0219]	1-19
A	(XU, ZX et al.) A 16-bp RBE element mediated Rep-dependent site-specific integration in AAVS1 transgenic mice for expression of hFIX. Gene Therapy. May 2009, Epub 26 February 2009, Vol. 16, No. 5; pages 589-595; page 589, 1st column, 1st to 2nd paragraphs; page 590, 2nd column, 3rd paragraph; DOI: 10.1038/gt.2009.9	1-14
A	(CONWAY, JE et al.) High-titer recombinant adeno-associated virus production utilizing a recombinant herpes simplex virus type I vector expressing AAV-2 Rep and Cap. Gene Therapy. June 1999, Vol. 6, No. 6; pages 986-993; page 987, 1st column, 4th paragraph; page 991, 2nd column, 3rd paragraph; page 992, 1st column, 2nd paragraph; DOI: 10.1038/sj.gt.3300937	15-19

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 June 2020 (01.06.2020)	17 JUN 2020
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas Telephone No. PCT Helpdesk: 571-272-4300