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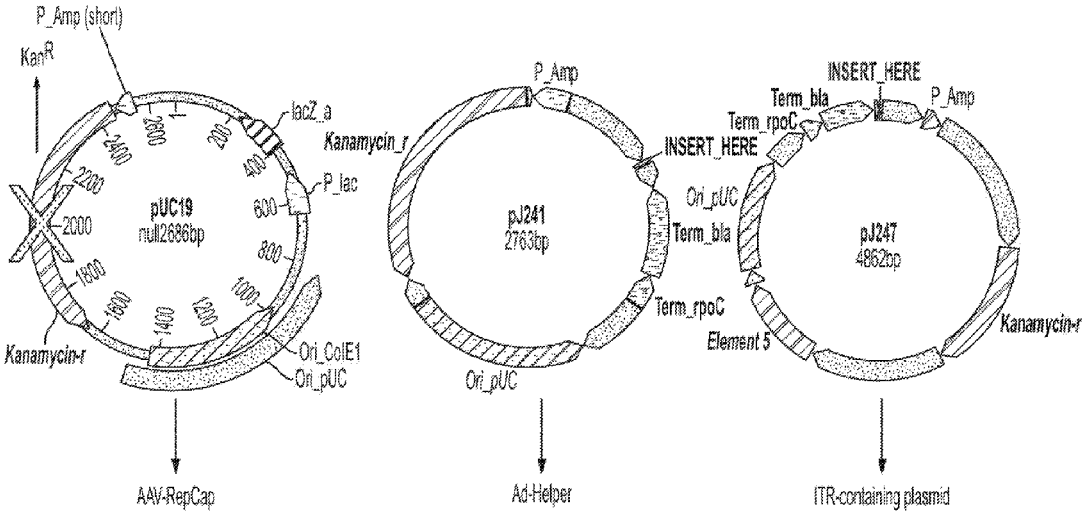

Figure 1

(57) Abstract: A triple-plasmid system for producing recombinant adeno-associated viruses is disclosed. In one aspect, the invention is directed to a plasmid system for Recombinant Adeno-Associated Viral Vector (rAAV) production comprising: (i) a transgene-containing plasmid comprising at least one heterologous nucleic acid sequence flanked by a 5' and 3' AAV inverted terminal repeat (ITR) and a stuffer sequence outside of the ITRs; (ii) a plasmid comprising AAV replication (Rep) and capsid (Cap) gene sequences; and (iii) an adenovirus (Ad) helper plasmid.

[Continued on next page]

WO 2020/086881 A1

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AAV TRIPLE-PLASMID SYSTEM

CROSS-REFERENCE

[0001] This application claims priority U.S. Provisional Patent Application No. 62/750,603, filed October 25, 2018, which is incorporated herein by reference in its entirety.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on October 22, 2019, is named 250478_001858_SL.txt and is 274,165 bytes in size.

BACKGROUND

[0003] Adeno-associated virus (AAV) is a DNA parvovirus that infects humans and various other animal species such as primates, bovine, feline, and canines. It belongs to the family *Parvoviridae* and is placed in the genus *Dependovirus*, because productive infection by AAV occurs only in the presence of a helper virus (e.g., adenovirus or herpes virus). This small non-enveloped virus contains a 4.6 kbases single stranded DNA genome that encodes sets of replication (Rep) and capsid (Cap) proteins. For example, Rep proteins (Rep78, Rep68, Rep52 and Rep40) are involved in replication, rescue and integration of the AAV genome, and Cap proteins (VP1, VP2 and VP3) provides structural function and form the virion capsid. Flanking the Rep and Cap open reading frames at the 5' and 3' ends are 145 bp inverted terminal repeats (ITRs). The ITRs function in cis as origins of nucleic acid replication and as packaging signals for the virus.

[0004] There are two stages to the AAV life cycle once infection has occurred: 1) the lytic stage and 2) the lysogenic stage. With the aid of the helper virus, the lytic stage begins. During this stage AAV commences productive infection resulting in genome replication, viral gene expression, and virion production. The case of the adenoviral helper, the adenoviral proteins that provide helper functions regarding AAV expression include E1a, E1b, E2a, E4, and VA RNA. The adenovirus helps regulate cellular gene expression by providing the proper milieu for AAV productive infection. *See* Daya and Berns *Clinical Microbiology Reviews* Oct 2008, p. 583-593.

[0005] AAV is a versatile virus that can be engineered for gene therapy. Recombinant Adeno-Associated Viral Vector (rAAV), which lacks viral genes in its DNA genome, used of gene therapy is primarily a protein-based nanoparticle engineered to cross the cell membrane in order to traffic

and deliver its DNA cargo into the nucleus of a cell. rAAV DNA genome can form circular concatemers persisting as episomes in the nucleus of a transduced cell. As the rAAV DNA does not integrate into the host genome, which contributes to the long-term gene expression and durability, which is one of the reasons rAAV is ideal for gene therapy.

[0006] Recombinant forms of AAV (rAAV) have been developed as vectors by replacing all viral genes with a therapeutic transgene expression cassette, while retaining the only cis elements, the ITRs, which is required for vector packaging and DNA replication. See, e.g., U.S. Pat. Nos. 4,797,368; 5,153,414; 5,139,941; 5,252,479; and 5,354,678; and International Publication Nos. WO1991/018088; WO1993/024641 and WO1994/13788. Early methods of rAAV production relied on a two-plasmid system comprising: 1) an AAV helper plasmid (generally encompassing AAV Rep and Cap coding regions, while lacking AAV ITRs so it cannot replicate or package itself) and 2) an ITR-containing plasmid (generally encompassing a selected transgene of interest bounded by AAV ITRs which provides for viral replication and packaging functions). Both the helper plasmid and the ITR-containing plasmid bearing the selected gene can be introduced into suitable cells for production by transient transfection. The transfected cell can then be infected with a helper virus, such as an adenovirus or herpes simplex virus, which transactivates the AAV promoters present on the helper plasmid that direct the transcription and translation of AAV Rep and Cap regions. Regarding the Ad helper virus, the E1a, E1b, E2a, E4, and VA RNA genes can supply the helper functions necessary for rAAV production. Infection of helper virus into producer cells to generate rAAV was effective in producing rAAV; however, a consequence is that it can also produce helper virus particles that can elicit immune responses from the host. In certain platforms, the viral helper genes necessary for AAV manufacturing can be stably transfected into the manufacturing cell line (e.g., HEK293 cells), thereby reducing the possibility of an anti-helper virus immune response by the host immune system coming from trace levels of residual helper virus.

[0007] More recently, a triple-plasmid transfection method has been developed. This method uses an AAV serotype-specific Rep and Cap plasmid as well as the transgene-containing plasmid but eliminated the use of helper virus infection by supplying the essential helper viral genes on a third plasmid (i.e., the viral coding sequences were removed or reduced), thus lowering the potential anti-helper virus immune response by the host immune system. Supplying the viral helper genes on the third plasmid greatly decreased helper viral production in the transfected cells, providing

only rAAV. Multiplasmid transient transfection of adherent HEK293 cells is a commonly used method for rAAV production.

[0008] In a multiplasmid system, it is important to maintain an appropriate plasmid size. Thus, it may be important to add nucleic acid sequences (a.k.a "stuffer sequences") to ensure that the plasmid is of an optimal size. For example, to discourage that the plasmid backbone of the ITR-containing plasmid is not packaged into the vector capsid, a stuffer sequence may need to be added such that the backbone is too large to be effectively packaged into the capsid. However, it is important that the stuffer sequence is "silent" and does not activate the immune system in the small chance that that plasmid does become packaged.

[0009] What is needed, therefore, is an improved triple-plasmid based system for producing rAAV. The plasmid system should provide improved transfection and lowered immunogenicity while still retaining optimum expression of the transgene. It is to such a plasmid system that embodiments of the present disclosure are directed.

BRIEF SUMMARY OF THE DISCLOSURE

[0010] As specified in the Background Section, there is a great need in the art to improve rAAV plasmid systems for rAAV-based gene therapies. The present disclosure satisfies this and other needs. Embodiments of the present disclosure relate generally to a plasmid system for the production of rAAV and more specifically to a triple-plasmid based system.

[0011] In one aspect, the invention is directed to a plasmid system for Recombinant Adeno-Associated Viral Vector (rAAV) production comprising: (i) a transgene-containing plasmid comprising at least one heterologous nucleic acid sequenceflanked by a 5' and 3' AAV inverted terminal repeat (ITR) and a stuffer sequence outside of the ITRs; (ii) a plasmid comprising AAV replication (Rep) and capsid (Cap) gene sequences; and (iii) an adenovirus (Ad) helper plasmid.

[0012] In certain embodiments, the stuffer sequence increases the size of the transgene-containing plasmid backbone. In certain embodiments, the stuffer sequence increases the size of the transgene-containing plasmid backbone such that the transgene-containing plasmid backbone is discouraged from being packaged into an rAAV capsid. In certain embodiments, the plasmid backbone incorporation into the rAAV is below the limit of detection. In certain embodiments,

the backbone of the transgene-containing plasmid is larger than a wild-type AAV genome following the addition of the stuffer sequence.

[0013] In certain embodiments, the stuffer sequence is devoid of enhancers, promoters, splicing regulators, noncoding RNAs, antisense sequences, coding sequences, or any combination thereof. In certain embodiments, the stuffer sequence is devoid of enhancers, promoters, splicing regulators, noncoding RNAs, antisense sequences, and coding sequences. In certain embodiments, the stuffer sequence comprises an inert intronic DNA sequence found in the human genome.

[0014] In certain embodiments, the stuffer sequence comprises a nucleic acid sequence of between 1000 and 5000 nucleotides in length or a nucleic acid sequence of between 1000 and 2000 nucleotides in length.

[0015] In certain embodiments, the stuffer sequence comprises GAPDH intron 2, fragment, or mutant thereof. In certain embodiments, the stuffer sequence comprises an inactivated gentamycin gene.

[0016] In certain embodiments, the stuffer sequence comprises a nucleic acid having at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 9. In certain embodiments, the stuffer sequences comprises SEQ ID NO: 9 or a fragment thereof. In certain embodiments, the fragment is between 800-1000 nucleotides long.

[0017] In certain embodiments, the stuffer sequence consists of a nucleic acid having at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 9. In certain embodiments, the stuffer sequences consists of SEQ ID NO: 9 or a fragment thereof. In certain embodiments, the fragment is between 800-1000 nucleotides long.

[0018] In certain embodiments, the transgene-containing plasmid comprises a plasmid with a structure in the same order as Figure 3A, wherein the eGFP and SEAP transgenes can be replaced with the at least one heterologous nucleic acid sequence.

[0019] In certain embodiments, the transgene-containing plasmid comprises a plasmid with a structure in the same order as Figure 3B, wherein the eGFP transgene can be replaced with the at least one heterologous nucleic acid sequence.

[0020] In certain embodiments, the transgene-containing plasmid comprises nucleic acid sequences in the 5′ to 3′ direction of: a 5' ITR (e.g., SEQ ID NOs: 2 or 43), a promoter (e.g., SEQ ID NO: 4), at least one heterologous nucleic acid sequence, a polyA sequence (e.g., SEQ ID NO: 8), a 3' ITR (e.g., SEQ ID NO: 3), and the stuffer sequence (e.g., SEQ ID NO: 9), wherein each nucleic acid sequence can be substituted with or encodes a corresponding functional fragment or derivative thereof or a sequence with at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity therewith.

[0021] In certain embodiments, the transgene-containing plasmid further comprises a DNA titer tag outside the expression cassette but between the 5' ITR and 3' ITR.

[0022] In certain embodiments, the transgene-containing plasmid further comprises a DNA titer tag i) upstream of the 3' ITR and downstream of a polyA sequence or ii) upstream of the 3' ITR and downstream of the at least one heterologous nucleic acid sequence; iii) or downstream of the 5' ITR and upstream of the at least one heterologous nucleic acid sequence; or iv) downstream of the 5' ITR and upstream of a promoter for the at least one heterologous nucleic acid sequence; or v) downstream of the 5' ITR and upstream of the 3' ITR.

[0023] In certain embodiments, the transgene-containing plasmid further comprises a DNA titer tag i) upstream of a 3' ITR (e.g., SEQ ID NO: 3) and downstream of a polyA sequence (e.g., SEQ ID NO: 8) or ii) upstream of a 3' ITR (e.g., SEQ ID NO: 3) and downstream of the at least one heterologous nucleic acid sequence; iii) or downstream of a 5' ITR (e.g., SEQ ID NOs: 2 or 43) and upstream of the at least one heterologous nucleic acid sequence; or iv) downstream of a 5' ITR (e.g., SEQ ID NOs: 2 or 43) and upstream of a promoter (e.g., SEQ ID NO: 4); or v) downstream of a 5' ITR (e.g., SEQ ID NOs: 2 or 43) and upstream of a 3' ITR (e.g., SEQ ID NO: 3).

[0024] In certain embodiments, the transgene-containing plasmid comprises nucleic acid sequences in the 5′ to 3′ direction of: a 5' ITR (e.g., SEQ ID NO: 2 or 43), a promoter (e.g., SEQ ID NO: 4), at least one heterologous nucleic acid sequence, a polyA sequence (e.g., SEQ ID NO: 8), a 3' ITR (e.g., SEQ ID NO: 3), and the stuffer sequence (e.g., SEQ ID NO: 9), wherein each

nucleic acid sequence can be substituted with or encodes a corresponding functional fragment or derivative thereof or a sequence with at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity therewith.

[0025] In certain embodiments, the transgene-containing plasmid further comprises a DNA titer tag outside the expression cassette but between the 5' ITR and 3' ITR.

[0026] In certain embodiments, the transgene-containing plasmid further comprises a DNA titer tag i) upstream of the 3' ITR and downstream of a polyA sequence or ii) upstream of the 3' ITR and downstream of the at least one heterologous nucleic acid sequence; iii) or downstream of the 5' ITR and upstream of the at least one heterologous nucleic acid sequence; or iv) downstream of the 5' ITR and upstream of a promoter for the at least one heterologous nucleic acid sequence; or v) downstream of the 5' ITR and upstream of the 3' ITR.

[0027] In certain embodiments, the transgene-containing plasmid further comprises a DNA titer tag i) upstream of a 3' ITR (e.g., SEQ ID NO: 3) and downstream of a polyA sequence (e.g., SEQ ID NO: 8) or ii) upstream of a 3' ITR (e.g., SEQ ID NO: 3) and downstream of the at least one heterologous nucleic acid sequence; iii) or downstream of a 5' ITR (e.g., SEQ ID NOs: 2 or 43) and upstream of the at least one heterologous nucleic acid sequence; or iv) downstream of a 5' ITR (e.g., SEQ ID NOs: 2 or 43) and upstream of a promoter (e.g., SEQ ID NO: 4); or v) downstream of a 5' ITR (e.g., SEQ ID NOs: 2 or 43) and upstream of a 3' ITR (e.g., SEQ ID NO: 3).

[0028] In certain embodiments, the AAV Rep gene sequence is from AAV serotype 2, 5, 8, 9, or hybrids thereof. In certain embodiments, the AAV Cap gene sequence is from AAV serotype 2, 5, 8, 9, or hybrids thereof. In certain embodiments, the plasmid comprising the Rep and Cap gene sequences further comprises a promoter. In certain embodiments, the promoter is an AAV promoter. In certain embodiments, the promoter is an AAV P5 promoter.

[0029] In certain embodiments, the Ad helper plasmid comprises one or more of Adenovirus genes selected from E1a, E1b, E2a, E4orf6, or VA RNA.

[0030] In certain embodiments, the Ad helper plasmid comprises nucleic acid sequences in the 5′ to 3′ direction of: SEQ ID NOs: 18, 17, 16, and 20, wherein each nucleic acid sequence can be substituted with a corresponding functional fragment or derivative thereof or a sequence with at

least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity therewith.

[0031] In certain embodiments, the Ad helper plasmid comprises nucleic acid sequences in the 5′ to 3′ direction of: SEQ ID NOs: 21, 16, 39, 40, 22, 23, and 20 wherein each nucleic acid sequence can be substituted with or encode a corresponding functional fragment or derivative thereof or a sequence with at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity therewith.

[0032] In certain embodiments, the Ad helper plasmid comprises a structure in the same order as either construct of Figure 5.

[0033] In certain embodiments, the Ad helper plasmid comprises a nucleic acid having at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 14.

[0034] In certain embodiments, the Ad helper plasmid comprises a nucleic acid having at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 15.

[0035] In certain embodiments, the heterologous nucleic acid sequence is a heterologous gene of interest encoding a peptide, polypeptide, or protein. In certain embodiments, the peptide, polypeptide, or protein is an enzyme, antibody, MHC molecule, T-cell receptor, B-cell receptor, aptamer, avimer, receptor-binding ligand, targeting peptides, a therapeutic agent, or gene editing molecule. In certain embodiments, the heterologous nucleic acid sequenceis a nucleic acid sequence such as an antisense, siRNA, shRNA, miRNA, EGSs, gRNA, sgRNA, ribozyme, or aptamer.

[0036] In another aspect, the invention is directed to a host cell comprising any one of the plasmid systems described herein.

[0037] In another aspect, the invention is directed to a rAAV produced by any one of the plasmid systems described herein.

[0038] In another aspect, the invention is directed to a DNA titer tag allowing for universal vector titering, comprising a nucleic acid tag sequence from about 60 nucleotides to about 100 nucleotides long either upstream or downstream from a nucleic acid sequence of a heterologous nucleic acid sequencewithin a transgene-containing plasmid, wherein the nucleic acid tag sequence can be used in at least two different transgene-containing plasmids to allow for universal vector genome titering between at least two different types of AAV vectors. In certain embodiments, the nucleic acid tag sequence is about 100 nucleotides long.

[0039] In certain embodiments, the nucleic acid tag sequence is upstream from a 3' ITR sequence of the transgene-containing plasmid but not within an expression cassette of the transgene-containing plasmid.

[0040] In certain embodiments, the nucleic acid tag sequence is downstream from a 5' ITR sequence of the transgene-containing plasmid but not within an expression cassette of the transgene-containing plasmid.

[0041] In certain embodiments, the DNA titer tag comprises any one of nucleic acid sequences of SEQ ID NOS: 61-70.

[0042] In another aspect, the invention is directed to a method for producing a rAAV comprising transducing a cell with the any one of the plasmid systems described herein and isolating the rAAV. In another aspect, the invention is directed to a rAAV produced by said method.

[0043] In another aspect, the invention is directed to a composition comprising the plasmid system of the invention.

[0044] In another aspect, the invention is directed to a pharmaceutical composition comprising the rAAV produced by the plasmid system of the invention.

[0045] In another aspect, the invention is directed to a method for delivering or transferring a nucleic acid sequence into a subject's cell, comprising administering the rAAV produced by the plasmid system of the invention to a subject thereby delivering the nucleic acid sequence into the cell. In certain embodiments, the subject's cell is in culture or is present in the subject.

[0046] In another aspect, the invention is directed to a method for treating or preventing a disease or disorder in a subject, comprising administering to a subject in need thereof a rAAV produced by the plasmid system of the invention.

[0047] In another aspect, the invention is directed to a host cell comprising contacting the host cell with a rAAV produced by the plasmid system of the invention.

[0048] These and other objects, features and advantages of the present disclosure will become more apparent upon reading the following specification in conjunction with the accompanying description, claims and drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0049] Figure 1 depicts an exemplary triple-plasmid system for the production of rAAV, in accordance with some embodiments of the present disclosure.

[0050] Figure 2 depicts an exemplary transgene-containing plasmid for rAAV production incorporating eGFP and SEAP as transgenes, in accordance with some embodiments of the present disclosure.

[0051] Figures 3A-3B: shows exemplary gene constructs of transgene-containing plasmids for single-stranded (ss) (Figure 3A) and self-complementary (sc) rAAV (Figure 3B) production.

[0052] Figures 4A-4B: Figure 4A depicts an exemplary AAV Rep-Cap plasmid incorporating different AAV Rep and Cap genes, in accordance with some embodiments of the present disclosure. Figure 4B depicts an exemplary AAV Rep-Cap plasmid incorporating a promoter from AAV serotype 2.

[0053] Figure 5 depicts exemplary Ad Helper Plasmids in short (top panel) and long (bottom panel) embodiments.

[0054] Figure 6 is a Western blot showing expression levels of Cap proteins from different AAV serotypes from a plasmid according to the disclosure.

[0055] Figure 7 is a Western blot showing expression levels of Cap proteins from different AAV serotypes from a plasmid according to the disclosure. A Monoclonal B1 clone was used for blot analysis.

[0056] Figure 8 is a Western blot showing AAV P5-driven expression levels of Cap proteins from different AAV serotypes from a plasmid according to the disclosure. - : plasmid constructs without the P5 promoter; + : plasmid constructs with the P5 promoter. A Monoclonal B1 clone was used for blot analysis.

[0057] Figure 9 shows the results of a qPCR assay of viral genome copy number using a short Ad Helper Plasmid according to the disclosure. 1: Negative Control 1 (pHelper+pAAV-RC2 (Agilent)); 2: Negative Control 2 (pHelper+pTRUF11); 3: Positive control (pHelper+pAAV-RC2+pTRUF11); 4: Short Ad Helper Test (Short-Helper (SEQ ID NO: 14)+pAAV-RC2 +pTRUF11).

[0058] Figure 10 shows the results of a qPCR assay of viral genome copy number using a long Ad Helper Plasmid according to the disclosure. 1: Negative Control 1 (pTRUF11+pAAV-RC2 (Rep2Cap2 (Agilent)); 2: Positive Control 2 (pHelper+ pAAV-RC2+pTRUF11); 3: Short Ad Helper Test (Short-Helper (SEQ ID NO: 14)+ pUC19-Rep2Cap8 +pITRss (SEQ ID NO: 1)); 4: Long Ad Helper Test (Long-Helper (SEQ ID NO: 15)+ pUC19-Rep2Cap8+pITRss (SEQ ID NO: 1)).

[0059] Figure 11 shows the viral genome copy number per ml cell lysate for rAAV containing a single-stranded (top panel) or self-complementary (bottom panel) DNA genome produced using the corresponding transgene-containing plasmids for rAAV production. For the top panel: 1: Negative Control (pHelper+AAV-RC2); 2: Positive Control (pHelper+pAAV-RC2+pTRUF11); 3: ssITR (pHelper+pAAV-RC2+ ssITR) (SEQ ID NO: 1). For the bottom panel: 1: Negative Control (pHelper+AAV-RC2); 2: Positive Control (pHelper+pAAV-RC2+pTRUF11); 3: scITR (pHelper+pAAV-RC2+ scITR (SEQ ID NO: 42).

[0060] Figure 12 shows the viral genome copy number per ml cell lysate of multiple capsid serotypes for a triple-plasmid system according to the disclosure, along with positive and negative controls. 1: Negative Control (pHelper+pTRUF11); 2: Positive Control (pHelper+pTRUF11+pAAV-RC2); 3: pHelper+pTRUF11+pUC19-P5-Rep2Cap2 (SEQ ID NO: 31); 4: pHelper+pTRUF11+pUC19-Rep2/5Cap5 (SEQ ID NO: 24); 5: pHelper+pTRUF11+pUC19-P5-Rep2Cap8 (SEQ ID NO: 35); 6: pHelper+pTRUF11+pUC19-P5-Rep2Cap9 (SEQ ID NO: 37); 7: Short-Helper (SEQ ID NO: 14)+ ssITR (SEQ ID NO: 1)+pUC19-P5-Rep2Cap2 (SEQ ID NO: 31); 8: Short-Helper (SEQ ID NO: 14)+ ssITR (SEQ ID NO: 1)+pUC19-Rep2/5Cap5 (SEQ ID NO: 24); 9: Short-Helper (SEQ ID NO: 14)+ ssITR (SEQ ID NO: 1)+pUC19-P5-Rep2Cap8 (SEQ ID NO: 35); 10: Short-Helper (SEQ ID NO: 14)+ ssITR (SEQ ID NO: 1)+pUC19-P5-Rep2Cap9 (SEQ ID NO: 37); 11: Short-Helper (SEQ ID NO: 14)+ ssITR (SEQ ID NO: 1)+pAAV-RC2.

[0061] Figure 13A-13B shows that viral genome copy number per ml lysate for the single strand ITR (ssITR) transgene plasmid (Figure 13A) and self complementary ITR (scITR) plasmid using both SV40 polyA and a 100 nucleotide long DNA titer tag for qPCR analysis.

[0062] Figures 14A-14B: Figure 14A depicts an exemplary AAV Rep-Cap plasmid incorporating different AAV Rep and Cap genes, in accordance with some embodiments of the present disclosure. Figure 14B depicts an exemplary AAV Rep-Cap plasmid incorporating different AAV Rep and Cap genes and incorporating the P5 promoter, in accordance with some embodiments of the present disclosure.

[0063] Figure 15: shows exemplary ene constructs of transgene-containing plasmids for single-stranded (ss) (Figure 15A) and self-complementary (sc) rAAV (Figure 15B) production. Both modified plasmids were containing improved plasmid backbones with higher developability.

[0064] Figure 16: shows that viral genome copy number per ml lysate for the modified single strand ITR (ssITR) transgene plasmid (Figure 16A) and modified self-complementary ITR (scITR) plasmid using a 100 nucleotide long DNA titer tag for qPCR analysis.

DETAILED DESCRIPTION OF THE DISCLOSURE

[0065] As specified in the Background section, there is a great need in the art to identify technologies for rAAV production to generate rAAV-based gene therapies. The present disclosure satisfies this and other needs. Embodiments of the present disclosure relate generally to a rAAV production and more specifically to a triple-plasmid based system to produce rAAV.

[0066] To facilitate an understanding of the principles and features of the various embodiments of the disclosure, various illustrative embodiments are explained below. Although exemplary embodiments of the disclosure are explained in detail, it is to be understood that other embodiments are contemplated. Accordingly, it is not intended that the disclosure is limited in its scope to the details of construction and arrangement of components set forth in the following description or examples. The disclosure is capable of other embodiments and of being practiced or carried out in various ways. Also, in describing the exemplary embodiments, specific terminology will be resorted to for the sake of clarity.

[0067] It is intended that each term contemplates its broadest meaning as understood by those skilled in the art and includes all technical equivalents which operate in a similar manner to

accomplish a similar purpose. It is to be understood that embodiments of the disclosed technology may be practiced without these specific details. In other instances, well-known methods, structures, and techniques have not been shown in detail in order not to obscure an understanding of this description. References to "one embodiment," "an embodiment," "example embodiment," "some embodiments," "certain embodiments," "various embodiments," etc., indicate that the embodiment(s) of the disclosed technology so described may include a particular feature, structure, or characteristic, but not every embodiment necessarily includes the particular feature, structure, or characteristic. Further, repeated use of the phrase "in one embodiment" does not necessarily refer to the same embodiment, although it may.

[0068] It must also be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the context clearly dictates otherwise. For example, reference to a component is intended also to include composition of a plurality of components. References to a composition containing "a" constituent is intended to include other constituents in addition to the one named. In other words, the terms "a," "an," and "the" do not denote a limitation of quantity, but rather denote the presence of "at least one" of the referenced item.

[0069] As used herein, the term "and/or" may mean "and," it may mean "or," it may mean "exclusive-or," it may mean "one," it may mean "some, but not all," it may mean "neither," and/or it may mean "both." The term "or" is intended to mean an inclusive "or."

[0070] Ranges may be expressed herein as from "about" or "approximately" or "substantially" one particular value and/or to "about" or "approximately" or "substantially" another particular value. When such a range is expressed, other exemplary embodiments include from the one particular value and/or to the other particular value. Further, the term "about" means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, i.e., the limitations of the measurement system. For example, "about" can mean within an acceptable standard deviation, per the practice in the art. Alternatively, "about" can mean a range of up to ±20%, preferably up to ±10%, more preferably up to ±5%, and more preferably still up to ±1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 2-fold, of a value. Where particular values are

described in the application and claims, unless otherwise stated, the term "about" is implicit and in this context means within an acceptable error range for the particular value.

[0071] By "comprising" or "containing" or "including" is meant that at least the named compound, element, particle, or method step is present in the composition or article or method, but does not exclude the presence of other compounds, materials, particles, method steps, even if the other such compounds, material, particles, method steps have the same function as what is named.

[0072] Throughout this description, various components may be identified having specific values or parameters, however, these items are provided as exemplary embodiments. Indeed, the exemplary embodiments do not limit the various aspects and concepts of the present disclosure as many comparable parameters, sizes, ranges, and/or values may be implemented. The terms "first," "second," and the like, "primary," "secondary," and the like, do not denote any order, quantity, or importance, but rather are used to distinguish one element from another.

[0073] It is noted that terms like "specifically," "preferably," "typically," "generally," and "often" are not utilized herein to limit the scope of the claimed disclosure or to imply that certain features are critical, essential, or even important to the structure or function of the claimed disclosure. Rather, these terms are merely intended to highlight alternative or additional features that may or may not be utilized in a particular embodiment of the present disclosure. It is also noted that terms like "substantially" and "about" are utilized herein to represent the inherent degree of uncertainty that may be attributed to any quantitative comparison, value, measurement, or other representation.

[0074] The dimensions and values disclosed herein are not to be understood as being strictly limited to the exact numerical values recited. Instead, unless otherwise specified, each such dimension is intended to mean both the recited value and a functionally equivalent range surrounding that value. For example, a dimension disclosed as "50 mm" is intended to mean "about 50 mm".

[0075] It is also to be understood that the mention of one or more method steps does not preclude the presence of additional method steps or intervening method steps between those steps expressly identified. Similarly, it is also to be understood that the mention of one or more components in a composition does not preclude the presence of additional components than those expressly identified.

[0076] As used herein, the terms "subject", "patient", "individual", and "animal" are used interchangeably herein and refer to mammals, including, without limitation, human and veterinary animals (e.g., cats, dogs, cows, horses, sheep, pigs, etc.) and experimental animal models. In a preferred embodiment, the subject is a human.

[0077] As used herein, the term "gene therapy" includes any therapeutic approach of providing a nucleic acid encoding a therapeutic gene (e.g., a Factor VIII/IX/X) to a patient to relieve, diminish, or prevent the reoccurrence of one or more symptoms (e.g., clinical factors) associated with a disease or condition. The term encompasses administering any compound, drug, procedure, or regimen comprising a nucleic acid encoding a therapeutic gene, including any modified form of the gene (e.g., a Factor VIII/IX/X variant), for maintaining or improving the health of an individual with the disease or condition. One skilled in the art will appreciate that either the course of gene therapy or the dose of a genetic therapeutic agent can be changed, e.g., based upon the results obtained in accordance with the present disclosure.

[0078] As used herein the term "therapeutically effective" applied to dose or amount refers to that quantity of a compound or pharmaceutical composition that when administered to a subject for treating (e.g., preventing or ameliorating) a state, disorder or condition, is sufficient to affect such treatment. For example, a therapeutically effective amount of a drug useful for treating hemophilia can be the amount that is capable of preventing or relieving one or more symptoms associated with hemophilia. The "therapeutically effective amount" will vary depending on the compound or bacteria or analogues administered as well as the disease and its severity and the age, weight, physical condition and responsiveness of the mammal to be treated. The exact dose will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques (see, e.g., Lieberman, Pharmaceutical Dosage Forms (vols. 1-3, 1992); Lloyd, The Art, Science and Technology of Pharmaceutical Compounding (1999); Pickar, Dosage Calculations (1999); and Remington: The Science and Practice of Pharmacy, 20th Edition, 2003, Gennaro, Ed., Lippincott, Williams & Wilkins).

[0079] As used herein, the term "vector" refers to any vehicle used to transfer a nucleic acid (e.g., encoding a gene therapy construct) into a host cell. In some embodiments, a vector includes a replicon, which functions to replicate the vehicle, along with the target nucleic acid. In some embodiments, a vector is a viral particle for introducing a target nucleic acid (e.g., a codon-altered

polynucleotide encoding a therapeutic gene or therapeutic gene variant). Many modified eukaryotic viruses useful for gene therapy are known in the art. For example, adeno-associated viruses (AAVs) are particularly well suited for use in human gene therapy because humans are a natural host for the virus, the native viruses are not known to contribute to any diseases, and the viruses elicit a mild immune response. "Recombinant AAV" (rAAV) and "AAV" are used interchangeably throughout the application.

[0080] The term "plasmid" refers to an extrachromosomal circular DNA capable of autonomous replication in a given bacterial cell. Exemplary plasmids include but are not limited to those derived from pBR322, pUC, pUC19, pUC57, pJ241, or pJ247, pBluescript, pREP4, pCEP4, pCI, and p Poly (Lathe et al., Gene 57 (1987), 193-201). Plasmids can also be engineered by standard molecular biology techniques (Sambrook et al., Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989), N.Y.). It may also comprise a selection gene in order to select or to identify the transfected cells (e.g., by complementation of a cell auxotrophy or by antibiotic resistance), stabilizing elements (e.g., cer sequence) or integrative elements (e.g., LTR viral sequences and transposons).

[0081] As used herein, the term "plasmid backbone" refers to a sequence of DNA that typically contains an origin of replication (e.g., SEQ ID NOs: 20 and 26), and an antibiotic selection gene, which are necessary for the specific growth of only the host that is transformed with the proper plasmid. In certain embodiments, these elements are not intended to be packaged in the rAAV capsid.

[0082] As used herein, the term "gene" refers to the segment of a DNA molecule that codes for a polypeptide chain (e.g., the coding region). In some embodiments, a gene is positioned by regions immediately preceding, following, and/or intervening the coding region that are involved in producing the polypeptide chain (e.g., regulatory elements such as a promoter, enhancer, polyadenylation sequence, 5′-untranslated region, 3′-untranslated region, or intron).

[0083] As used herein, the term "regulatory elements" refers to nucleic acid sequences, such as promoters, enhancers, terminators, polyadenylation sequences, introns, etc…, that provide for the expression of a coding sequence in a cell.

[0084] As used herein, the term "promoter element" refers to a nucleic acid sequence that assists with controlling expression of a coding sequence. Generally, promoter elements are located 5′ of

the translation start site of a gene. However, in certain embodiments, a promoter element may be located within an intron sequence, or 3′ of the coding sequence. In some embodiments, a promoter useful for gene therapy is derived from the native gene of the target protein. In some embodiments, a promoter useful for gene therapy is specific for expression in a particular cell or tissue of the target organism (e.g., a liver-specific promoter) (Wu Z et al. Molecular Therapy 16(2):280-9. Choi VW et al. Molecular Therapy Methods & Clinical Development 2015. 2:15022), both of which are incorporated herein in their entirety for all intended purposes. In yet other embodiments, one of a plurality of well characterized promoter elements is used in gene therapy described herein. Non-limiting examples of well-characterized promoter elements include the CMV early promoter (e.g., hCMVie (SEQ ID NO: 4))), the 3-actin promoter, and the methyl CpG binding protein 2 (MeCP2) promoter. In some embodiments, the promoter is a constitutive promoter, which drives substantially constant expression of the target protein. In other embodiments, the promoter is an inducible promoter, which drives expression of the target protein in response to a particular stimulus (e.g., exposure to a particular treatment or agent). For a review of designing promoters for AAV-mediated gene therapy, see Gray et al. (Human Gene Therapy 22:1143-53 (2011)), the contents of which are expressly incorporated by reference in their entirety for all purposes.

[0085] As used herein, the term “transgene” broadly refers to any nucleic acid that is introduced into the genome of an animal, including but not limited to genes or nucleic acid having sequences which are perhaps not normally present in the genome, genes which are present but not normally transcribed and translated (“expressed”) in a given genome, or any other gene or nucleic acid which one desires to introduce into the genome. This may include genes which may normally be present in the non-transgenic genome, but which one desires to have altered in expression, or which one desires to introduce in a non-mutated form or an altered or variant form. The transgene may be specifically targeted to a defined genetic locus, may be randomly integrated within a chromosome, or it may be extrachromosomally replicating DNA. A transgene may include one or more transcriptional regulatory sequences and any other nucleic acid, such as introns, that may be necessary for optimal expression of a selected nucleic acid. A transgene can be as few as a couple of nucleotides long, but is preferably at least about 50, 100, 150, 200, 250, 300, 350, 400, or 500 nucleotides long or even longer and can be, e.g., an entire viral genome. A transgene can be coding or non-coding sequences, or a combination thereof. A transgene usually comprises a regulatory

element that is capable of driving the expression of one or more transgenes under appropriate conditions.

[0086] As used herein, the term "heterologous" as it relates to nucleic acid sequences, such as coding sequences and/or control sequences, denotes sequences that are not normally joined together and/or are not normally associated with a particular cell. Thus, a "heterologous" nucleic acid sequence means that the nucleic acid sequence is from an organism other than AAV or is synthetically derived. In certain embodiments, the heterologous nucleic acid sequence (e.g., a heterologous gene of interest) can encode a polypeptide such as, but not limited to, a clotting factor, an enzyme, an antibody or other polypeptide of interes. In certain embodiments, the heterologous nucleic acid sequence can encode an RNA having a structural or therapeutic function such as, but not limited to, an antisense, siRNA, shRNA, miRNA, EGSs, gRNA, sgRNA, ribozyme, or aptamer. Similarly, a cell transformed with a construct which is not normally present in the cell would be considered heterologous for purposes of this invention.

[0087] "Operably-linked" refers to the association of two or more nucleic acid sequence elements that are physically linked so that the function of one of the sequences is affected by another. For example, a regulatory DNA sequence is said to be "operably linked to" or "associated with" a DNA sequence that codes for an RNA or a polypeptide if the two sequences are situated such that the regulatory DNA sequence affects expression of the coding DNA sequence (i.e., that the coding sequence or functional RNA is under the transcriptional control of the promoter). Coding sequences can be operably-linked to regulatory sequences in sense or antisense orientation.

[0088] As used herein, the term "nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form and complements thereof. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, and peptide-nucleic acids (PNAs).

[0089] The term "amino acid" refers to naturally occurring and non-natural amino acids, including amino acid analogs and amino acid mimetics that function in a manner similar to the naturally

occurring amino acids. Naturally occurring amino acids include those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, y-carboxyglutamate, and O-phosphoserine. Naturally occurring amino acids can include, e.g., D-and L-amino acids. The amino acids used herein can also include non-natural amino acids. Amino acid analogs refer to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., any carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, or methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refer to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid. Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0090] The term "derivative" as used herein refers to a nucleic acid, peptide, or protein or a variant or analog thereof comprising one or more mutations and/or chemical modifications as compared to a corresponding full-length wild type nucleic acid, peptide or protein. Non-limiting examples of chemical modifications involving nucleic acids include, for example, modifications to the base moiety, sugar moiety, phosphate moiety, phosphate-sugar backbone, or a combination thereof.

[0091] The nucleic acid sequences that encode mutant gene constructs that may be useful with the plasmid system described herein may be identical to a wildtype (i.e., unmutated) sequence or may be a different coding sequence, which sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same polypeptides as the wildtype coding sequence. One of ordinary skill in the art will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each variation of a nucleic acid which encodes a same polypeptide is implicit in each described sequence with respect to the expression product, but not with respect to actual gene therapy constructs.

[0092] As to amino acid sequences, one of ordinary skill in the art will recognize that individual substitutions, deletions or additions to a nucleic acid or peptide sequence that alters, adds or deletes

a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the disclosure. Conservative amino acid substitutions providing functionally similar amino acids are well known in the art. Dependent on the functionality of the particular amino acid, e.g., catalytic, structural, or sterically important amino acids, different groupings of amino acid may be considered conservative substitutions for each other.

[0093] The terms "identical" or percent (%) "identity," in the context of two or more nucleic acids or peptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (i.e., about 60% identity, preferably 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified region, when compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual inspection.

[0094] As is known in the art, a number of different programs may be used to identify whether a protein (or nucleic acid as discussed below) has sequence identity or similarity to a known sequence. Sequence identity and/or similarity is determined using standard techniques known in the art, including, but not limited to, the local sequence identity algorithm of Smith & Waterman, Adv. Appl. Math., 2:482 (1981), by the sequence identity alignment algorithm of Needleman & Wunsch, J. Mol. Biol., 48:443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Natl. Acad. Sci. U.S.A., 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, Wis.), the Best Fit sequence program described by Devereux et al., Nucl. Acid Res., 12:387-395 (1984), preferably using the default settings, or by inspection. Preferably, percent identity is calculated by FastDB based upon the following parameters: mismatch penalty of 1; gap penalty of 1; gap size penalty of 0.33; and joining penalty of 30, "Current Methods in Sequence Comparison and Analysis," Macromolecule

Sequencing and Synthesis, Selected Methods and Applications, pp 127-149 (1988), Alan R. Liss, Inc, all of which are incorporated by reference.

[0095] In accordance with the present disclosure there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.,* Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual,* Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook *et al.,* 1989"); *DNA Cloning: A Practical Approach,* Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds.(1985); *Transcription and Translation* (B.D. Hames & S.J. Higgins, eds. (1984); *Animal Cell Culture* (R.I. Freshney, ed. (1986); *Immobilized Cells and Enzymes* (IRL Press, (1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology,* John Wiley & Sons, Inc. (1994); among others.

Plasmid Systems of the Disclosure

[0096] In one aspect, the disclosure provides a triple-plasmid system for engineering and producing Recombinant Adeno Associated Viral Vector (rAAV). In certain embodiments, the three plasmid backbones are all the same. In certain embodiments, at least one of the three plasmid backbones are different. In certain embodiments, all three plasmid backbones are different. In certain embodiments, all three plasmid backbones are different to prevent recombination occurring that can lead to the reconstruction of the complete AAV genome. In certain embodiments, the three plasmids comprise plasmid backbones based on, for example and without limitation, pUC19, pBR322, pUC57, pJ241, or pJ247. In certain embodiments, the three plasmids comprise plasmid backbones based on pUC19, pJ241, and pJ247.

[0097] In certain embodiments, one plasmid serves as the transgene-containing plasmid for rAAV production construct, a second plasmid serves as the AAV Rep-Cap construct, and a third plasmid serves as the Adenovirus (Ad) Helper construct. Exemplary plasmids of each type are shown in Figure 1.

Transgene-Containing Plasmid for rAAV Production

[0098] The transgene-containing plasmid for rAAV production is engineered to carry at least one heterologous nucleic acid sequence of interest (e.g., an anti-sense RNA molecule, shRNA, miRNA, a ribozyme, or a gene encoding a polypeptide of interest) in which the internal portion of the AAV genome is replaced with a heterologous nucleic acid sequence of interest within an expression cassette. "Expression cassette" as used herein means a nucleic acid sequence capable of directing expression of a particular heterologous nucleic acid sequence in an appropriate host cell (e.g., mammal), which may include a promoter operably linked to the nucleic acid sequence of interest that may be operably linked to termination signals. The expression cassette including the heterologous nucleic acid sequence of interest may be chimeric. The expression cassette may also be one that is naturally occurring but has been obtained in a recombinant form useful for heterologous expression.

[0099] In certain embodiments, the transgene-containing plasmid does not comprise an antibiotic resistance gene. In certain embodiments, the transgene-containing plasmid does not comprise an ampicillin resistance gene (e.g., SEQ ID NOs: 71 and 73). While antibiotic resistance genes are commonly used as selection markers for plasmid production, the inclusion of an antibiotic resistance gene (e.g., ampicillin resistance gene) can raise safety concerns. For example, there can be a horizontal gene transfer to patient's bacteria, which would be prevented if the gene is not present in the plasmid. It is particularly important to avoid using antibiotic selection markers involving antibiotics that are i n significant clinical use, in order to avoid unnecessary risk of spread of antibiotic resistance traits to environmental microbes (e.g., ampicillin). One should also avoid using antibiotic resistance genes for antibiotics that cause serious hypersensitivity reactions in patients as there could be residual antibiotic in the pharmaceutical composition (e.g., penicillin and other β-lactam antibiotics).

[0100] Exemplary transgene-containing plasmids according to the invention is shown in Figures 2, 3A, 3B, 15A, and 15B and SEQ ID NOs: 1, 42, 71, and 73, or a plasmid with at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NOs: 1, 42, 71, and 73. Figures 2, 3A, 3B, 15A, and 15B provides an example of the order of the elements of the transgene-containing plasmids of the invention.

[0101] The transgene-containing plasmids according to SEQ ID NOs: 71 and 73 are advantageous because they remove all traces of the ampicillin resistance gene and also include an inactivated gentamycin resistance gene (e.g., the start codon from the open reading frame was removed), which acts as an additional stuffer sequence.

[0102] The transgene-containing plasmid is constructed using known techniques to at least provide operatively linked components in the direction of transcription, control elements including a transcriptional initiation region, the DNA of interest and a transcriptional termination region. The control elements are selected to be functional in a mammalian cell. The resulting construct, which contains the operatively linked components, is flanked (5′ and 3′) with functional AAV inverted terminal repeat (ITR) sequences. Termination signals, such as polyadenylation sites, can also be included in the plasmid.

[0103] The ITRs have been shown to be the only cis elements required for packaging allowing for complete gutting of viral genes to create rAAV. Even though the rolling-circle DNA replication mechanism primarily amplifies (i.e., replicates) the transgene expression cassette DNA sequence flanked by the ITRs due to the presence of the D sequence within the ITRs, the plasmid DNA backbone (e.g., origin of replication, antibiotic resistance gene expression cassette, etc…) can also be packaged into the vector capsid, albeit at a lower frequency due to the absence of the flanking D sequence domain. AAV is efficient in packaging a genome size similar to or smaller than the wildtype virus genome (~4.7 kbases). One could discourage the packaging of the plasmid backbone by increasing the size of the backbone to such a degree that it is unfavorable for the backbone to be packaged into the capsid. Enlargement of the backbone can be achieved by additional "stuffer" sequences (i.e., filler component), resulting in a plasmid backbone size larger than the wild-type AAV genome. Without wishing to be bound by theory, it is suggested that the presence of an enlarged plasmid backbone can reduce the probability of the rAAV packaging the plasmid backbone into the vector capsid. In some embodiments, the enlarged plasmid backbone is created by use of the stuffer sequence.

[0104] In certain embodiments, the stuffer sequence is silent in terms of biological activity, in that it is devoid of at least one of enhancers, promoters, splicing regulators, noncoding RNAs, antisense sequences, and/or coding sequences. In certain embodiments, each of enhancers, promoters, splicing regulators, noncoding RNAs, antisense sequences, and coding sequences are absent.

[0105] In certain embodiments, the stuffer sequence comprises an inert intronic DNA sequence found in the human genome. By utilizing a DNA sequence from the human genome, there will be lower probability that the stuffer sequence will elicit an immune response in case the plasmid becomes packaged into the capsid. It is also important that the stuffer sequence does not include an open reading frame.

[0106] The stuffer sequence should be large enough that the size of the plasmid backbone is larger than the optimal packaging size of rAAV such that the plasmid backbone is not packaged into the vector capsid. The stuffer sequence can consist of at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 200, at least 300, at least 400, at least 500, at least 600, at least 700, at least 800, at least 900, at least 1000, at least 2000, at least 3000, at least 4000, at least 5000, at least 6000, at least 7000, at least 8000, at least 9000 or at least 10000 nucleotides. In certain embodiments, the stuffer sequence comprises a nucleic acid of between 1000 and 5000 nucleotides in length. In certain embodiments, the stuffer sequence comprises a nucleic acid of between 1000 and 2000 nucleotides in length. In certain embodiments, the stuffer sequence comprises a nucleic acid of between 800 and 1500 nucleotides in length. In certain embodiments, the stuffer sequence comprises a nucleic acid of between 800 and 1000 nucleotides in length.

[0107] In a preferred embodiment, the stuffer sequence comprises human GAPDH intron 2 (NG007073.2). Without wishing to be bound by theory, the use of human GAPDH intron 2 has lower immunogenicity as it is present in the human genome already and thus should not elicit an immune response if it is by chance packaged. GAPDH intron 2 is ideal as a stuffer sequence as it is a single naturally occurring sequence. There is no need to include any additional nucleotides or to link more than one sequence together, which would result in an unnatural buttressing of DNA sequences.

[0108] In certain embodiments, the stuffer sequence comprises, consists of, or consists essentially of a nucleic acid having at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 9 or a fragment thereof. In certain embodiments, the stuffer sequence comprises, consists of, or consists essentially of SEQ ID NO: 9 or a fragment thereof.

[0109] In certain embodiments, the stuffer sequence comprises an inactivated gentamycin gene. In certain embodiments, the gentamycin gene is modified so that it is not expressed. For example, the start codon could be removed.

[0110] In certain embodiments, the stuffer sequence comprises, consists of, or consists essentially of a nucleic acid having at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 72 or a fragment thereof. In certain embodiments, the stuffer sequence comprises, consists of, or consists essentially of SEQ ID NO: 72 or a non-functional fragment thereof.

[0111] The transgene-containing plasmid can be constructed using ITRs from any of the various AAV serotypes. These ITRs base pair to allow for synthesis of the complementary DNA strand. The ITRs remain functional in such plasmids to allow replication and packaging of the rAAV containing the heterologous nucleic acid sequence of interest. Mutations within the terminal repeat sequences of AAV plasmids are well tolerated in generating functional AAV vectors. *See e.g.*, Samulski et al, 1983; Muzyczka et al, 1984; and U.S. Patent No. 9,163,259, which of which as incorporated herein in their entirety for all purposes. Even plasmids with one of the two ITRs deleted, the AAV sequences could be rescued, replicated, and infectious virions be produced, as long as the existing ITR in the construct contains the full AAV ITR sequence.

[0112] The nucleic acid sequences of AAV ITR regions are known. The ITR need not have the wild-type nucleic acid sequence, but may be altered, e.g., by the insertion, deletion or substitution of nucleotides. Additionally, the AAV ITR may be derived from any of several AAV serotypes, including without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, or a chimera thereof. Furthermore, 5' and 3' ITRs which flank a selected nucleic acid sequence in an AAV vector need not necessarily be identical or derived from the same AAV serotype or isolate, so long as they function as intended, i.e., to allow for excision and rescue of the sequence of interest from a host cell genome. Even though SEQ ID NOs: 2 and 43 is used as an example of the 5′ ITR sequence of the rAAV described in this document, it is expected that any 5′ ITR sequence that carries the terminal resolution site would produce vectors with the same functionality. Likewise, even though SEQ ID NO: 3 is used as an example of the 3′ ITR sequence

of the rAAV described in this document, it is expected that any 3′ ITR sequence that carries the terminal resolution site would produce vectors with the same functionality.

[0113] In certain embodiments, the 5′ ITR sequence comprises, consists of, or consists essentially of a nucleic acid having at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 2 or SEQ ID NO 43 or a functional fragment or derivative thereof. In certain embodiments, the 5′ ITR comprises, consists of, or consists essentially of SEQ ID NO: 2 or SEQ ID NO 43, or a functional fragment or derivative thereof.

[0114] In certain embodiments, the 3′ ITR sequence comprises, consists of, or consists essentially of a nucleic acid having at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 3 or a functional fragment or derivative thereof. In certain embodiments, the 3′ ITR comprises, consists of, or consists essentially of SEQ ID NO: 3, or a functional fragment or derivative thereof.

[0115] In certain embodiments, the transgene-containing plasmid comprising the stuffer sequence as described above is operably linked to an expression cassette.

[0116] In certain embodiments, the expression cassette comprises a promoter. In certain embodiments, the at least one heterologous nucleic acid sequence (e.g., heterologous gene of interest) is operably linked to a pol II promoter (constitutive, cell-specific, or inducible) such that the heterologous nucleic acid sequence is capable of being expressed in the patient's target cells under appropriate or desirable conditions. Numerous examples of constitutive, cell-specific, and inducible promoters are known in the art, and one of skill could readily select a promoter for a specific intended use, e.g., the selection of the muscle-specific skeletal α-actin promoter or the muscle-specific creatine kinase promoter/enhancer for muscle cell-specific expression, the selection of the constitutive CMV promoter for strong levels of continuous or near-continuous expression (e.g., hCMVie (SEQ ID NO: 4)), or the selection of the inducible ecdysone promoter for induced expression. Induced expression allows the skilled artisan to control the amount of protein that is synthesized. In this manner, it is possible to vary the concentration of therapeutic product. Other examples of well-known inducible promoters are: steroid promoters (e.g., estrogen

and androgen promoters) and metallothionein promoters. In certain embodiments, the promoter is a pol III promoter. In certain embodiments, the promoter is a U6 promoter. In certain embodiments, the promoter is an H1 promoter. In certain embodiments, the gene expression cassette is without a promoter.

[0117] In certain embodiments, the transgene-containing plasmid is multicistronic, i.e., carries more than one gene. Unlike promoters which will create unique mRNA transcripts for each gene that is expressed, multicistronic plasmids simultaneously express two or more separate proteins from the same mRNA. In such cases, the multiple genes are separated by an element that allows for separate translation for each gene (e.g., internal ribosomal entry sites (IRES) or 2A peptides).

[0118] Even though SEQ ID NO: 6 is used as an example of an IRES sequence of the rAAV described in this document, it is expected that any 5′ ITR sequence that carries the terminal resolution site would produce vectors with the same functionality.

[0119] IRES allow for initiation of translation from an internal region of the mRNA by acting as another ribosome recruitment site. In certain embodiments, the IRES sequence comprises, consists of, or consists essentially of a nucleic acid having at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 6 or a functional fragment or derivative thereof. In certain embodiments, IRES comprises, consists of, or consists essentially of SEQ ID NO: 6 or a functional fragment or derivative thereof.

[0120] In certain embodiments, the transgene-containing plasmid encodes a 2A peptide. 2A peptides (see non-limiting examples in Table 1 below) were created to overcome some of the disadvantages of the IRES element. In particular 2A peptides are “self-cleaving” in that these peptides are thought to function by making the ribosome skip the synthesis of a peptide bond at the C-terminus of a 2A element, leading to separation between the end of the 2A sequence and the next peptide downstream. The “cleavage” occurs between the Glycine and Proline residues found on the C-terminus meaning the upstream cistron will have a few additional residues added to the end, while the downstream cistron will start with the Proline. 2A cleavage is universal in eukaryotic cells, and, some scientists report close to 100% cleavage. The choice of specific 2A peptide will ultimately depend on a number of factors such as cell type or experimental conditions, which one of ordinary skill would be understand which one to choose.

Table 1 Examples of four common 2A peptides.

Peptide	Amino acid sequence*
T2A:	(GSG) E G R G S L L T C G D V E E N P G P (SEQ ID NO: 74)
P2A:	(GSG) A T N F S L L K Q A G D V E E N P G P (SEQ ID NO: 75)
E2A:	(GSG) Q C T N Y A L L K L A G D V E S N P G P (SEQ ID NO: 76)
F2A:	(GSG) V K Q T L N F D L L K L A G D V E S N P G P (SEQ ID NO: 77)

* (GSG) residues can be added to the 5' end of the peptide to improve cleavage efficiency.

[0121] In an embodiment, the plasmid comprises 5' and 3' ITRs from an AAV, wherein the ITRs surround at least one gene. In certain embodiments, a stuffer sequence is located downstream of the 3' ITR. In certain embodiments, the stuffer sequence is upstream of the 5' ITR. ITRs can be from AAV serotypes AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, and/or AAV11, or a chimera thereof. In certain embodiments, the ITRs are from AAV serotypes AAV2 and/or AAV5. In certain embodiments, the ITRs can be SEQ ID NO: 2, 3, or 43, or a functional fragment or derivative thereof. In some embodiments, the gene is a reporter gene, such as for example and not limitation, eGFP (e.g., SEQ ID NO: 5) and/or SEAP (e.g., SEQ ID NO: 7). In some embodiments, the stuffer sequence is GAPDH intron 2 or a fragment or variant thereof. In some embodiments, the stuffer sequence is SEQ ID NO: 9 or a fragment thereof. Exemplary gene constructs are shown in Figure 3 for use in plasmids to generate ssAAV (Figure 3A) and scAAV (Figure 3B) rAAV.

Rep-Cap Plasmid

[0122] The second plasmid comprises AAV replication (Rep) and capsid (Cap) gene sequences. The AAV Rep-Cap plasmid includes both of the major AAV genes open reading frames (ORFs), Rep gene, and Cap gene. Rep proteins have been shown to possess many functions, including, among others: recognition, binding and nicking of the AAV origin of DNA replication; DNA helicase activity; and modulation of transcription from AAV (or other heterologous) promoters. Cap proteins supply necessary packaging functions and assemble into the viral capsid shell. AAV helper functions are used herein to complement AAV functions in trans that are missing from AAV vectors. Rep and Cap genes are translated to produce multiple distinct proteins (Rep78, Rep68,

Rep52, Rep40 - required for the AAV life cycle; VP1, VP2, VP3 - capsid proteins). The Rep and/or Cap genes can be derived from AAV serotypes AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, and/or AAV11, or a chimera thereof. In certain embodiments, the AAV Rep and/or Cap genes encode genetically engineered AAV and/or chemically modified AAV. *See e.g.,* AAV virions mutated to be less immunogenic such as those recited in U.S. 7,259,151, incorporated herein by reference for all intended purposes. The selection of the AAV serotype can be selected on the tropism of the AAV serotype. Table 2 below provides examples, without limitation, of tropism of the most widely used AAV serotypes. The tropism of AAV can also be modified via pseudotyping (i.e., the mixing of a capsid and genome from ITRs from a different viral serotypes). These serotypes are denoted using a slash, so that AAV2/5 indicates a virus containing the genome carrying ITR of serotype 2 packaged in the capsid from serotype 5. Use of these pseudotyped viruses can improve transduction efficiency, as well as alter tropism. For example, neurons that are not efficiently transduced by AAV2, one can use AAV2/5, which is distributed more widely in the brain and shown to have improved transduction efficiency. One can also use hybrid capsids derived from multiple different serotypes, which also alter viral tropism. For example, AAV-DJ, which contains a hybrid capsid derived from eight serotypes, displays a higher transduction efficiency *in vitro* than any wild type serotype; *in vivo*, it displays very high infectivity across a broad range of cell types. The mutant AAV-DJ8 displays the properties of AAV-DJ, but with enhanced brain uptake. A number of AAV helper plasmids have been described, such as the commonly used plasmids pAAV/Ad and pIM29+45 which encode both Rep and Cap gene expression products. See, e.g., Samulski et al. (1989) J. Virol. 63:3822-3828; and McCarty et al. (1991) J. Virol. 65:2936-2945 and U.S. Pat. Nos. 5,139,941; 6,001,650; 6,376,237; 7,259,151, each of which are incorporated herein by reference in their entirety for all purposes.

Table 2 Tissue Tropism of AAV Serotypes

Tissue	Optimal Serotype
Heart	AAV1, AAV8, AAV9
Kidney	AAV2
Liver	AAV7, AAV8, AAV9

Nervous System	AAV1, AAV2, AAV4, AAV5, AAV8, AAV9
Lung	AAV4, AAV5, AAV6, AAV9
Pancreas	AAV8
Photoreceptor Cells	AAV2, AAV5, AAV8
RPE (Retinal Pigment Epithelium)	AAV1, AAV2, AAV4, AAV5, AAV8
Skeletal Muscle	AAV1, AAV6, AAV7, AAV8, AAV9

[0123] An exemplary Rep-Cap plasmid according to the invention is shown in Figures 4A, 4B, 14A, and 14B; and SEQ ID NOs: 24, 31, 33, 35, 37, 41, 59, and 60, or a plasmid with at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NOs: 24, 31, 33, 35, 37, 41, 59, or 60. Figures 4A, 4B, 14A, and 14B; provide examples of the order of the elements in the plasmids of AAV Rep-Cap plasmids of the invention.

[0124] In certain embodiments, the Rep genes can be derived from AAV serotypes AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, and/or AAV11, or a chimera thereof. In certain embodiments, the AAV Rep gene is genetically engineered AAV and/or chemically modified AAV. In certain embodiments, the Rep gene includes genes from AAV serotype 2 (Rep2) and/or Rep5, which includes chimeras (e.g., AAV Rep2/5).

[0125] In certain embodiments, the Cap genes can be derived from AAV serotypes AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, and/or AAV11, or a chimera thereof. In certain embodiments, the AAV Cap gene is genetically engineered AAV and/or chemically modified AAV. In any of the foregoing embodiments, the Cap gene may be from the same AAV serotype as the Rep gene or a different AAV serotype from the Rep gene. In any of the foregoing embodiments, the plasmid further comprises a Cap gene from any of AAV serotypes 2, 5, 8, and/or 9 (Cap2, Cap5, Cap8, and Cap9, respectively), including chimeric proteins comprising hybrids of Cap proteins from those serotypes.

[0126] In certain embodiments, the Rep-Cap plasmid includes, but is not limited to, Rep gene sequence from AAV serotypes 2 and as a chimeric Rep protein combined from more than 1 serotypes, for example Rep2/5, and capsid gene sequence from any AAV capsid serotypes including AAV2, AAV5, AAV8, and/or AAV9.

[0127] In certain embodiments, the Rep gene sequence comprises, consists of, or consists essentially of a nucleic acid having at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NOs: 11, 12, 28, or 30, or a functional fragment or derivative thereof. In certain embodiments, Rep gene sequence comprises, consists of, or consists essentially of SEQ ID NOs: 11, 12, 28, or 30, or a functional fragment or derivative thereof.

[0128] In certain embodiments, the Cap gene sequence comprises, consists of, or consists essentially of a nucleic acid having at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NOs: 13, 29, 32, or 36, or a functional fragment or derivative thereof. In certain embodiments, Cap gene sequence comprises, consists of, or consists essentially of SEQ ID NOs: 13, 29, 32, or 36, or a functional fragment or derivative thereof.

[0129] In certain embodiments, the promoter sequence comprises, consists of, or consists essentially of a nucleic acid having at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 34, or a functional fragment or derivative thereof. In certain embodiments, promoter sequence comprises, consists of, or consists essentially of SEQ ID NO: 34, or a functional fragment or derivative thereof.

[0130] In an embodiment, the Rep-Cap plasmid further comprises an AAV promoter to control expression of the AAV Rep and Cap proteins described herein. The promoter can be any desired promoter, selected by known considerations, such as the level of expression of a nucleic acid functionally linked to the promoter and the cell type in which the vector is to be used. That is, the promoter can be tissue/cell- specific. Promoters can be prokaryotic, eukaryotic, fungal, nuclear,

mitochondrial, viral or plant promoters. Promoters can be exogenous or endogenous to the cell type being transduced by the vector. Promoters can include, for example, bacterial promoters, known strong promoters such as SV40 or the inducible metallothionein promoter, or an AAV promoter, such as an AAV P5 promoter. Additionally, chimeric regulatory promoters for targeted gene expression can be utilized. Examples of these regulatory systems, which are known in the art, include the tetracycline based regulatory system which utilizes the tet transactivator protein (tTA), a chimeric protein containing the VPl 6 activation domain fused to the tet repressor of Escherichia coli, the EPTG based regulatory system, the CID based regulatory system, and the Ecdysone based regulatory system. Other promoters include promoters derived from actin genes, immunoglobulin genes, cytomegalovirus (CMV) (e.g., hCMVie (SEQ ID NO: 4), adenovirus, bovine papilloma virus, adenoviral promoters, such as the adenoviral major late promoter, an inducible heat shock promoter, respiratory syncytial virus, Rous sarcomas virus (RSV), etc. The promoter can be the promoter of any of the AAV serotypes and can be the pl9 promoter or the p40 promoter. In certain embodiments, the promoter can be an AAV2 P5 promoter or an AAV5 P5 promoter or an AAV P5 promoter. Furthermore, smaller fragments of the P5 promoter that retain promoter activity can readily be determined by standard procedures including, for example, constructing a series of deletions in the P5 promoter, linking the deletion to a reporter gene, and determining whether the reporter gene is expressed, i.e., transcribed and/or translated. Examples of potential promoter ca be found in WO2005017101, incorporated by reference herein for all intended purposes. In certain embodiments, the AAV promoter is from AAV serotype 2. Exemplary P5-Rep-Cap plasmids comprising the AAV2 promoter P5 are shown in Figures 4B and 14B and in SEQ ID NO: 34.

[0131] Suitable plasmid backbones for the Rep-Cap plasmid includes but is not limited to. pHLP19, pUC18, pUC19, and pAAV-RC2, see also plasmid backbones described in U.S. Pat. Nos. 6,001,650 and 6,156,303, the entirety of both incorporated herein by reference for all purposes. In certain embodiments, the Rep-Cap plasmid backbone is pUC19.

Ad Helper Plasmid

[0132] In an embodiment, the Ad helper plasmid comprises adenovirus genes including, but not limited to, Ad2 and/or Ad5. In an embodiment, the Ad helper plasmid comprises Ad5 genes. The Ad5 gene sequence is used because Ad5 is an efficient helper virus to rAAV. It is known that the

full-complement of adenovirus genes is not required for helper function. In fact, it is more desirable to not have the full compliment. For example, adenovirus mutants incapable of DNA replication and late gene synthesis have been shown to be permissive for AAV replication. Ito et al., (1970) J. Gen. Virol. 9: 243; Ishibashi et al, (1971) Virology 45: 317. Thus, the Ad Helper Plasmid is designed to be of minimal size to only carry the required Ad genes required for rAAV production and to serve as a reduced plasmid size construct. It has been shown that adenoviruses defective in the E1 region, or having a deleted E4 region, are unable to support AAV replication. Thus, E1A and/or E4 regions are likely required for AAV replication, either directly or indirectly. Laughlin et al., (1982) J. Virol. 41: 868; Janik et al., (1981) Proc. Natl. Acad. Sci. USA 78: 1925; Carter et al., (1983) Virology 126: 505. Other characterized Ad mutants include: E1B (Laughlin et al. (1982), supra; Janik et al. (1981), supra; Ostrove et al., (1980) Virology 104: 502); E2A (Handa et al., (1975) J. Gen. Virol. 29: 239; Strauss et al., (1976) J. Virol. 17: 140; Myers et al., (1980) J. Virol. 35: 665; Jay et al., (1981) Proc. Natl. Acad. Sci. USA 78: 2927; Myers et al., (1981) J. Biol. Chem. 256: 567); E2B (Carter, Adeno-Associated Virus Helper Functions, in I CRC Handbook of Parvoviruses (P. Tijssen ed., 1990)); E3 (Carter et al. (1983), supra); and E4 (Carter et al. (1983), supra; Carter (1995)). Although studies of the accessory functions provided by adenoviruses having mutations in the E1B coding region have produced conflicting results, Samulski et al., (1988) J. Virol. 62: 206-210, recently reported that E1B55k is required for AAV virion production, while E1B19k is not. In addition, International Publication WO 97/17458 and Matshushita et al., (1998) Gene Therapy 5: 938-945, describe accessory proteins encoding various Ad genes. Particularly preferred accessory function plasmids comprise an adenovirus VA RNA coding region, an adenovirus E4 ORF6 coding region, an adenovirus E2A 72 kD coding region, an adenovirus E1A coding region, and an adenovirus E1B region lacking an intact E1B55k coding region. Examples of these plasmids are described in International Publication No. WO 01/83797. Each reference recited in this paragraph are incorporated herein by reference in their entirety for all purposes.

[0133] Exemplary Ad helper plasmids according to the invention is shown in Figure 5 and SEQ ID NOs: 14 and 15, or a plasmid with at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NOs: 14

and 15. Figure 5 provides examples of the order of the elements in the plasmids of Ad helper plasmids of the invention.

[0134] In certain embodiments, the Ad helper plasmid can include, without limitation, adenoviral gene sequences for E2a, E4 (orf6), the VA1 RNA gene, and the parvovirus VP capsid gene unit. In certain embodiments, the Ad Helper Plasmid can include VA, E4, and E2A genes. As there is a limitation of how much plasmid can be efficiently transfected to cells for rAAV production, having a reduced size plasmid carrying these Ad genes could help increase the molar content of all three plasmids used in the transfection, thus increasing the probability of producing higher yield rAAV.

[0135] In an embodiment, the Ad helper plasmid comprises E2A, E4 ORFs 1, 2, 3, 4, and 6/7, and VA ("short Ad helper plasmid"). An exemplary short Ad Helper Plasmid is shown in the top panel of Figure 5. The shorter plasmid described here is to reduce "plasmid load" during the step of transfection so that the overall copy number of plasmids of all three plasmids can be increased to give higher number of plasmid templates for gene expression and replication for rAAV production. The reduced plasmid load is surprisingly useful for larger batches. This may not be a crucial parameter in small research scale production but could be much more critical when scaled up. This exemplary short Ad Helper Plasmid is approximately 12 kb. In another embodiment, the Ad Helper Plasmid comprises E2A, E4 ORFs 1, 2, 3, 4, and 6/7, and VA, as well as genes encoding a protease and a fiber and promoter pVIII ("long Ad helper plasmid"). An exemplary long Ad Helper Plasmid is shown in the bottom panel of Figure 5. This exemplary long Ad Helper Plasmid is approximately 18 kb.

[0136] The differences between the short and long constructs are shown in Figure 5. The orientations of the three essential gene elements are different. The long version carries additional elements from the adenovirus genome that may have functions that influence rAAV production. The short version contains the minimal gene sequence that is known to be able to support rAAV production.

[0137] In certain embodiments, the VA sequence comprises, consists of, or consists essentially of a nucleic acid having at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NOs: 16 or 48-50, or a functional fragment or derivative thereof. In certain embodiments, VA sequence comprises,

consists of, or consists essentially of SEQ ID NOs: 16 or 48-50, or a functional fragment or derivative thereof.

[0138] In certain embodiments, the E4 sequence comprises, consists of, or consists essentially of a nucleic acid having at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NOs: 17, 40, 47, or 55-58, or a functional fragment or derivative thereof. In certain embodiments, E4 sequence comprises, consists of, or consists essentially of SEQ ID NOs: 17, 40, 47, or 55-58, or a functional fragment or derivative thereof.

[0139] In certain embodiments, the E2A sequence comprises, consists of, or consists essentially of a nucleic acid having at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NOs: 18, 39, 46, or 51, or a functional fragment or derivative thereof. In certain embodiments, E2A sequence comprises, consists of, or consists essentially of SEQ ID NOs: 18, 39, 46, or 51, or a functional fragment or derivative thereof.

[0140] Suitable plasmids for the Ad Helper Plasmid include, but are not limited to, pJ241, see also plasmids described in U.S. Pat. Nos. 6,001,650 and 6,156,303, the entirety of both incorporated by reference herein. In certain embodiments, the Ad Helper Plasmid backbone is pUC57.

Additional Genes

[0141] In a further embodiment, all three plasmids contain a selection marker. An example of a selection marker includes, but is not limited, to positive selection markers such as drug resistance genes including, but not limited to, G418 (with neor), puromycin (with puror), hygromycin B (with hygr), blasticidin S (with bsrr), mycophenolic acid and 6-thio(guanine) (with gpt) and gancyclovir or 1 (2'-deoxy-2'-fluoro-beta-D-arabinofuranosyl)-5-iodouracil (FIAU) (with HSV-tk), gentamycin, and/or kanamycin (with kanr). In a further embodiment, the drug selection marker on all three plasmids is kanamycin. In certain embodiments, the kanamycin gene comprises or consists of SEQ ID NOs: 19 or 25, or functional fragments or derivatives thereof. In certain embodiments, the gentamycin gene comprises or consists of SEQ ID NOs: 44 or 72, or functional fragments or derivatives thereof.

[0142] In an embodiment, one or more of the three plasmids carries one or more reporter genes. Several reporter genes are known in the art and some are commercially available (see, Alam and Cook, supra). The reporter gene can be inserted within a plasmid that is particularly suited for an organism and molecular biology manipulations. Promoters of interest can be inserted into cloning sites so that the expression of the reporter gene is under the control of the promoter (see, Rosenthal, N., Methods Enzymol. 152: 704-720 (1987); and Shiau, A. and Smith, J. M., Gene 67: 295-299 (1988)). Known methods are used to introduce these plasmids into a cell type or whole organism (see, Sambrook et al., Molecular Biology, A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989); and Nolan, In: Molecular Cloning, Cold Spring Harbor Laboratory Press, (1989)). Examples of reporter genes include, without limitation, β-galactosidase (LacZ), firefly luciferase, Renilla luciferase, Gaussia luciferase, chloramphenicol acetyltransferase (CAT), secreted embryonic alkaline phosphatase (SEAP), cyan fluorescent protein (CFP), green fluorescent protein (GFP), enhanced GFP (eGFP), yellow fluorescent protein (YFP), enhanced YFP (eYFP), blue fluorescent protein (BFP), enhanced BFP (eBFP), red fluorescent protein from the Discosoma coral (DsRed), and/or MmGFP (Zemicka-Goetz et al. (1997) Development 124: 1133-1137) or others familiar to those of ordinary skill. In another embodiment, one or more of the three plasmids carries a reporter construct comprising both eGFP and SEAP, with an internal ribosome entry site (IRES) located between eGFP and SEAP. In such an embodiment, eGFP, which localizes in the nucleus, can be used for determining vector transduction tropism of the rAAV, while SEAP, which is secreted outside of the cell, can permit quantitative measurement of transduction efficiency, either in culture medium in an *in vitro* setting, or in the subject's bloodstream in an *in vivo* setting. LacZ can enable color-based selection of desired clones, based on disruption of the *lacZ* gene by a cloned gene.

[0143] In an embodiment, each plasmid comprises a unique DNA titer tag. In certain embodiments, the DNA titer tag only appears in the transgene-containing plasmid. In certain embodiments the DNA titer tag appears in all of the plasmid systems. This unique DNA titer tag can be included to enable universal vector genome titering, e.g., via a qPCR (or ddPCR)-based vector genome titering assay, to quantify the amount of vector present. In certain embodiments, the DNA titer tag can be outside the expression cassette but between the 2 ITR's to ensure that it becomes packaged. For example, the DNA titer tag can be upstream of the 3'ITR sequence. As another example, the DNA titer tag can be downstream of the 5'ITR sequence. In certain

embodiments, the DNA titer tag is constructed such that it does not appear endogenously within the subject's genome. For example, the sequence can be compared against the subject's DNA (e.g., via a Blast search or other alignment search tool). The primers used to run the qPCR analysis can also be analyzed to ensure that they do not identify any sequence found in the host cells used to package the virion.

[0144] The DNA titer tag can be of a size that allows for efficient qPCR analysis but also takes up the least amount of genome space in the plasmid. In certain embodiments, the DNA titer tag sequence is about 60 nucleotides to about 100 nucleotides in length (e.g. SEQ ID NO: 10) and designed based on a sequence that does not exist in humans or standard laboratory animals. In certain embodiments, the DNA titer tag sequence is about 60 nucleotides to about 80 nucleotides, about 65 nucleotides to about 95 nucleotides, about 70 nucleotides to about 90 nucleotides, or about 75 nucleotides to about 85 nucleotides. In certain embodiments, the DNA titer tag sequence is about 60 nucleotides to about 70 nucleotides, about 65 nucleotides to about 75 nucleotides, about 70 nucleotides to about 80 nucleotides, about 75 nucleotides to about 85 nucleotides, about 80 nucleotides to about 90 nucleotides, about 85 nucleotides to about 95 nucleotides, or about 90 nucleotides to about 100 nucleotides. In certain embodiments, the DNA titer tag sequence is at least about 60 nucleotides, at least about 65 nucleotides, at least about 70 nucleotides, at least about 75 nucleotides, at least about 80 nucleotides, at least about 85 nucleotides, at least about 90 nucleotides, at least about 95 nucleotides, or at least about 100 nucleotides. In certain embodiments, the stretch of DNA titer sequence is 100 nucleotides in length. In certain embodiments, a titer tag of 100 nucleotides can be advantageous in rapid qPCR assays and allow for efficient packaging due to the overall plasmid size and packaging limitations.

[0145] Non-limiting examples of nucleic acid sequences that encode DNA titer tags includes SEQ ID NO: 61-70.

Heterologous Nucleic Acid Sequence

[0146] Recombinant AAV made by the plasmids of the invention can be administered to one or more cells or tissue of a subject. Thus, the invention embraces the delivery of heterologous nucleic acid sequence that can be useful to modulate the cells or tissue of the subject. For example, rAAV can upregulate or downregulate an activity or product of a cell or tissue.

[0147] In certain embodiments, the heterologous nucleic acid sequence can be a heterologous gene of interest encoding one or more peptide, polypeptide, or protein. In certain embodiments, the heterologous nucleic acid sequence can encode a peptide, polypeptide, or protein that binds to a specific target of interest, which can be useful for the treatment or prevention of disease in a subject. Examples of such heterologous nucleic acid sequences and associated peptides, polypeptides, or proteins include, but are not limited to, a gene encoding antibodies, MHC molecules, T-cell receptors, B-cell receptors, aptamers, avimers, receptor-binding ligands, or targeting peptides. Antibodies useful in the present invention can encompass monoclonal antibodies, polyclonal antibodies, antibody fragments (e.g., Fab, Fab′, F(ab′)2, Fv, Fc, etc.), chimeric antibodies, bispecific antibodies, heteroconjugate antibodies, single chain (ScFv), mutants thereof, fusion proteins comprising an antibody portion, humanized antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site of the required specificity, including glycosylation variants of antibodies, amino acid sequence variants of antibodies, and covalently modified antibodies. The antibodies may be murine, rat, human, or any other origin (including chimeric or humanized antibodies). An antibody includes an antibody of any class, such as IgG, IgA, or IgM (or sub-class thereof), and the antibody need not be of any particular class. Depending on the antibody amino acid sequence of the constant domain of its heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

[0148] In certain embodiments, the heterologous nucleic acid sequence (e.g., heterologous gene of interest) can encode a peptide, polypeptide, or protein that can be useful for the treatment or prevention of disease in a subject. For example, the heterologous nucleic acid sequencecan encode a protein X for the treatment of disease Y. Protein X can, for example, substitute for a mutated protein or act to block a mutated protein. Such nucleic acid sequences and associated diseases include, but are not limited to, nucleic acid sequences encoding glucose-6-phosphatase, associated with glycogen storage deficiency type 1A; DNA encoding phosphoenolpyruvate-carboxykinase, associated with Pepck deficiency; DNA encoding galactose-1 phosphate uridyl transferase,

associated with galactosemia; DNA encoding phenylalanine hydroxylase, associated with phenylketonuria; DNA encoding branched chain alpha-ketoacid dehydrogenase, associated with Maple syrup urine disease; DNA encoding fumarylacetoacetate hydrolase, associated with tyrosinemia type 1; DNA encoding methylmalonyl-CoA mutase, associated with methylmalonic acidemia; DNA encoding medium chain acyl CoA dehydrogenase, associated with medium chain acetyl CoA deficiency; DNA encoding ornithine transcarbamylase, associated with ornithine transcarbamylase deficiency; DNA encoding argininosuccinic acid synthetase, associated with citrullinemia; DNA encoding low density lipoprotein receptor protein, associated with familial hypercholesterolemia; DNA encoding UDP-glucouronosyltransferase, associated with Crigler-Najjar disease; DNA encoding adenosine deaminase, associated with severe combined immunodeficiency disease; DNA encoding hypoxanthine guanine phosphoribosyl transferase, associated with Gout and Lesch-Nyan syndrome; DNA encoding biotinidase, associated with biotinidase deficiency; DNA encoding alpha-galactosidase-A, associated with Fabry disease; DNA encoding beta-glucocerebrosidase, associated with Gaucher disease; DNA encoding beta-glucuronidase, associated with Sly syndrome; DNA encoding peroxisome membrane protein 70 kDa, associated with Zellweger syndrome; DNA encoding porphobilinogen deaminase, associated with acute intermittent porphyria; DNA encoding alpha-1 antitrypsin for treatment of alpha-1 antitrypsin deficiency (emphysema); DNA encoding C1-esterase for the treatment of hereditary angioedema (HAE); DNA encoding phenylalanine hydroxylase for the treatment of phenylketonuria; DNA encoding acid alpha-glucosidase for the treatment of with Pompe disease; DNA encoding ATP7B for the treatment of Wilson's disease; DNA encoding alpha-L-iduronidase for the treatment of mucopolysaccharidose type I (MPSI); DNA encoding iduronate sulfatase for the treatment of mucopolysaccharidose type II (MPSII); DNA encoding heparan sulfamidase for the treatment of mucopolysaccharidose type IIIA (MPSIIIA); DNA encoding N-acetylglucosaminidase for the treatment of mucopolysaccharidose type IIIB (MPSIIIB); DNA encoding heparan-alpha-glucosaminide N-acetyltransferase for the treatment of mucopolysaccharidose type IIIC (MPSIIIC); DNA encoding N-acetylglucosamine 6-sulfatase for the treatment of mucopolysaccharidose type IIID (MPSIIID); DNA encoding galactose-6-sulfate sulfatase for the treatment of mucopolysaccharidose type IVA (MPSIVA); DNA encoding beta-galactosidase for the treatment of mucopolysaccharidose type IVB (MPSIVB); DNA encoding N-acetylgalactosamine-4-sulfatase for the treatment of mucopolysaccharidose type VI (MPSVI);

DNA encoding beta-glucuronidase for the treatment of mucopolysaccharidose type VII (MPSVII); DNA encoding hyaluronidase for the treatment of mucopolysaccharidose type IX (MPSIX); DNA encoding erythropoietin for treatment of anemia due to thalassemia or to renal failure; DNA encoding vascular endothelial growth factor, DNA encoding angiopoietin-1, and DNA encoding fibroblast growth factor for the treatment of ischemic diseases; DNA encoding thrombomodulin and tissue factor pathway inhibitor for the treatment of occluded blood vessels as seen in, for example, atherosclerosis, thrombosis, or embolisms; DNA encoding aromatic amino acid decarboxylase (AADC), and DNA encoding tyrosine hydroxylase (TH) for the treatment of Parkinson's disease; DNA encoding the beta adrenergic receptor, DNA encoding anti-sense to, or DNA encoding a mutant form of, phospholamban, DNA encoding the sarco(endo)plasmic reticulum adenosine triphosphatase-2 (SERCA2), and DNA encoding the cardiac adenylyl cyclase for the treatment of congestive heart failure; DNA encoding a tumor suppressor gene such as p53 for the treatment of various cancers; DNA encoding a cytokine such as one of the various interleukins for the treatment of inflammatory and immune disorders and cancers; DNA encoding dystrophin or minidystrophin and DNA encoding utrophin or miniutrophin for the treatment of muscular dystrophies; DNA encoding ABCA4 for the treatment of Stargardt's disease; and, DNA encoding insulin for the treatment of diabetes.

[0149] In certain embodiments, the heterologous nucleic acid sequence (e.g., heterologous gene of interest) can encode a peptide, polypeptide, or protein that encodes a blood coagulation protein, which proteins may be delivered to the cells of a subject having a blood disorder (e.g., hemophilia). Examples of such nucleic acids and associated peptides, polypeptides, or proteins include, but are not limited to, DNA encoding Factor IX to a subject for treatment of hemophilia B, Factor VIII to a subject for treatment of hemophilia A, Factor VII for treatment of Factor VII deficiency, Factor X for treatment of Factor X deficiency, Factor XI for treatment of Factor XI deficiency, Factor XIII for treatment of Factor XIII deficiency, and Protein C for treatment of Protein C deficiency.

[0150] The invention also includes the expression of engineered artificial DNA binding domain peptide, transcriptional activator or transcriptional repressor and nucleases that can interact with the host cell genome to affect up or down gene expression level for genetic and/or acquired diseases.

[0151] The invention also includes the expression of heterologous nucleic acid sequences, including but not limited to, antisense, siRNA, shRNA, miRNA, EGSs, gRNA, sgRNA, ribozymes, or aptamers, which could interact with cellular DNA, RNA and/or proteins that can change the gene expression or activities of proteins for genetic and/or acquired diseases.

[0152] The invention also includes the expression of intermediate and/or critical raw material for cellular therapy, including but not limited to rAAV, to be used to infect cells to generate genetically engineered cell therapy materials or drug product.

[0153] The invention also includes a heterologous nucleic acid sequence that is a gene editing molecule used for modifying a genomic locus of interest (i.e., target) in a cell. Such modifications include, but are not limited to a disruption, deletion, repair, mutation, addition, alteration, or modification of a gene sequence at a target locus in a gene. Examples of gene-editing molecules include, but are not limited to, endonucleases such as zinc finger nucleases (ZFns), transcription activator-like effector nucleases (TALENs), meganucleases, restriction endonucleases, recombinases, and Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) proteins.

Delivery of rAAV

[0154] Recombinant AAV described herein, can be used at a therapeutically useful concentration for the treatment and/or prevention of a disease of interest, by administering to a subject in need thereof, an effective amount of the rAAV made by the plasmids of the invention. Subjects to be treated with rAAV made by the plasmids of the present invention can also be administered with other therapeutic agents or devices with known efficacy for treating or preventing the disease.

[0155] Delivery of the rAAV to a subject may be by intramuscular injection or by administration into the bloodstream of the subject. Administration into the bloodstream may be by injection into a vein, an artery, or any other vascular conduit the mutant virions into the bloodstream by way of isolated limb perfusion, a technique well known in the surgical arts, the method essentially enabling the artisan to isolate a limb from the systemic circulation prior to administration of the rAAV. Moreover, for certain conditions, it may be desirable to deliver the mutant virions to the CNS of a subject. By "CNS" is meant all cells and tissue of the brain and spinal cord of a vertebrate. Thus, the term includes, but is not limited to, neuronal cells, glial cells, astrocytes, cerebrospinal fluid (CSF), interstitial spaces, bone, cartilage, intracerebral ventricular, intracranial, cisterna

magna injection, intrathecal, intracatorid, intranasal and the like. rAAV or cells transduced *in vitro* may be delivered directly to the CNS or brain by injection into, e.g., the ventricular region, as well as to the striatum (e.g., the caudate nucleus or putamen of the striatum), spinal cord and neuromuscular junction, or cerebellar lobule, with a needle, catheter or related device, using neurosurgical techniques known in the art, such as by stereotactic injection. *See*, e.g., Stein et al., J Virol 73:3424-3429, 1999; Davidson et al., PNAS 97:3428-3432, 2000; Davidson et al., Nat. Genet. 3:219-223, 1993; and Alisky and Davidson, Hum. Gene Ther. 11:2315-2329, 2000, each of which are incorporated herein in their entirety for all purposes. For administration to the eye, methods can include, subretinal, intravitreal, trans-scleral, or intracranial.

Table 3 Exemplary Sequences for Use in the Plasmids of the Invention

Sequence ID	Description
SEQ ID NO: 1	Single-strand ITR transgene plasmid
SEQ ID NO: 2	5' ITR
SEQ ID NO: 3	3' ITR
SEQ ID NO: 4	hCMVie
SEQ ID NO: 5	eGFP
SEQ ID NO: 6	IRES
SEQ ID NO: 7	SEAP
SEQ ID NO: 8	SV40 polyA
SEQ ID NO: 9	GAPDH stuffer sequence
SEQ ID NO: 10	DNA titer tag
SEQ ID NO: 11	Rep2/5
SEQ ID NO: 12	Rep2
SEQ ID NO: 13	Cap9
SEQ ID NO: 14	Complete short ad helper plasmid
SEQ ID NO: 15	Complete long Ad helper plasmid
SEQ ID NO: 16	VA gene
SEQ ID NO: 17	E4 gene
SEQ ID NO: 18	E2A
SEQ ID NO: 19	Kanamycin resistance gene (complement)

SEQ ID NO: 20	pUC origin (complement)
SEQ ID NO: 21	P_Amp (complement)
SEQ ID NO: 22	Term_bla (complement)
SEQ ID NO: 23	Rpo_C (complement)
SEQ ID NO: 24	pRep2/5-Cap5 plasmid
SEQ ID NO: 25	Kanamycin resistance gene (complement)
SEQ ID NO: 26	origin of replication; RNaseH cleavage point
SEQ ID NO: 27	lac promoter (complement)
SEQ ID NO: 28	Rep5 BamHI fragment (complement)
SEQ ID NO: 29	Cap5/VP1 fragment (complement)
SEQ ID NO: 30	Start site of Rep2 (complement)
SEQ ID NO: 31	pRep2-Cap2 plasmid
SEQ ID NO: 32	Cap2
SEQ ID NO: 33	pUC19-Kan-Rep2Cap2 plasmid
SEQ ID NO: 34	AAV2/P5 promoter (complement)
SEQ ID NO: 35	pRep2-Cap8 plasmid
SEQ ID NO: 36	Cap8 gene (complement)
SEQ ID NO: 37	pRep2-Cap9 plasmid
SEQ ID NO: 38	lac promoter
SEQ ID NO: 39	E2A gene (complement)
SEQ ID NO: 40	E4 gene (complement)
SEQ ID NO: 41	pUC19-Kan-Rep2Cap9 plasmid
SEQ ID NO: 42	Self-complementary ITR transgene plasmid
SEQ ID NO: 43	Truncated 5' ITR
SEQ ID NO: 44	Gentamycin resistance gene (complement)
SEQ ID NO: 45	pHelper plasmid
SEQ ID NO: 46	E2A complement
SEQ ID NO: 47	E4 (complement)
SEQ ID NO: 48	VA (complement)
SEQ ID NO: 49	VA2 RNA (complement)

SEQ ID NO: 50	VA1 RNA (complement)
SEQ ID NO: 51	E2A-BP
SEQ ID NO: 52	L4 100K
SEQ ID NO: 53	hAdV2, 33-100kD
SEQ ID NO: 54	Incomplete L4 pVIII
SEQ ID NO: 55	E4 orf 6/7 (complement)
SEQ ID NO: 56	E4 orf 4 (complement)
SEQ ID NO: 57	E4 orf 3
SEQ ID NO: 58	E4 orf 2 (complement)
SEQ ID NO: 59	pAAV-RC
SEQ ID NO: 60	pUC19-Kan-Rep2Cap8 plasmid
SEQ ID NO: 61	DNA Titer Tag
SEQ ID NO: 62	DNA Titer Tag
SEQ ID NO: 63	DNA Titer Tag
SEQ ID NO: 64	DNA Titer Tag
SEQ ID NO: 65	DNA Titer Tag
SEQ ID NO: 66	DNA Titer Tag
SEQ ID NO: 67	DNA Titer Tag
SEQ ID NO: 68	DNA Titer Tag
SEQ ID NO: 69	DNA Titer Tag
SEQ ID NO: 70	DNA Titer Tag
SEQ ID NO: 71	Single-strand ITS transgene plasmid
SEQ ID NO: 72	Gentamycin resistance gene – inactivated (complement)
SEQ ID NO: 73	Self-complementary ITS transgene plasmid
SEQ ID NO: 74	T2A
SEQ ID NO: 75	P2A
SEQ ID NO: 76	E2A
SEQ ID NO: 77	F2A

EXAMPLES

[0156] The present disclosure is also described and demonstrated by way of the following examples. However, the use of these and other examples anywhere in the specification is illustrative only and in no way limits the scope and meaning of the disclosure or of any exemplified term. Likewise, the disclosure is not limited to any preferred embodiments described here. Indeed, many modifications and variations of the disclosure may be apparent to those skilled in the art upon reading this specification, and such variations can be made without departing from the disclosure in spirit or in scope. The disclosure is therefore to be limited only by the terms of the appended claims along with the full scope of equivalents to which those claims are entitled.

EXAMPLE 1: *In Vitro* Expression of Cap Proteins

[0157] This example investigated the *in vitro* expression of the capsid proteins in AAV293 (Agilent) cells from pUC19-based plasmids as compared to expression levels of the same capsid proteins from control pAAV-RC2 based plasmids carrying Rep2 and Cap2 genes (Agilent) (Figure 4A).

[0158] A first set of four plasmids with varied Rep and Cap genes were created in the pAAV-RC background, starting with Rep2Cap2-pAAV-RC (i.e., pAAV-RC2 as shown in Figure 4A). The pAAV-RC2 was used to generate Rep2/5Cap5-pAAV-RC, Rep2Cap8-pAAV-RC, and Rep2Cap9-pAAV-RC (Figure 4A).

[0159] A second set of four plasmids were created in the pUC19-Kan background, with the same replication and capsid proteins as the first set. Thus, Rep2Cap2-pUC19-Kan, Rep2/5Cap5-pUC19-Kan, Rep2Cap8-pUC19-Kan, and Rep2Cap9-pUC19-Kan (Figures 4A and 14A).

[0160] For the experiments, each of the 8 plasmids were separately transfected along with the Ad Helper plasmid, pHelper (Agilent) (e.g., SEQ ID NO: 45), at a ratio of 1:1.

[0161] Expression levels of the Cap protein from the pUC19-Kan-based plasmids were compared to expression levels of the same Cap protein from the pAAV-RC2-based plasmids via Western blotting using a monoclonal B1 antibody (Figure 6). Positive controls AAV2 reference standard material (RSM) and AAV8 RSM are reference standard materials containing the AAV2 and AAV8 Cap proteins, while the negative control was a cell lysate from HEK293 without any Cap-bearing

plasmid. The expression level of capsid protein was AAV5 > AAV8 > AAV9 > AAV2 for both the pUC19-based plasmids and the pAAV-RC2-based plasmids.

[0162] Figure 7 is a Western blot analysis conducted with reduced sample to specifically analyze the amounts of Cap proteins VP1-VP3 more clearly.

[0163] Next, the AAV2 P5 promoter was added to the Rep2Cap2 pUC19-Kan, Rep2Cap8 pUC19-Kan, and Rep2Cap9 pUC19-Kan plasmids (e.g., Figures 4B and 14B). Figure 8 shows expression levels of Cap proteins from AAV serotypes 2, 8 and 9 expressed using the P5 promoter tested under the same conditions as described above in comparison with those without the P5 promoter. It was found that the P5 promoter gives higher levels of capsid protein expression. The transgene-containing plasmid and Ad helper plasmid were administered at a ratio of 1:1.

EXAMPLE 2: Functional Testing of Short and Long Ad Helper Plasmids

[0164] The purpose of this example was to test the function of a short Ad helper plasmid and a long Ad helper plasmid versus the commercial pHelper. Each plasmid was tested individually to ensure each one is functional before using them in combination.

[0165] Figure 9 shows the positive test results using the short Ad helper plasmid (SEQ ID NO: 14) in the HEK293 host cell system. The short Ad helper plasmid (SEQ ID NO: 14) was tested by co-transfecting the short helper plasmid along with a pTRUF11 transgene-containing plasmid carrying GFP as the transgene between the ITRs and the Agilent RC2 plasmid carrying AAV Rep2 and Cap2 genes. The negative controls consisted of 1) a commercial Ad helper plasmid (pHelper) and the Agilent plasmid RC2 and 2) pHelper and pTRUF11; the positive control consisted of pHelper, pTRUF11, and Agilent RC2 plasmid. After 48 hours, the HEK293 cells were lysed with Triton X-100 and treated with benzonase nuclease to degrade DNA and RNA. The cell lysates, containing AAV particles, were treated with DNase I and serially diluted before undergoing qPCR to determine the viral genome copy number per ml cell lysate. Figure 9 shows the results of the qPCR assay, with columns 1 and 2 representing the negative controls, column 3 showing the positive control, and column 4 showing the viral genome copy number obtained when the short Ad helper plasmid was used together with 2 other plasmids to produce rAAV.

[0166] A similar experiment was performed to test a long Ad helper plasmid (SEQ ID NO: 15) according to the disclosure (Figure 10). Figure 10 shows the viral genome copy number per ml

cell lysate, as determined by qPCR, of a negative control (column 1), a positive control (column 2), the short Ad helper plasmid (SEQ ID NO: 14) + Rep-Cap bearing plasmid + ITR-GFP bearing plasmid (column 3), and the long Ad Helper Plasmid + Rep-Cap bearing plasmid + ITR-GFP bearing plasmid (column 4). Therefore, the long Ad Helper Plasmid also resulted in the production of AAV.

EXAMPLE 3: rAAV Virion Production Using the Triple-Plasmid System

[0167] The ability of the single strand (ss)- and self-complementary (sc)-ITR-bearing plasmids according to the disclosure to form rAAV virions was tested. In this experiment, the plasmids were co-transfected into HEK293 cells (Agilent). For each transfection, Ad-helper plasmid, Rep-Cap plasmid, and transgene-containing plasmid were used at 1:1:1 molar ratio and 10ug of total DNA was used per 10cm plate. The negative control was the commercially available Ad helper plasmid (Agilent) and the commercially available Rep-Cap-bearing plasmid (Agilent), while the positive control used a different ITR-bearing plasmid (ATCC). The top panel of Figure 11 shows the viral genome copy number per ml cell lysate for the ss-ITR-bearing plasmid (measured by qPCR as above), while the bottom panel shows the viral genome copy number per ml cell lysate for the sc-ITR-bearing plasmid. In both panels, column 1 shows the copy number for the negative control, column 2 represents the positive control, and column 3 represents the plasmid according to the disclosure.

[0168] Next, three plasmids according to the disclosure were co-transfected into HEK293 cells (again, 1:1:1 ratio) for qPCR assays. The negative control (column 1 of Figure 12) was a commercially available Ad helper plasmid and a ITR-bearing plasmid. The positive control (column 2 of Figure 12) included a commercially available Rep-Cap-bearing plasmid. Columns 3-6 correspond to AAV genome copy numbers from cells transfected with the same commercially available Ad helper plasmid and ITR-bearing plasmid along with a pUC19-based plasmid encoding Rep and Cap proteins from AAV serotypes 2, 5, 8, or 9 (noted across the top of the figure). Columns 7-10 of Figure 12 correspond to AAV genome copy numbers from cells transfected with Ad helper plasmids, pUC19-based Rep-Cap-bearing plasmids, and ITR-bearing plasmids according to the disclosure. Column 11 of Figure 12 is another positive control corresponding to AAV genome copy number from cells transfected with an Ad helper plasmid and

ss-ITR plasmid according to the disclosure and a commercially available Rep-Cap-bearing plasmid.

EXAMPLE 4: Purification and Production of rAAVs

[0169] HEK293 cells were co-transfected with a plasmid system comprising three plasmids according to the disclosure. The cells were chemically lysed, and the cell pellet and medium were collected. The cell lysate was clarified and treated with benzonase. The clarified lysate was run on an appropriate affinity column (e.g., for a plasmid system comprising AAV8 capsid, the affinity column was AVB; for a plasmid system comprising AAV9, the affinity column was AAV9-POROS CaptureSelect). Following a buffer exchange, the rAAV was eluted from the column. The rAAV was then characterized, by way of example and not limitation, by qPCR to determine the viral genome copy number (see Figures 9-13, 16). The rAAV can further be evaluated by silver stain to determine purity and identity, by Limulus amebocyte lysate (LAL) assay to measure endotoxin activity and microbial contamination, and by an *in vitro* transduction assay to determine biological activity. Other characterization assays include alkaline electrophoresis to test the size and integrity of the viral genome, ELISA to examine the capsids, infectious center assays to determine the infectivity of the rAAV particles, and electron microscopy to observe the rAAV particles. Western blotting for specific proteins may also be performed by using appropriate antibodies (see Figures 6-8).

EXAMPLE 5: Use of Tag to Titer Vector Genome

[0170] While sequences such as polyA sequences can be used for qPCR quantification, it is not ideal to use such sequences for universal titering. For example, each transgene may use a different polyA sequence (e.g., SV40, bGH polyA, etc…), thereby precluding its use to quantitate vectors across all transgene platforms. Therefore, a separate DNA titer tag outside the transgene cassette (i.e., not transcribed as part of the transgene mRNA transcript) was tested for its ability to universally quantitate any transgene cassette.

[0171] A 100 nucleotides DNA titer tag was included upstream of the 3' ITR sequence. This same titer tag can be used in any transgene-containing plasmid for rAAV production to allow for universal vector genome titering via qPCR techniques, which can be used as a single reference

standard for any project. The qPCR titration results were compared for the same batch of AAV using either SV40 polyA or the 100 nucleotides DNA titer tag as the target sequence.

[0172] Two different viral vectors: rAAV8-ssITR (SEQ ID NO: 1) and rAAV8-scITR (SEQ ID NO: 42) were produced with the transgene-containing plasmid being either single-stranded (SEQ ID NO: 1) or self-complementary (SEQ ID NO: 42) transgene-containing plasmids. Similar qPCR titers were obtained using the two different target sequences, indicating the 100 nucleotides DNA titer tag works equally well as the SV40 polyA, which has been widely used in the field for qPCR-based vector titration (Figure 13A (rAAV8-ssITR) and 13B (rAAV8-scITR)).

EXAMPLE 6: Use of Tag to Titer Vector Genome

[0173] To further confirm the utility of the DNA titer tag, the same 100 nucleotides DNA titer tag used in Example 5 was included upstream of the 3' ITR sequence in two additional viral vectors: rAAV9-ssITR (SEQ ID NO: 71) and rAAV9-scITR (SEQ ID NO: 73).

[0174] While several possible embodiments are disclosed above, embodiments of the present disclosure are not so limited. These exemplary embodiments are not intended to be exhaustive or to unnecessarily limit the scope of the disclosure, but instead were chosen and described in order to explain the principles of the present disclosure so that others skilled in the art may practice the disclosure. Indeed, various modifications of the disclosure in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims. Further, the terminology employed herein is used for the purpose of describing exemplary embodiments only and the terminology is not intended to be limiting since the scope of the various embodiments of the present disclosure will be limited only by the appended claims and equivalents thereof. The scope of the disclosure is therefore indicated by the following claims, rather than the foregoing description and above-discussed embodiments, and all changes that come within the meaning and range of equivalents thereof are intended to be embraced therein.

[0175] All patents, applications, publications, test methods, literature, and other materials cited herein are hereby incorporated by reference in their entirety as if physically present in this specification.

CLAIMS

What is claimed is:

1. A plasmid system for Recombinant Adeno-Associated Viral Vector (rAAV) production comprising:

(i) a transgene-containing plasmid comprising at least one heterologous nucleic acid flanked by a 5' and 3' AAV inverted terminal repeat (ITR) and a stuffer sequence outside of the ITRs;

(ii) a plasmid comprising AAV replication (Rep) and capsid (Cap) gene sequences; and

(iii) an adenovirus (Ad) helper plasmid.

2. The plasmid system of claim 1, wherein the stuffer sequence increases the size of the transgene-containing plasmid backbone such that the transgene-containing plasmid is not packaged into an rAAV capsid.

3. The plasmid system of claim 1 or claim 2, wherein the backbone of the transgene-containing plasmid is larger than a wild-type AAV genome.

4. The plasmid system of any one of claims 1-3, wherein the stuffer sequence is devoid of enhancers, promoters, splicing regulators, noncoding RNAs, antisense sequences, coding sequences, or any combination thereof.

5. The plasmid system of claim 4, wherein the stuffer sequence is devoid of enhancers, promoters, splicing regulators, noncoding RNAs, antisense sequences, and coding sequences.

6. The plasmid system of any one of claims 1-5, wherein the stuffer sequence comprises an inert intronic DNA sequence found in the human genome.

7. The plasmid system of any one of claims 1-6, wherein the stuffer sequence comprises a nucleic acid sequence of between 1000 and 5000 nucleotides in length or a nucleic acid sequence of between 1000 and 2000 nucleotides in length.

8. The plasmid system of any one of claims 1-7, wherein the stuffer sequence comprises GAPDH intron 2, fragment, or mutant thereof.

9. The plasmid system of any one of claims 1-8, wherein the stuffer sequence comprises a nucleic acid having at least about 40%, about 50%, about 60%, about 65%, about

70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 9.

10. The plasmid system of any one of claims 1-9, wherein the stuffer sequence consists of a nucleic acid having at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 9.

11. The plasmid system of any one of claims 1-8, wherein the stuffer sequences comprises a SEQ ID NO: 9 or a fragment thereof.

12. The plasmid system of any one of claims 1-8, wherein the stuffer sequences consists of a SEQ ID NO: 9 or a fragment thereof.

13. The plasmid system of any one of claims 1-12, wherein the transgene-containing plasmid comprises a plasmid with a structure in the same order as Figure 3A, wherein the eGFP and SEAP transgenes can be replaced with the at least one heterologous nucleic acid.

14. The plasmid system of any one of claims 1-12, wherein the transgene-containing plasmid comprises a plasmid with a structure in the same order as Figure 3B, wherein the eGFP transgene can be replaced with the at least one heterologous nucleic acid.

15. The plasmid system of any one of claims 1-12, wherein the transgene-containing plasmid comprises nucleic acid sequences in the 5′ to 3′ direction of: SEQ ID NOs: 2, 4, at least one heterologous nucleic acid, 8, 3, and the stuffer sequence, wherein each nucleic acid sequence can be substituted with or encodes a corresponding functional fragment or derivative thereof or a sequence with at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity therewith.

16. The plasmid system of any one of claims 1-15, wherein the transgene-containing plasmid further comprises a DNA titer tag outside the expression cassette but between the 5’ ITR and 3’ ITR.

17. The plasmid system of any one of claims 1-15, wherein the transgene-containing plasmid further comprises a DNA titer tag i) upstream of the 3’ ITR and downstream of a polyA sequence or ii) upstream of the 3’ ITR and downstream of the at least one heterologous nucleic acid; iii) or downstream of the 5’ ITR and upstream of the at least one heterologous nucleic acid

sequence; or iv) downstream of the 5' ITR and upstream of a promoter for the at least one heterologous nucleic acid sequence; or v) downstream of the 5' ITR and upstream of the 3' ITR.

18. The plasmid system of any one of claims 1-15, wherein the transgene-containing plasmid further comprises a DNA titer tag i) upstream of SEQ ID NO: 3 and downstream of SEQ ID NO: 8 or ii) upstream of SEQ ID NO: 3 and downstream of the at least one heterologous nucleic acid sequence; iii) or downstream of SEQ ID NO: 2 and upstream of the at least one heterologous nucleic acid sequence; or iv) downstream of SEQ ID NO: 2 and upstream of SEQ ID NO: 4; or v) downstream of SEQ ID NO: 2 and upstream of SEQ ID NO: 3.

19. The plasmid system of any one of claims 1-12, wherein the transgene-containing plasmid comprises nucleic acid sequences in the 5′ to 3′ direction of: SEQ ID NOs: 43, 4, at least one heterologous nucleic acid sequence, 8, 3, and the stuffer sequence, wherein each nucleic acid sequence can be substituted with or encodes a corresponding functional fragment or derivative thereof or a sequence with at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity therewith.

20. The plasmid system of claim 19, wherein the transgene-containing plasmid further comprises a DNA titer tag outside the expression cassette but between the 5' ITR and 3' ITR.

21. The plasmid system of claim 19 or claim 20, wherein the transgene-containing plasmid further comprises a DNA titer tag i) upstream of the 3' ITR and downstream of a polyA sequence or ii) upstream of the 3' ITR and downstream of the at least one heterologous nucleic acid sequence; iii) or downstream of the 5' ITR and upstream of the at least one heterologous nucleic acid sequence; or iv) downstream of the 5' ITR and upstream of a promoter for the at least one heterologous nucleic acid sequence; or v) downstream of the 5' ITR and upstream of the 3' ITR.

22. The plasmid system of any one of claims 19-20, wherein the transgene-containing plasmid further comprises a DNA titer tag i) upstream of SEQ ID NO: 3 and downstream of SEQ ID NO: 8 or ii) upstream of SEQ ID NO: 3 and downstream of the at least one heterologous nucleic acid sequence; iii) or downstream of SEQ ID NO: 43 and upstream of the at least one heterologous nucleic acid sequence; or iv) downstream of SEQ ID NO: 43 and upstream of SEQ ID NO: 4; or v) downstream of SEQ ID NO: 43 and upstream of SEQ ID NO: 3.

23. The plasmid system of any one of claims 1-22, wherein the AAV Rep gene sequence is from AAV serotype 2, 5, 8, 9, or hybrids thereof.

24. The plasmid system of any one of claims 1-23, wherein the AAV Cap gene sequence is from AAV serotype 2, 5, 8, 9, or hybrids thereof.

25. The plasmid system of any one of claims 1-24, wherein the plasmid comprising the Rep and Cap gene sequences further comprises a promoter.

26. The plasmid system of claim 25, wherein the promoter is an AAV promoter.

27. The plasmid system of claim 26, wherein the promoter is an AAV P5 promoter.

28. The plasmid system of any one of claims 1-27, wherein the Ad helper plasmid comprises one or more of Adenovirus genes selected from E1a, E1b, E2a, E4orf6, or VA RNA.

29. The plasmid system of claim 28, wherein the Ad helper plasmid comprises nucleic acid sequences in the 5′ to 3′ direction of: SEQ ID NOs: 18, 17, 16, and 20, wherein each nucleic acid sequence can be substituted with a corresponding functional fragment or derivative thereof or a sequence with at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity therewith.

30. The plasmid system of claim 28, wherein the Ad helper plasmid comprises nucleic acid sequences in the 5′ to 3′ direction of: SEQ ID NOs: 21, 16, 39, 40, 22, 23, and 20 wherein each nucleic acid sequence can be substituted with or encode a corresponding functional fragment or derivative thereof or a sequence with at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity therewith.

31. The plasmid system of any one of claims 1-28, wherein the Ad helper plasmid comprises a structure in the same order as either construct of Figure 5.

32. The plasmid system of any one of claims 1-28, wherein the Ad helper plasmid comprises a nucleic acid having at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 14.

33. The plasmid system of any one of claims 1-28, wherein the Ad helper plasmid comprises a nucleic acid having at least about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 15.

34. The plasmid system of any one of claims 1-33, wherein the heterologous nucleic acid sequence is a heterologous gene of interest encoding a peptide, polypeptide, or protein.

35. The plasmid system of claim 34, wherein the peptide, polypeptide, or protein is an enzyme, antibody, MHC molecule, T-cell receptor, B-cell receptor, aptamer, avimer, receptor-binding ligand, targeting peptides, a therapeutic agent, or gene editing molecule.

36. The plasmid system of any one of claims 1-35, wherein the heterologous nucleic acid is a nucleic acid sequence such as an antisense, siRNA, shRNA, miRNA, EGSs, gRNA, sgRNA, ribozyme, or aptamer.

37. A host cell comprising the plasmid system of any one of claims 1-36.

38. A Recombinant Adeno-Associated Viral Vector (rAAV) produced by the host cell of claim 37.

39. A DNA titer tag allowing for universal vector titering, comprising a nucleic acid tag sequence from about 60 nucleotides to about 100 nucleotides long either upstream or downstream from a nucleic acid sequence of a heterologous nucleic acid sequencewithin a transgene-containing plasmid, wherein the nucleic acid tag sequence can be used in at least two different transgene-containing plasmids to allow for universal vector genome titering between at least two different types of AAV vectors.

40. The DNA titer tag of claim 39, wherein the nucleic acid tag sequence is about 100 nucleotides long.

41. The DNA titer tag of claim 39 or claim 40, wherein the nucleic acid tag sequence is upstream from a 3' ITR sequence of the transgene-containing plasmid but not within an expression cassette of the transgene-containing plasmid.

42. The DNA titer tag of claim 39 or claim 40, wherein the nucleic acid tag sequence is downstream from a 5' ITR sequence of the transgene-containing plasmid but not within an expression cassette of the transgene-containing plasmid.

43. The DNA titer tag of any one of claims 39-42, wherein the DNA titer tag comprises any one of nucleic acid sequences of SEQ ID NOS: 61-70.

44. A method for producing a Recombinant Adeno-Associated Viral Vector (rAAV) comprising transducing a cell with the plasmid system of any one of claims 1-36 and isolating the rAAV.

45. A Recombinant Adeno-Associated Viral Vector (rAAV) produced by the method of claim 44.

46. A composition comprising the plasmid system of any one of claims 1-36.

47. A pharmaceutical composition comprising the rAAV of claim 38 or claim 45.

48. A method for delivering or transferring a nucleic acid sequence into a subject's cell, comprising administering the rAAV of claim 38 or claim 45 to a subject thereby delivering the nucleic acid sequence into the cell.

49. The method of claims 48, wherein the subject's cell is in culture or is present in the subject.

50. A method for treating or preventing a disease or disorder in a subject, comprising administering to a subject in need thereof, a rAAV according to claim 38 or claim 45.

51. A method of transducing a host cell comprising contacting the host cell with a rAAV according to claim 38 or claim 45.

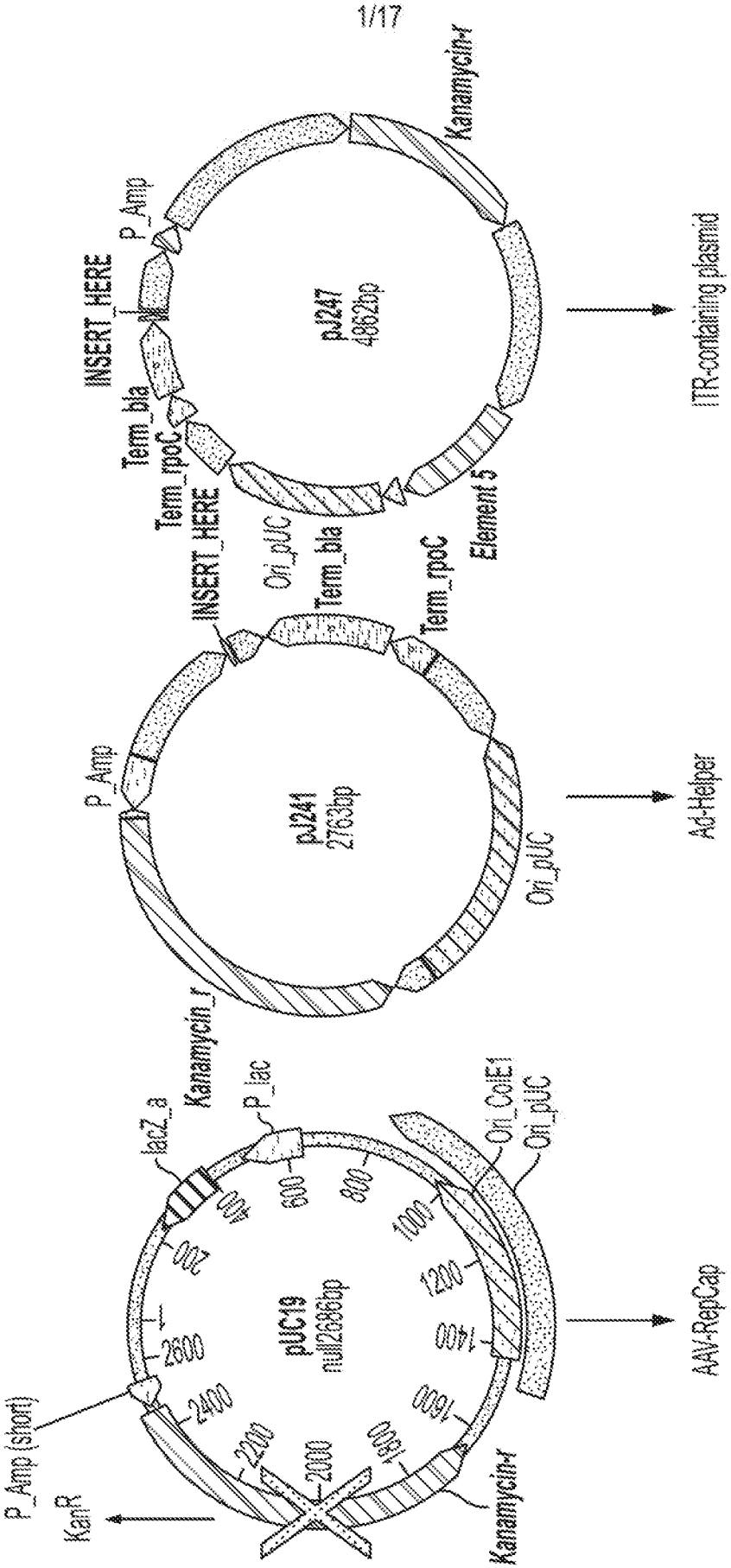

pJ247
4862bp
Kanamycin-r
P_Amp
INSERT_HERE
Tem_bla
Tem_rpoC
Ori_pUC
Element 5
ITR-containing plasmid
pJ241
2763bp
P_Amp
INSERT_HERE
Ori_pUC
Tem_bla
Tem_rpoC
Kanamycin_r
Ad-Helper
pUC19
null2686bp
lacZ_a
P_lac
Ori_ColE1
Ori_pUC
P_Amp (short)
KanR
Kanamycin-r
AAV-RepCap
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400
2600

Figure 1

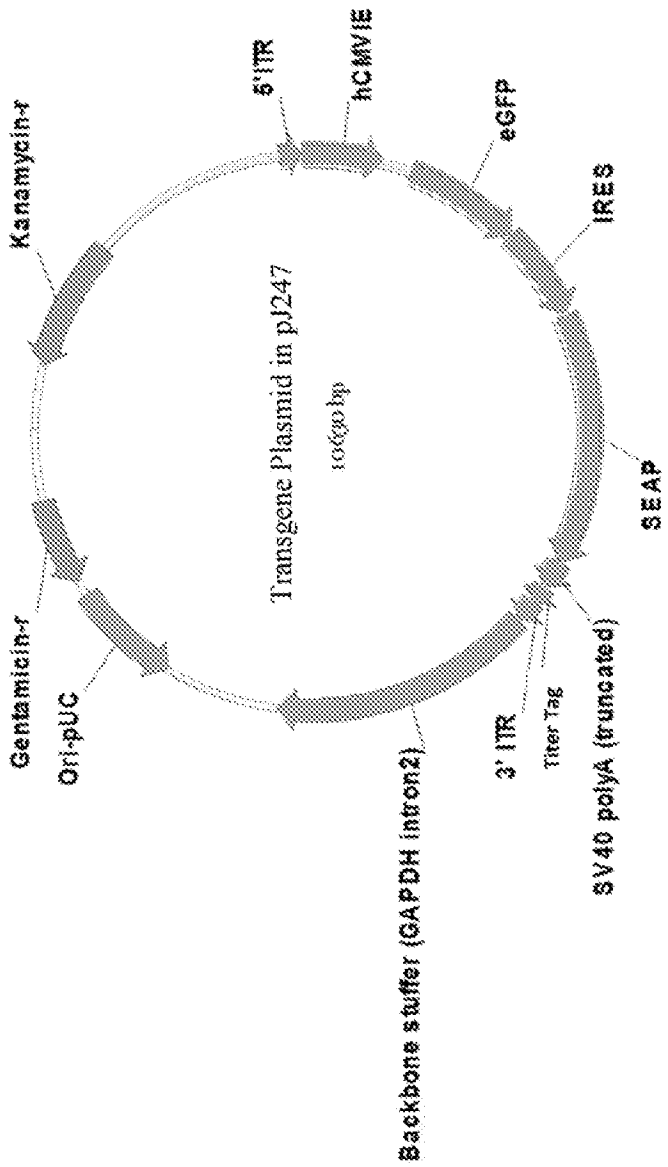
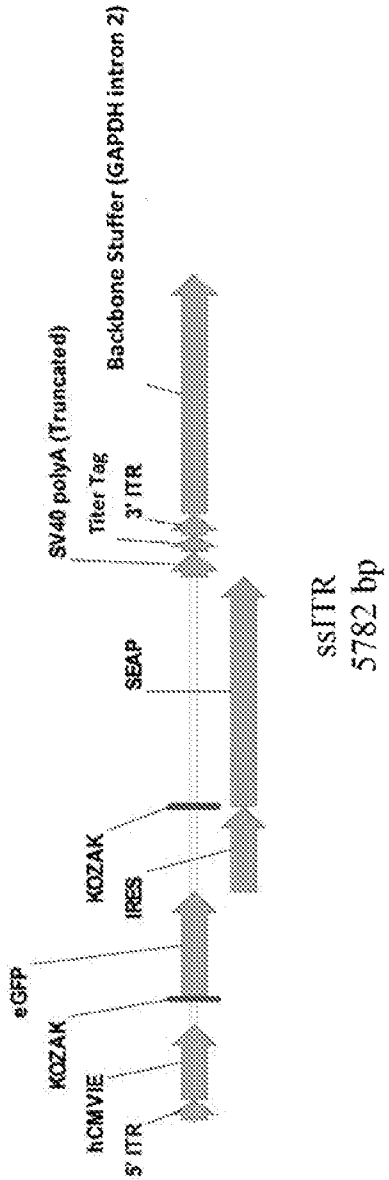
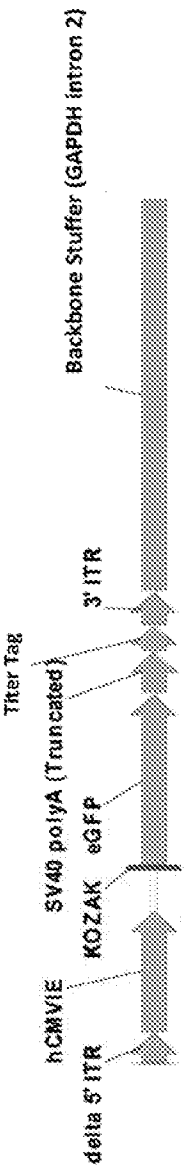

Transgene Plasmid in pJ247
10650 bp
5'ITR
hCMVIE
eGFP
IRES
SEAP
SV40 polyA (truncated)
Titer Tag
3' ITR
Backbone stuffer (GAPDH intron2)
Ori-pUC
Gentamicin-r
Kanamycin-r

Figure 2



ssITR
5782 bp

Figure 3A



scITR
3621 bp

Figure 3B

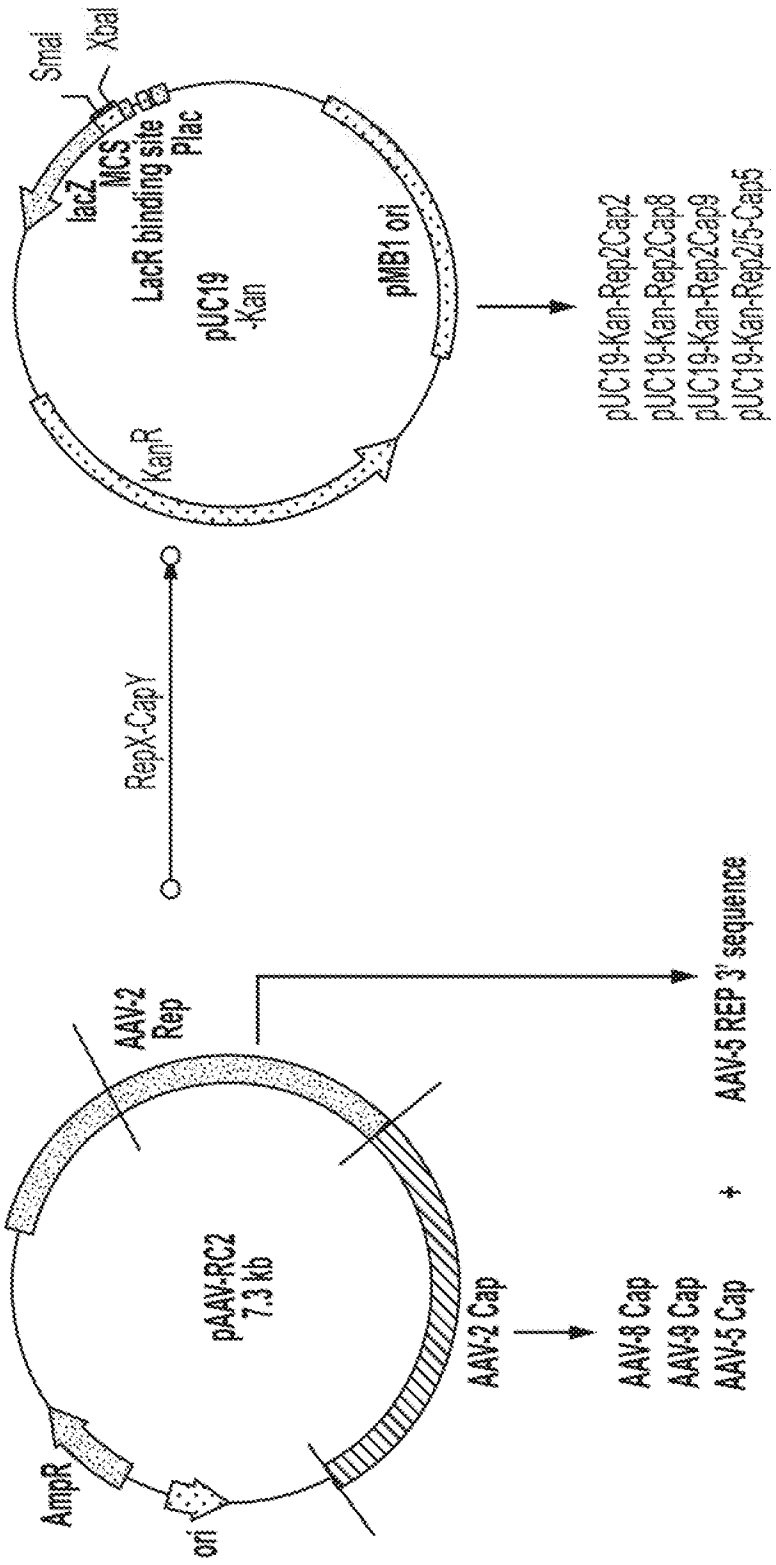

SmaI
XbaI
lacZ
MCS
LacR binding site
Plac
pUC19
-Kan
pMB1 ori
KanR
pUC19-Kan-Rep2Cap2
pUC19-Kan-Rep2Cap8
pUC19-Kan-Rep2Cap9
pUC19-Kan-Rep2/5-Cap5
RepX-CapY
AAV-2
Rep
pAAV-RC2
7.3 kb
AAV-2 Cap
AAV-8 Cap
AAV-9 Cap
AAV-5 Cap
+
AAV-5 REP 3' sequence
AmpR
ori

Figure 4A

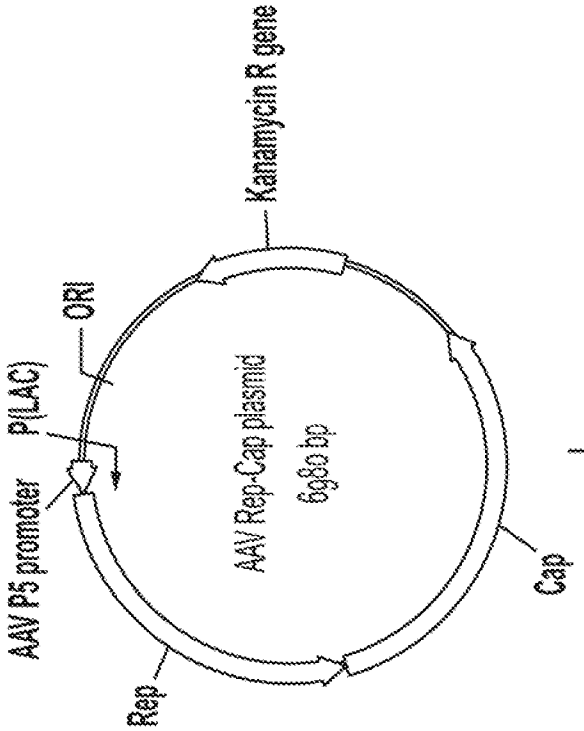

Kanamycin R gene
ORI
P(LAC)
AAV P5 promoter
AAV Rep-Cap plasmid
6g6o bp
Rep
Cap
pUC19-Kan-P5-Rep2Cap2
pUC19-Kan-P5-Rep2Cap8
pUC19-Kan-P5-Rep2Cap9

Figure 4B

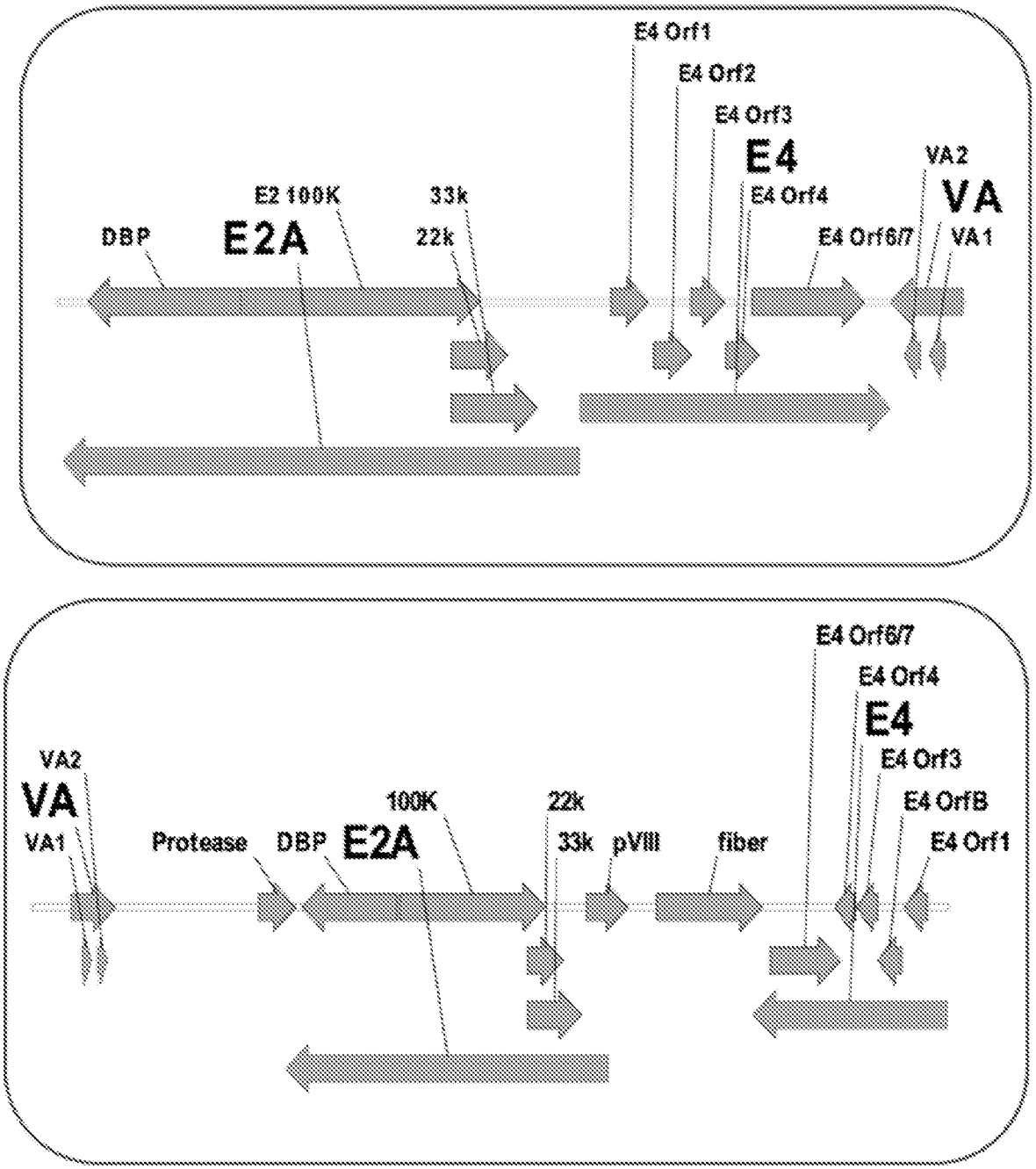

E4 Orf1
E4 Orf2
E4 Orf3
E4
VA2
VA
E2 100K
33k
E4 Orf4
DBP
E2A
22k
E4 Orf6/7
VA1
E4 Orf6/7
E4 Orf4
E4
VA2
E4 Orf3
VA
100K
22k
E4 OrfB
VA1
Protease
DBP
E2A
33k
pVIII
fiber
E4 Orf1

Figure 5

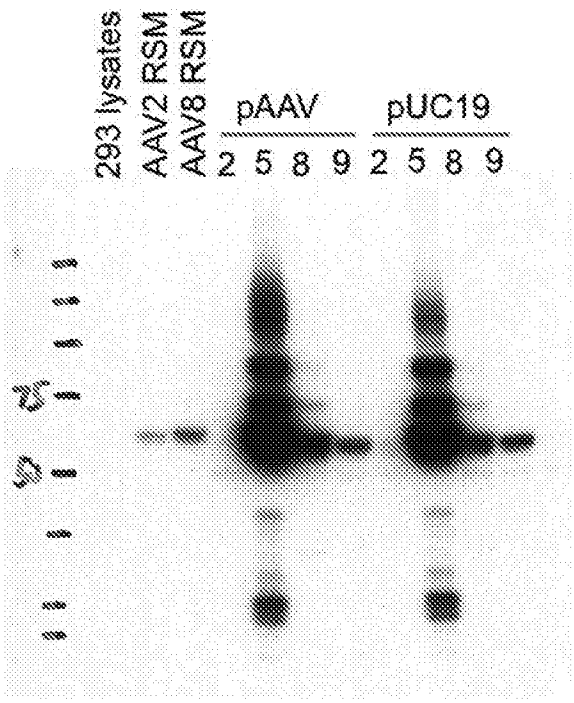


Figure 6

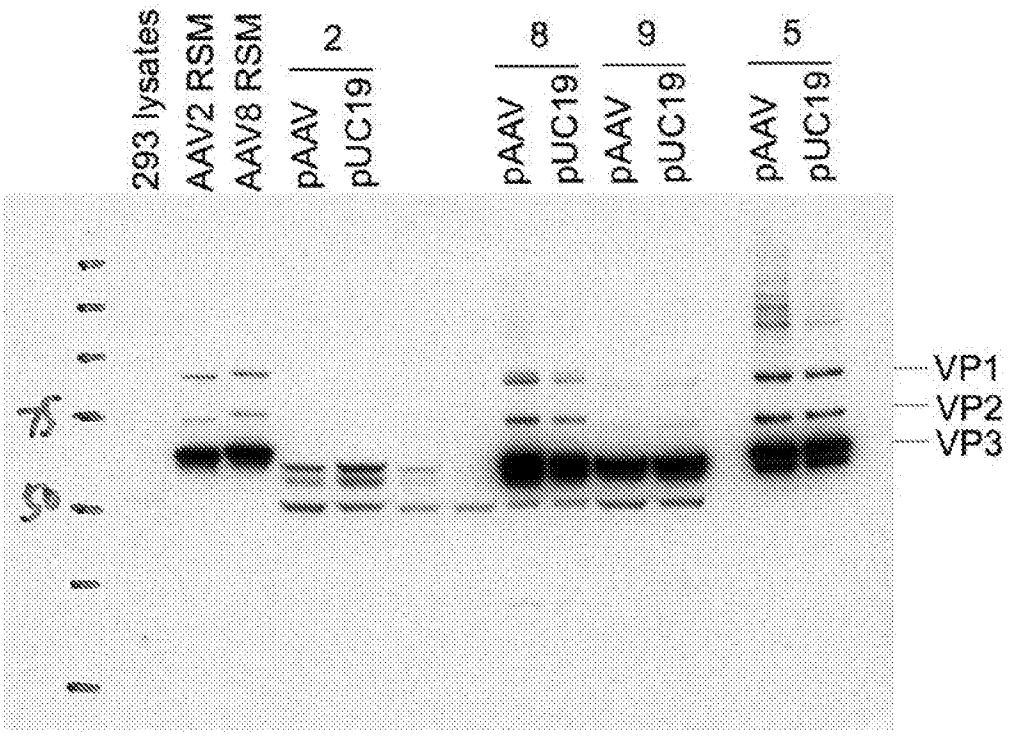

293 lysates
AAV2 RSM
AAV8 RSM
2
pAAV
pUC19
8
pAAV
pUC19
9
pAAV
pUC19
5
pAAV
pUC19
VP1
VP2
VP3

Figure 7

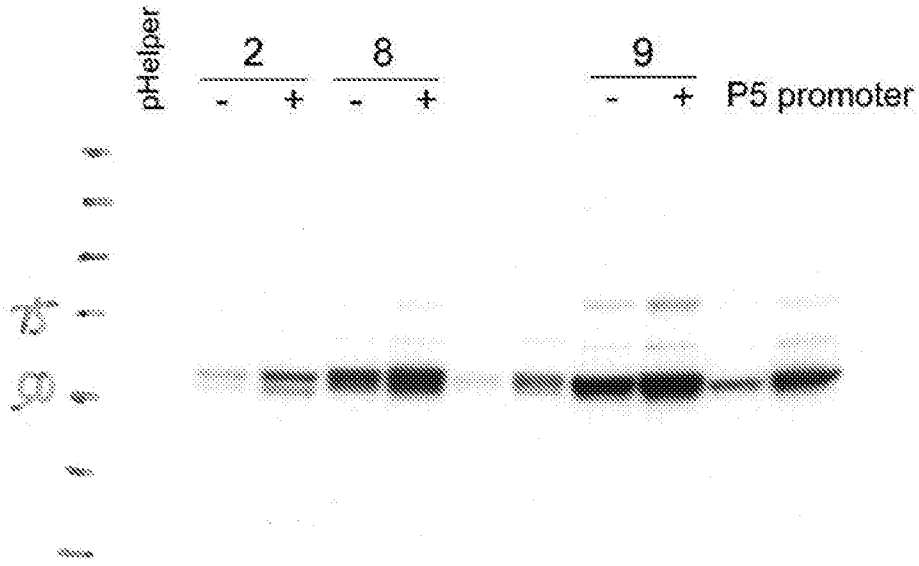


Figure 8

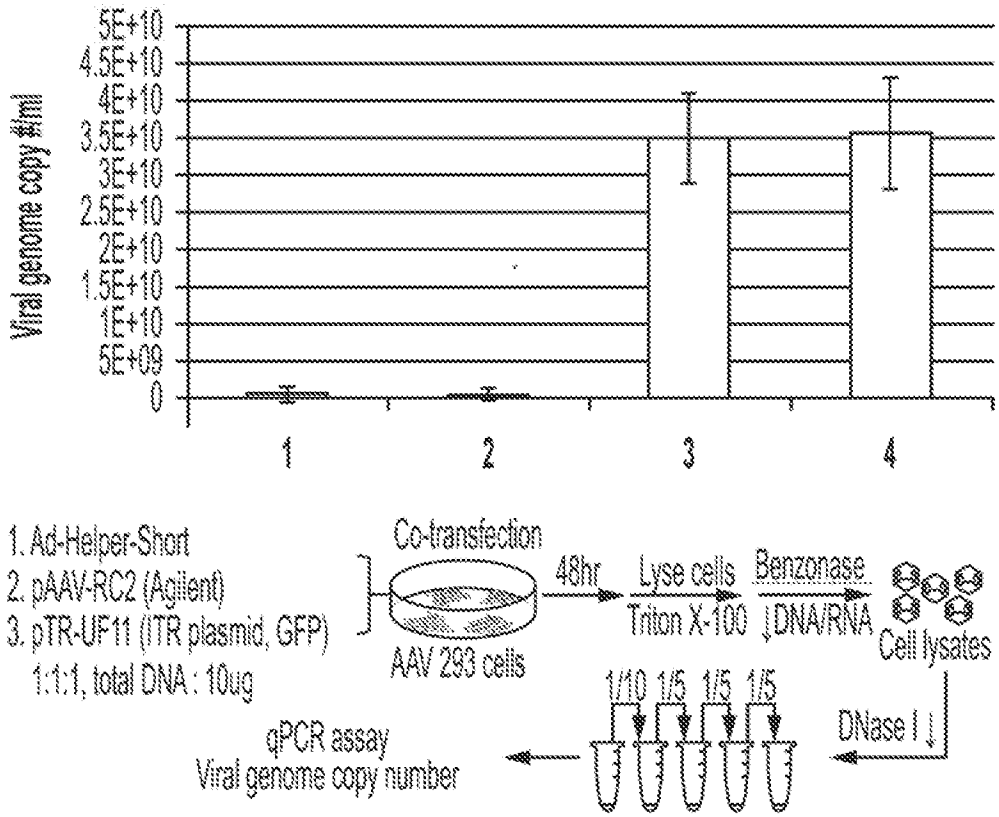


Figure 9

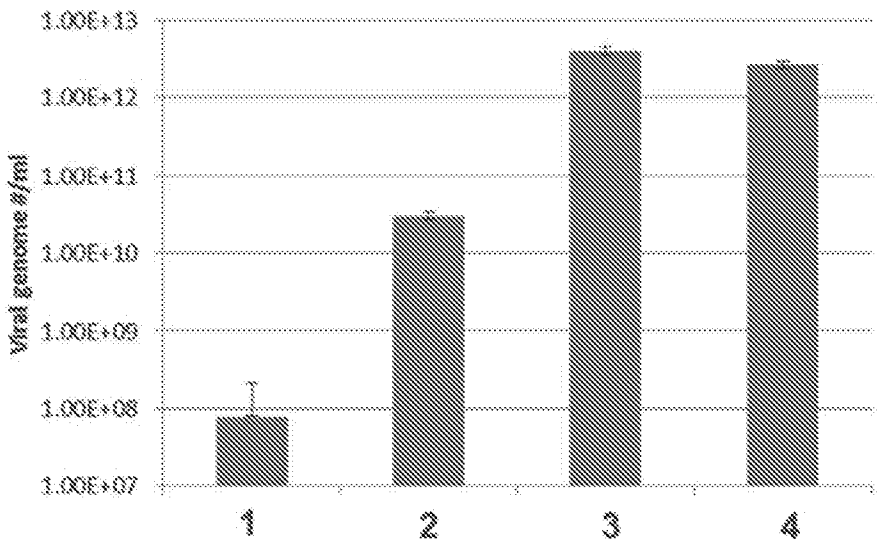


Figure 10

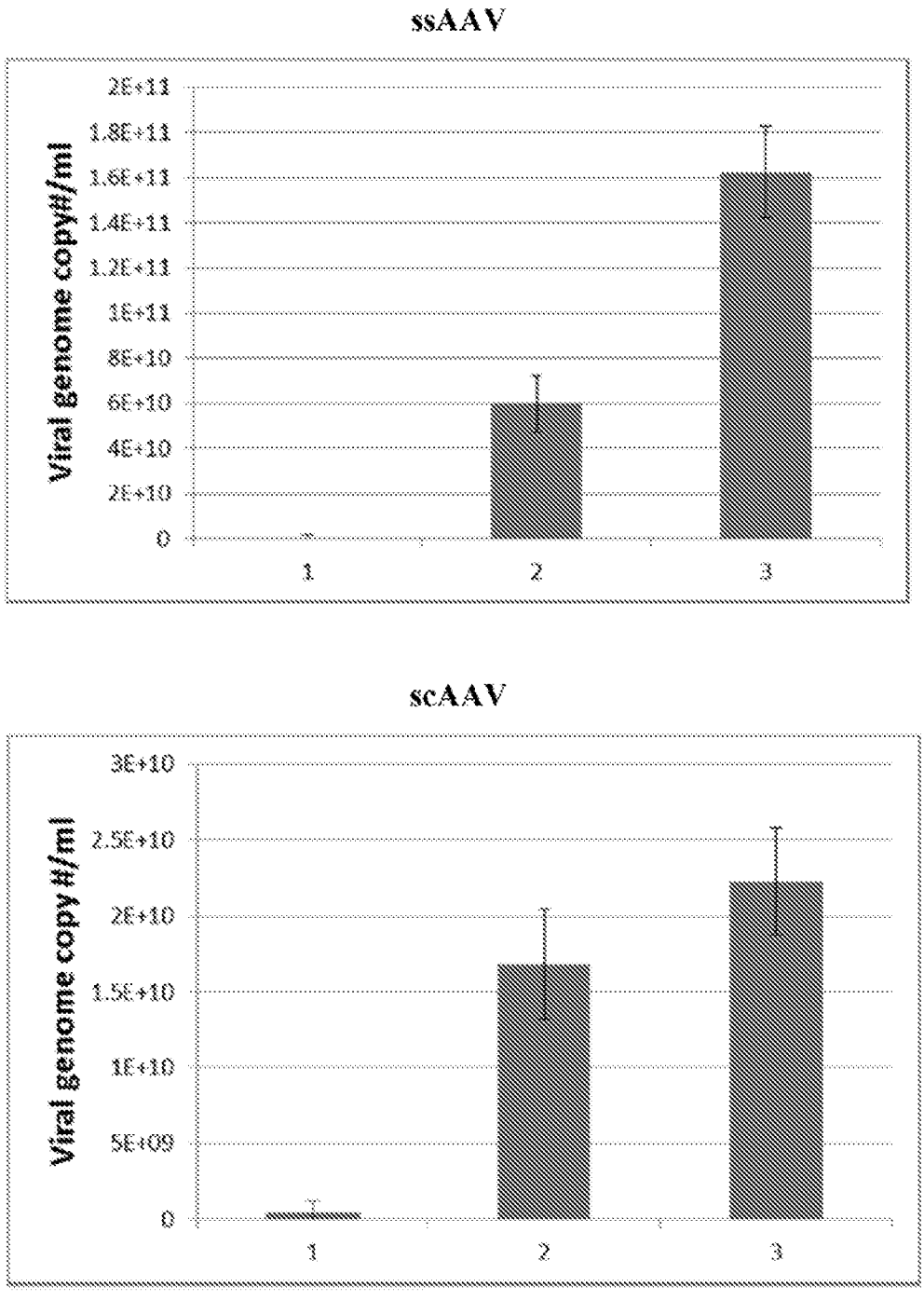

ssAAV
Viral genome copy#/ml
2E+11
1.8E+11
1.6E+11
1.4E+11
1.2E+11
1E+11
8E+10
6E+10
4E+10
2E+10
0
1
2
3
scAAV
Viral genome copy #/ml
3E+10
2.5E+10
2E+10
1.5E+10
1E+10
5E+09
0
1
2
3

Figure 11

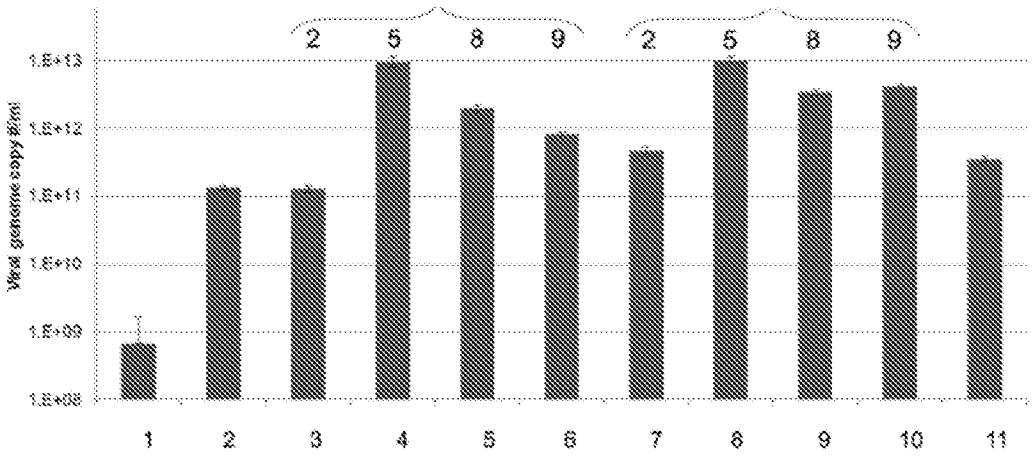
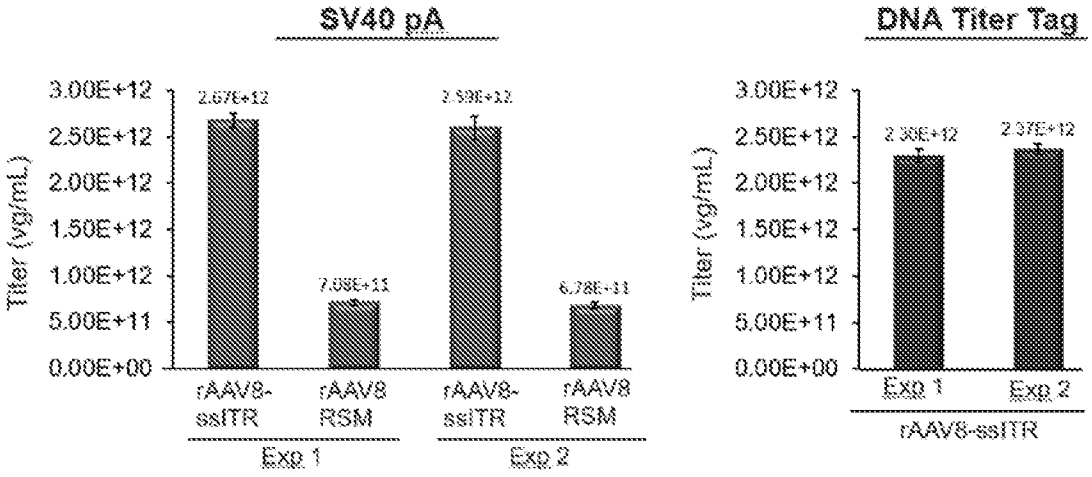
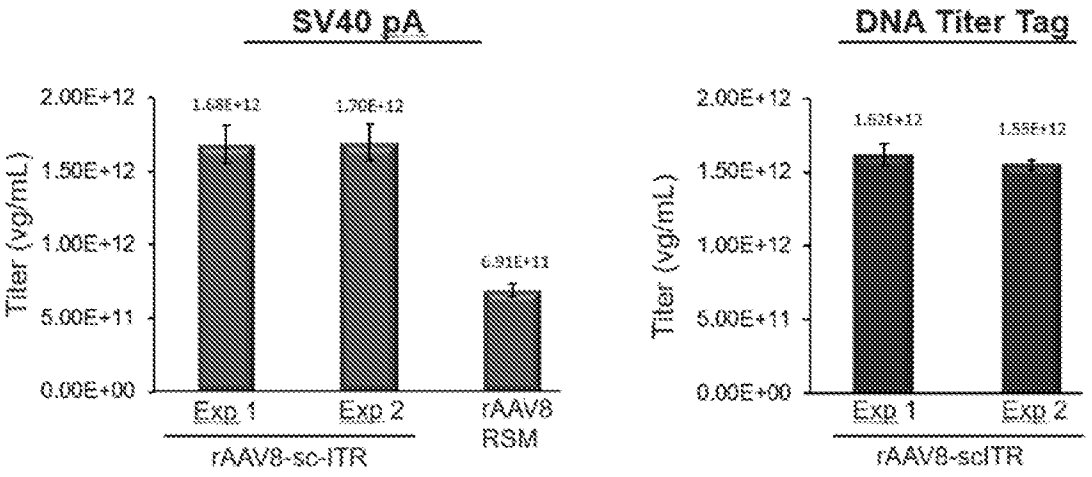


Figure 12



Target sequence	rAAV8-ssITR vg titer Mean
SV40 pA	2.63E+12
DNA titer tag	2.33E+12

Figure 13A



Target sequence	rAAV8-scITR vg titer Mean
SV40 pA	1.69E+12
Dna titer tag	1.58E+12

Figure 13B

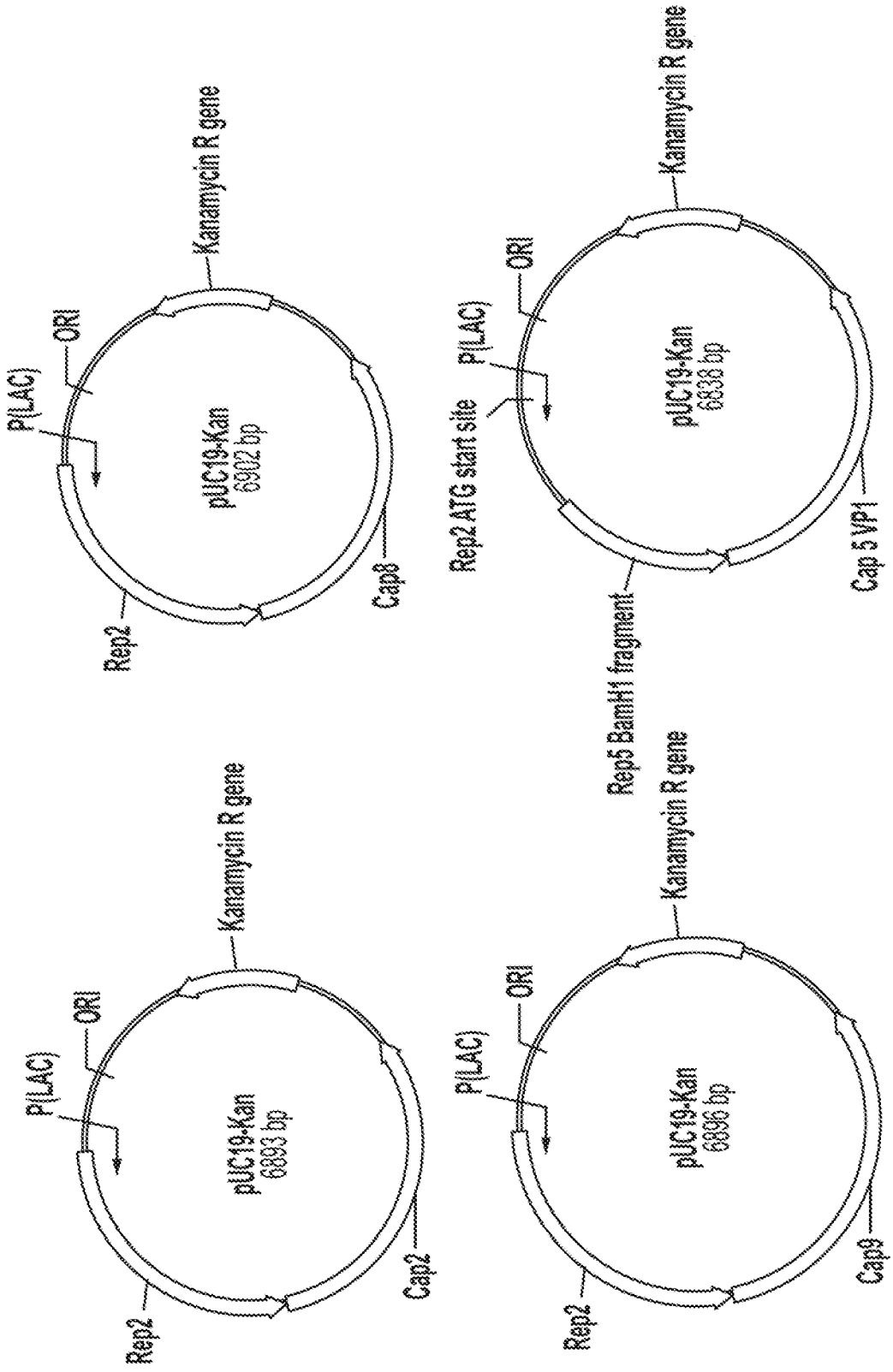

P(LAC)
ORI
Kanamycin R gene
pUC19-Kan
6902 bp
Rep2
Cap3
P(LAC)
ORI
Kanamycin R gene
pUC19-Kan
6838 bp
Rep2 ATG start site
Rep5 BamH1 fragment
Cap 5 VP1
P(LAC)
ORI
Kanamycin R gene
pUC19-Kan
6893 bp
Rep2
Cap2
P(LAC)
ORI
Kanamycin R gene
pUC19-Kan
6896 bp
Rep2
Cap9

Figure 14A

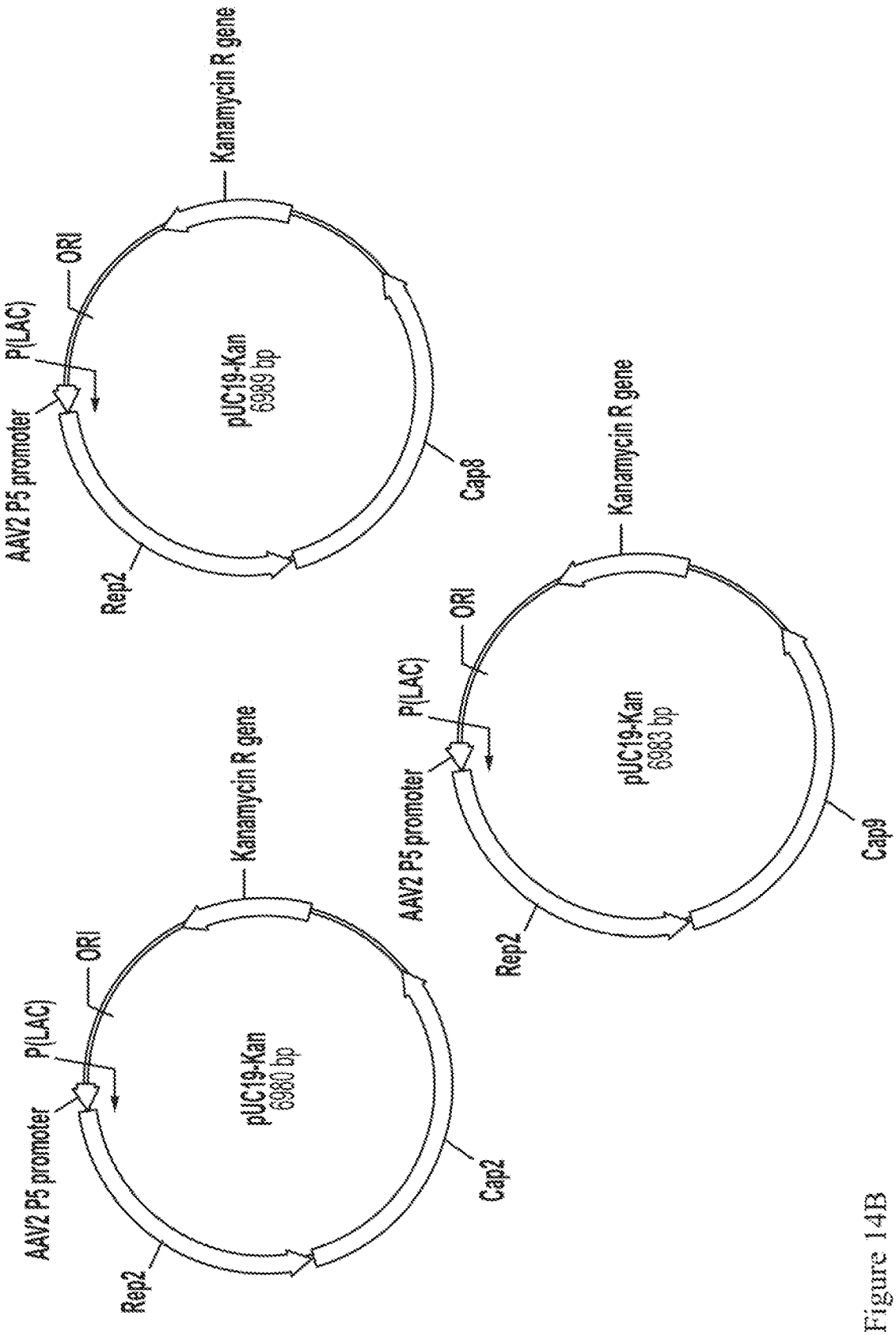

Kanamycin R gene
ORI
P(LAC)
AAV2 P5 promoter
pUC19-Kan
6980 bp
Cap8
Rep2
Kanamycin R gene
ORI
P(LAC)
AAV2 P5 promoter
pUC19-Kan
6983 bp
Cap9
Rep2
Kanamycin R gene
ORI
P(LAC)
AAV2 P5 promoter
pUC19-Kan
6980 bp
Cap2
Rep2

Figure 14B

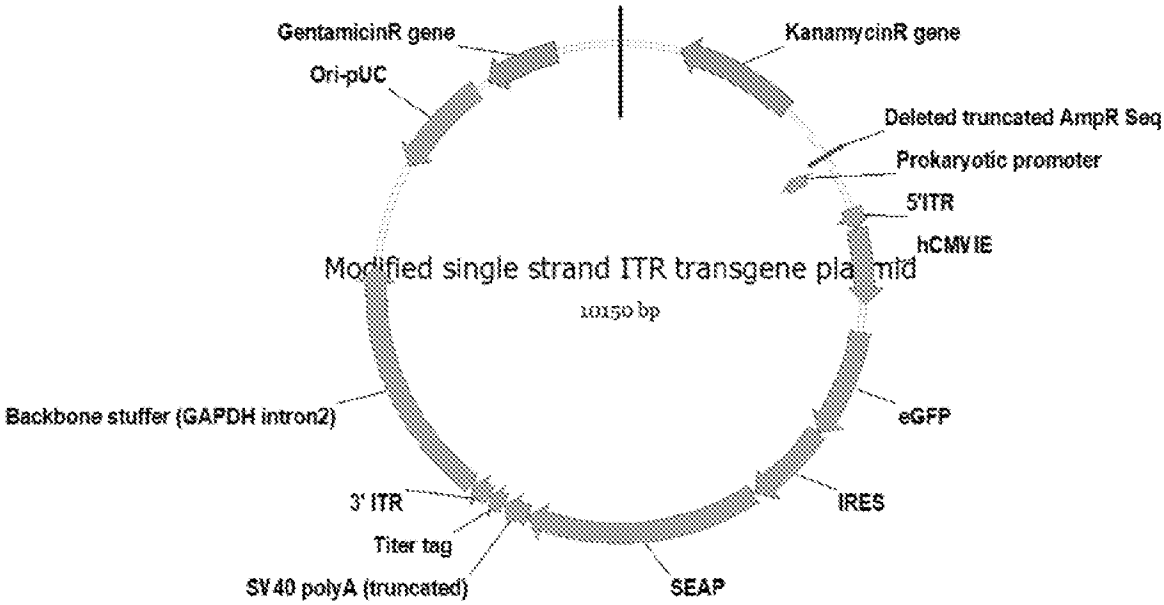


Figure 15B

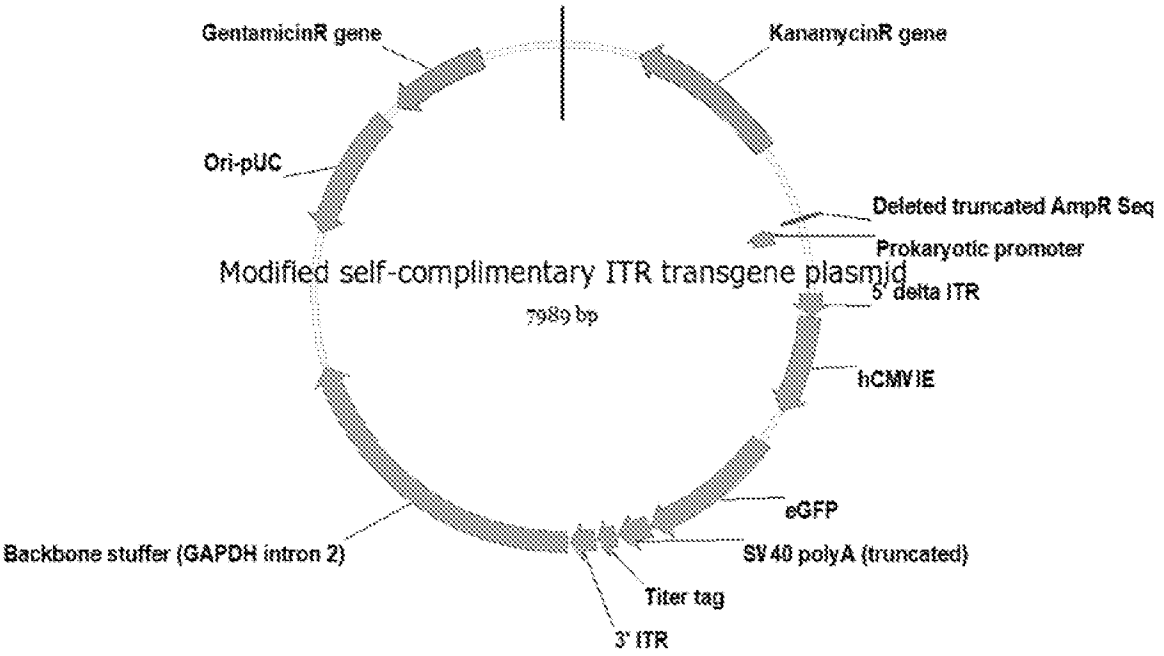
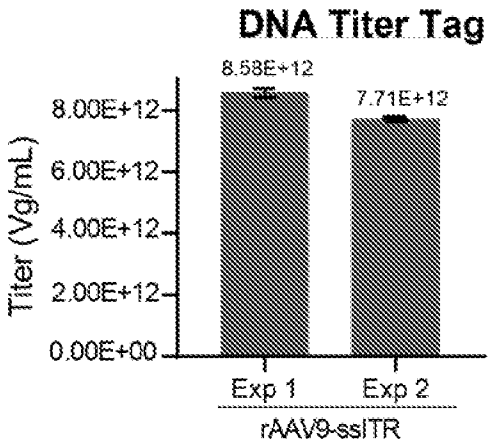
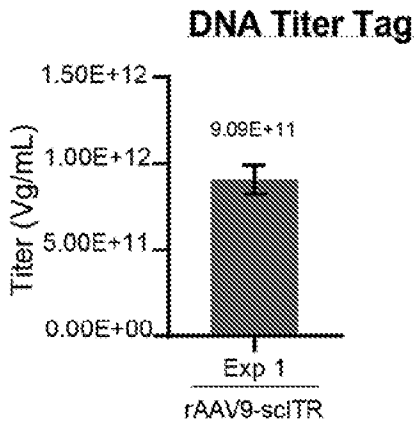


Figure 15B



	rAAV9-ssITR vg titer
Target sequence	Mean
DNA titer tag	8.14E+12

Figure 16A



	rAAV9-scITR vg titer
Target sequence	Mean
DNA titer tag	9.09E+11

Figure 16B

SEQUENCE LISTING

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BAXALTA GMBH

<120> AAV TRIPLE-PLASMID SYSTEM

<130> 250478.001858

<140>
<141>

<150> 62/750,603
<151> 2018-10-25

<160> 77

<170> PatentIn version 3.5

<210> 1
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<212> DNA
<213> Artificial Sequence

<220>
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polynucleotide

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<222> (6316)..(6415)
<223> a, c, t, g, unknown or other

<220>
<221> misc_feature
<222> (6316)..(6415)
<223> This region may encompass 60-100 nucleotides

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actgccggaa atcgtcgtgt gcactcatgg aaaacggtgt aacaagggtg aacactatcc 120

catatcacca gctcaccgtc tttcattgcc atacggaact ccggatgagc attcatcagg 180

cgggcaagaa tgtgaataaa ggccggataa aacttgtgct tatttttctt tacggtcttt 240

aaaaaggccg taatatccag ctgaacggtc tggttatagg tacattgagc aactgactga 300

aatgcctcaa aatgttcttt acgatgccat tgggatatat caacggtggt atatccagtg 360

atttttttct ccatttttt ttcctccttt agaaaaactc atcgagcatc aaatgaaact 420

gcaatttatt catatcagga ttatcaatac catatttttg aaaaagccgt ttctgtaatg 480

aaggagaaaa ctcaccgagg cagttccata ggatggcaag atcctggtat cggtctgcga 540

ttccgactcg tccaacatca atacaaccta ttaatttccc ctcgtcaaaa ataaggttat 600

caagtgagaa atcaccatga gtgacgactg aatccggtga gaatggcaaa agtttatgca 660

tttctttcca gacttgttca acaggccagc cattacgctc gtcatcaaaa tcactcgcat 720

caaccaaacc gttattcatt cgtgattgcg cctgagcgag gcgaaatacg cgatcgctgt 780

taaaaggaca attacaaaca ggaatcgagt gcaaccggcg caggaacact gccagcgcat 840

caacaatatt ttcacctgaa tcaggatatt cttctaatac ctggaacgct gtttttccgg 900

ggatcgcagt ggtgagtaac catgcatcat caggagtacg gataaaatgc ttgatggtcg 960

gaagtggcat aaattccgtc agccagttta gtctgaccat ctcatctgta acatcattgg 1020

caacgctacc tttgccatgt ttcagaaaca actctggcgc atcgggcttc ccatacaagc 1080

gatagattgt cgcacctgat tgcccgacat tatcgcgagc ccatttatac ccatataaat 1140

cagcatccat gttggaattt aatcgcggcc tcgacgtttc ccgttgaata tggctcattt 1200

ttttttcctc ctttaccaat gcttaatcag tgaggcacct atctcagcga tctgtctatt 1260

tcgttcatcc atagttgcct gactccccgt cgtgtagata actacgatac gggagggctt 1320

accatctggc cccagcgctg cgatgatacc gcgagaacca cgctcaccgg ctccggattt 1380

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cataattctc ttactgtcat gccatccgta agatgctttt ctgtgactgg tgagtactca 1740

accaagtcat tctgagaata gtgtatgcgg cgaccgagtt gctcttgccc ggcgtcaata 1800

cgggataata ccgcgccaca tagcagaact ttaaaagtgc tcatcattgg aaaacgttct 1860

tcggggcgaa aactctcaag gatcttaccg ctgttgagat ccagttcgat gtaacccact 1920

cgtgcaccca actgatcttc agcatctttt actttcacca gcgtttctgg gtgagcaaaa 1980

acaggaaggc aaaatgccgc aaaaaaggga ataagggcga cacggaaatg ttgaatactc 2040

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tttgcctggc ggcagtagcg cggtggtccc acctgacccc atgccgaact cagaagtgaa 2280

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atcaaataaa acgaaaggct cagtcgaaag actgggcctt tcgcccgggc taattgaggg 2400

gtgtcgccct tattcgactc ggggctcgag ctgcgcgctc gctcgctcac tgaggccgcc 2460

cgggcaaagc ccgggcgtcg ggcgaccttt ggtcgcccgg cctcagtgag cgagcgagcg 2520

cgcagagagg gagtggccaa ctccatcact aggggttcct ttaattaaac gcgtttacat 2580

aacttacggt aaatggcccg cctggctgac cgcccaacga cccccgccca ttgacgtcaa 2640

taatgacgta tgttcccata gtaacgccaa tagggacttt ccattgacgt caatgggtgg 2700

actatttacg gtaaactgcc cacttggcag tacatcaagt gtatcatatg ccaagtacgc 2760

cccctattga cgtcaatgac ggtaaatggc ccgcctggca ttatgcccag tacatgacct 2820

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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

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ggtcgcccgg cctcagtgag cgagcgagcg cgcagagagg gagtggccaa ctccatcact 120

aggggttcct 130

<210> 3
<211> 133
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

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gagcgcgcag aga 133

<210> 4

<211> 503
<212> DNA
<213> Human cytomegalovirus

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gtgtacggtg ggaggtctat ata 503

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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

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ggcatcaagg tgaacttcaa gatccgccac aacatcgagg acggcagcgt gcagctcgcc 540

gaccactacc agcagaacac ccccatcggc gacggccccg tgctgctgcc cgacaaccac 600

tacctgagca cccagtccgc cctgagcaaa gaccccaacg agaagcgcga tcacatggtc 660

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tag 723

<210> 6
<211> 575
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

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<210> 7
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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

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<211> 168
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic

polynucleotide

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attctagttg tggtttgtcc aaactcatca atgtatctta tcatgtct 168

<210> 9
<211> 1632
<212> DNA
<213> Homo sapiens

<400> 9
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tcagttgcag ccatgcctta agccaggcca gcctggcagg gaagctcaag ggagataaaa 1500

ttcaacctct tgggccctcc tgggggtaag gagatgctgc attcgccctc ttaatgggga 1560

ggtggcctag ggctgctcac atattctgga ggagcctccc ctcctcatgc cttcttgcct 1620

cttgtctctt ag 1632

<210> 10
<211> 100
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

<220>
<221> modified_base
<222> (1)..(100)
<223> a, c, t, g, unknown or other

<220>
<221> misc_feature
<222> (1)..(100)
<223> This sequence may encompass 60-100 nucleotides

<400> 10
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nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 100

<210> 11
<211> 1845
<212> DNA
<213> Adeno-associated virus

<400> 11
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tctgacatgg atctgaatct gattgagcag gcacccctga ccgtggccga gaagctgcag 180

cgcgactttc tgacggaatg gcgccgtgtg agtaaggccc cggaggccct tttctttgtg 240

caatttgaga agggagagag ctacttccac atgcacgtgc tcgtggaaac caccggggtg 300

aaatccatgg ttttgggacg tttcctgagt cagattcgcg aaaaactgat tcagagaatt 360

taccgcggga tcgagccgac tttgccaaac tggttcgcgg tcacaaagac cagaaatggc 420

gccggaggcg ggaacaaggt ggtggatgag tgctacatcc ccaattactt gctccccaaa 480

acccagcctg agctccagtg ggcgtggact aatatggaac agtatttaag cgcctgtttg 540

aatctcacgg agcgtaaacg gttggtggcg cagcatctga cgcacgtgtc gcagacgcag 600

gagcagaaca aagagaatca gaatcccaat tctgatgcgc cggtgatcag atcaaaaact 660

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cagtggatcc aggaaaatca ggagagctac ctctccttca actccaccgg caactctcgg 780

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aatgaaaact ttccctttaa tgactgtgtg gacaaaatgc tcatttggtg ggaggaggga 1140

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gtcgatcaga aatgtaaatc ctctgttcaa attgattcta cccctgtcat tgtaacttcc 1260

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ttgtcagatt ttggggattt tgacgatgcc aataaagaac agtaa 1845

<210> 12
<211> 1866
<212> DNA
<213> Adeno-associated virus

<400> 12
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tctgacatgg atctgaatct gattgagcag gcacccctga ccgtggccga gaagctgcag 180

cgcgactttc tgacggaatg gcgccgtgtg agtaaggccc cggaggccct tttctttgtg 240

caatttgaga agggagagag ctacttccac atgcacgtgc tcgtggaaac caccggggtg 300

aaatccatgg ttttgggacg tttcctgagt cagattcgcg aaaaactgat tcagagaatt 360

taccgcggga tcgagccgac tttgccaaac tggttcgcgg tcacaaagac cagaaatggc 420

gccggaggcg ggaacaaggt ggtggatgag tgctacatcc ccaattactt gctccccaaa 480

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ccagacgctt gcactgcctg cgatctggtc aatgtggatt tggatgactg catctttgaa 1860

caataa 1866

<210> 13
<211> 2211
<212> DNA
<213> Adeno-associated virus

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aacgctcgag gtcttgtgct tccgggttac aaataccttg gacccggcaa cggactcgac 180

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<210> 14
<211> 12003
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

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gcgtaatgct ctgcttttag aaaaactcat cgagcatcaa atgaaactgc aatttattca 180

tatcaggatt atcaatacca tatttttgaa aaagccgttt ctgtaatgaa ggagaaaact 240

caccgaggca gttccatagg atggcaagat cctggtatcg gtctgcgatt ccgactcgtc 300

caacatcaat acaacctatt aatttcccct cgtcaaaaat aaggttatca agtgagaaat 360

caccatgagt gacgactgaa tccggtgaga atggcaaaag tttatgcatt tctttccaga 420

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cacctgaatc aggatattct tctaatacct ggaacgctgt ttttccgggg atcgcagtgg 660

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<223> Description of Artificial Sequence: Synthetic
polynucleotide

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<211> 810
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

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taggatggca agatcctggt atcggtctgc gattccgact cgtccaacat caatacaacc 180

tattaatttc ccctcgtcaa aaataaggtt atcaagtgag aaatcaccat gagtgacgac 240

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tagtctgacc atctcatctg taacatcatt ggcaacgcta cctttgccat gtttcagaaa 660

caactctggc gcatcgggct tcccatacaa gcgatagatt gtcgcacctg attgcccgac 720

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<210> 20
<211> 674
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
polynucleotide

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cgaaacccga caggactata aagataccag gcgtttcccc ctggaagctc cctcgtgcgc 180

tctcctgttc cgaccctgcc gcttaccgga tacctgtccg cctttctccc ttcgggaagc 240

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tatcgtcttg agtccaaccc ggtaagacac gacttatcgc cactggcagc agccactggt 420

aacaggatta gcagagcgag gtatgtaggc ggtgctacag agttcttgaa gtggtgggct 480

aactacggct acactagaag aacagtattt ggtatctgcg ctctgctgaa gccagttacc 540

ttcggaaaaa gagttggtag ctcttgatcc ggcaaacaaa ccaccgctgg tagcggtggt 600

ttttttgttt gcaagcagca gattacgcgc agaaaaaaag gatctcaaga agatcctttg 660

atcttttcta cggg 674

<210> 21
<211> 118
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

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tgtatttaga aaaataaaca aatagggggtc agtgttacaa ccaattaacc aattctga 118

<210> 22
<211> 301
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

<400> 22

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tcgttgacat gtataatttt gatatcaaaa actgattttc cctttattat tttcgagatt 120

tattttctta attctcttta acaaactaga aatattgtat atacaaaaaa tcataaataa 180

tagatgaata gtttaattat aggtgttcat caatcgaaaa agcaacgtat cttatttaaa 240

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<210> 23
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<212> DNA
<213> Artificial Sequence

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<223> Description of Artificial Sequence: Synthetic
polynucleotide

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ccgaagcggg tttttacgtt atttgcggat taacgattac tcgttatcag aaccgcccag 120

<210> 24
<211> 6838
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

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gcctggggtg cctaatgagt gagctaactc acattaattg cgttgcgctc actgcccgct 180

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aagcaaaaaa gtccttgact tcctgcttag taatcttgcc aaaatctggc gggagccgct 5400

tagtcagttc aaatttgaac atgcggtcct ccagcggctg ctggtgttca aaggtcgtgg 5460

aattcccatc caccaccaca cacatgtttg tattggaagt tacaatgaca ggggtagaat 5520

caatttgaac agaggattta catttctgat cgacccgcac ctttgagccc cccaggatgg 5580

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ggaccttaat cacaatctcg taaaaccccg gcatggcggc tgcgcagatc agaagttcct 6780

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<210> 25
<211> 816
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

<400> 25
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taggatggca agatcctggt atcggtctgc gattccgact cgtccaacat caatacaacc 180

tattaatttc ccctcgtcaa aaataaggtt atcaagtgag aaatcaccat gagtgacgac 240

tgaatccggt gagaatggca aaagtttatg catttctttc cagacttgtt caacaggcca 300

gccattacgc tcgtcatcaa aatcactcgc atcaaccaaa ccgttattca ttcgtgattg 360

cgcctgagcg agacgaaata cgcgatcgct gttaaaagga caattacaaa caggaatcga 420

atgcaaccgg cgcaggaaca ctgccagcgc atcaacaata ttttcacctg aatcaggata 480

ttcttctaat acctggaatg ctgttttccc agggatcgca gtggtgagta accatgcatc 540

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tagtctgacc atctcatctg taacatcatt ggcaacgcta cctttgccat gtttcagaaa 660

caactctggc gcatcgggct tcccatacaa tcgatagatt gtcgcacctg attgcccgac 720

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cctagagcaa gacgtttccc gttgaatatg gctcat 816

<210> 26
<211> 673
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

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gaaacccgac aggactataa agataccagg cgtttccccc tggaagctcc ctcgtgcgct 180

ctcctgttcc gaccctgccg cttaccggat acctgtccgc ctttctccct tcgggaagcg 240

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tcggaaaaag agttggtagc tcttgatccg gcaaacaaac caccgctggt agcggtggtt 600

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tcttttctac ggg 673

<210> 27
<211> 143
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

<400> 27
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tggggtgcct aatgagtgag ctaactcaca ttaattgcgt tgcgctcact gcccgctttc 120

cagtcgggaa acctgtcgtg cca 143

<210> 28
<211> 5121
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

<400> 28
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gctggtagcg gtggtttttt tgtttgcaag cagcagatta cgcgcagaaa aaaaggatct 120

caagaagatc ctttgatctt ttctacgggg tctgacgctc agtggaacga aaactcacgt 180

taagggattt tggtcatgtg attatcaaaa aggatcttca cctagatcct tttaaattaa 240

aaatgaagtt ttaaatcaat ctaaagtata tatgagtaaa cttggtctga cagttagaaa 300

aactcatcga gcatcaaatg aaactgcaat ttattcatat caggattatc aataccatat 360

ttttgaaaaa gccgtttctg taatgaagga gaaaactcac cgaggcagtt ccataggatg 420

gcaagatcct ggtatcggtc tgcgattccg actcgtccaa catcaataca acctattaat 480

ttcccctcgt caaaaataag gttatcaagt gagaaatcac catgagtgac gactgaatcc 540

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gcgagacgaa atacgcgatc gctgttaaaa ggacaattac aaacaggaat cgaatgcaac 720

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accatctcat ctgtaacatc attggcaacg ctacctttgc catgtttcag aaacaactct 960

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caagacgttt cccgttgaat atggctcata ctcttccttt ttcaatatta ttgaagcatt 1140

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gcgaaagggg gatgtgctgc aaggcgatta agttgggtaa cgccagggtt ttcccagtca 1680

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cggagctccc cacgaggtag tccaccgcgc tttttgtcag actcataatt ttggtcgcgt 5040

tgtcgagcgc ggccttgatc tggctccgag agttgccggt ggagttgaag gagaggtagc 5100

tctcctgatt ttcctggatc c 5121

<210> 29
<211> 2175
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

<400> 29
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tgggttccac ctcttggagt tttccttctt gagctcccac tccatctcca cggtgacctg 180

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tccgggcaca ggcgtgttct tgatgagcat catgggcggt gggtgtttga gtccgaatcc 300

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gttgacaggc tctcctcgat cgagaccgtt tccgggtccg agatagttat aaccaggcag 2040

cacaagacca cgggcttgat cttgatgctg ctgattgggt tttggtttcg gtgggcccgc 2100

ttcaaggccc aaaaactcgc gaagaccttc accaacttct tccaaccaat ctggagggtg 2160

atcaacaaaa gacat 2175

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<211> 3
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
oligonucleotide

<400> 30
cat 3

<210> 31
<211> 6980
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

<400> 31
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gcctggggtg cctaatgagt gagctaactc acattaattg cgttgcgctc actgcccgct 180

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<223> Description of Artificial Sequence: Synthetic
oligonucleotide

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<210> 39
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polynucleotide

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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic polynucleotide

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polynucleotide

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tacatatttg aatgtattta gaaaaataaa caaatagggg ttccgcgcac atttccccga 11160

aaagtgccac ctaaattgta agcgttaata ttttgttaaa attcgcgtta aatttttgtt 11220

aaatcagctc atttttaac caataggccg aaatcggcaa aatcccttat aaatcaaaag 11280

aatagaccga gatagggttg agtgttgttc cagtttggaa caagagtcca ctattaaaga 11340

acgtggactc caacgtcaaa gggcgaaaaa ccgtctatca gggcgatggc ccactacgtg 11400

aaccatcacc ctaatcaagt tttttggggt cgaggtgccg taaagcacta aatcggaacc 11460

ctaaagggag cccccgattt agagcttgac ggggaaagcc ggcgaacgtg gcgagaaagg 11520

aagggaagaa agcgaaagga gcgggcgcta gggcgctggc aagtgtagcg gtcacgctgc 11580

gcgtaaccac cacacccgcc gcgcttaatg cgccgctaca gggcgcgatg gatcc 11635

<210> 46
<211> 5336
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

<400> 46
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acagctctac agcttcctgg agcgccactc gccctacttc cgcagccaca gtgcgcagat 120

taggagcgcc acttcttttt gtcacttgaa aaacatgtaa aaataatgta ctaggagaca 180

ctttcaataa aggcaaatgt ttttatttgt acactctcgg gtgattattt acccccccacc 240

cttgccgtct gcgccgttta aaaatcaaag gggttctgcc gcgcatcgct atgcgccact 300

ggcagggaca cgttgcgata ctggtgttta gtgctccact taaactcagg cacaaccatc 360

cgcggcagct cggtgaagtt ttcactccac aggctgcgca ccatcaccaa cgcgtttagc 420

aggtcgggcg ccgatatctt gaagtcgcag ttggggcctc cgccctgcgc gcgcgagttg 480

cgatacacag ggttgcagca ctggaacact atcagcgccg ggtggtgcac gctggccagc 540

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gtcaactttg gtagctgcct tcccaaaaag ggtgcatgcc caggctttga gttgcactcg 660

caccgtagtg gcatcagaag gtgaccgtgc ccggtctggg cgttaggata cagcgcctgc 720

atgaaagcct tgatctgctt aaaagccacc tgagcctttg cgccttcaga gaagaacatg 780

ccgcaagact tgccggaaaa ctgattggcc ggacaggccg cgtcatgcac gcagcacctt 840

gcgtcggtgt tggagatctg caccacattt cggccccacc ggttcttcac gatcttggcc 900

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accaggaaag tcccgctccc accactgtgg tacttcccag agacgcccag gccgaagttc 5280

agatgactaa ctcaggggcg cagcttgcgg gcggctttcg tcacagggtg cggtcg 5336

<210> 47
<211> 3201
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

<400> 47
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ggaagtgacg tatcgtggga aaacggaagt gaagatttga ggaagttgtg ggtttttgg 120

ctttcgtttc tgggcgtagg ttcgcgtgcg gttttctggg tgtttttttgt ggactttaac 180

cgttacgtca ttttttagtc ctatatatac tcgctctgta cttggccctt tttacactgt 240

gactgattga gctggtgccg tgtcgagtgg tgtttttttaa taggtttttt tactggtaag 300

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gtcttacatt atgactccgg acatgaccga ggaactgtcg gtggtgcttt ttaatcacgg 600

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attctttatt cctgggcaat gtaggagaag gtgtaagagt tggtagcaaa agtttcagtg 3120

gtgtattttc cactttccca ggaccatgta aaagacatag agtaagtgct tacctcgcta 3180

gtttctgtgg attcactaga a 3201

<210> 48
<211> 743
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

<400> 48
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tgccgctgct cttgctcttg ccgctgctga ggagggggggc gcatctgccg cagcaccgga 120

tgcatctggg aaaagcaaaa aaggggctcg tccctgtttc cggaggaatt tgcaagcggg 180

gtcttgcatg acggggaggc aaacccccgt tcgccgcagt ccggccggcc cgagactcga 240

accgggggtc ctgcgactca acccttggaa aataaccctc cggctacagg gagcgagcca 300

cttaatgctt tcgctttcca gcctaaccgc ttacgccgcg cgcggccagt ggccaaaaaa 360

gctagcgcag cagccgccgc gcctggaagg aagccaaaag gagcgctccc ccgttgtctg 420

acgtcgcaca cctgggttcg acacgcgggc ggtaaccgca tggatcacgg cggacggccg 480

gatccggggt tcgaaccccg gtcgtccgcc atgataccct tgcgaattta tccaccagac 540

cacggaagag tgcccgctta caggctctcc ttttgcacgg tctagagcgt caacgactgc 600

gcacgcctca ccggccagag cgtcccgacc atggagcact ttttgccgct gcgcaacatc 660

tggaaccgcg tccgcgactt tccgcgcgcc tccaccaccg ccgccggcat cacctggatg 720

tccaggtaca tctacggatt acg 743

<210> 49
<211> 160
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

<400> 49
aaggggctcg tccctgtttc cggaggaatt tgcaagcggg gtcttgcatg acggggaggc 60

aaacccccgt tcgccgcagt ccggccggcc cgagactcga accgggggtc ctgcgactca 120

acccttggaa aataaccctc cggctacagg gagcgagcca 160

<210> 50
<211> 162
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

<400> 50
aaaggagcgc tcccccgttg tctgacgtcg cacacctggg ttcgacacgc gggcggtaac 60

cgcatggatc acggcggacg gccggatccg gggttcgaac cccggtcgtc cgccatgata 120

cccttgcgaa tttatccacc agaccacgga agagtgcccg ct 162

<210> 51
<211> 1590
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

<400> 51
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atactggtgt ttagtgctcc acttaaactc aggcacaacc atccgcggca gctcggtgaa 120

gttttcactc cacaggctgc gcaccatcac caacgcgttt agcaggtcgg gcgccgatat 180

cttgaagtcg cagttggggc ctccgccctg cgcgcgcgag ttgcgataca cagggttgca 240

gcactggaac actatcagcg ccgggtggtg cacgctggcc agcacgctct tgtcggagat 300

cagatccgcg tccaggtcct ccgcgttgct cagggcgaac ggagtcaact ttggtagctg 360

ccttcccaaa aagggtgcat gcccaggctt tgagttgcac tcgcaccgta gtggcatcag 420

aaggtgaccg tgcccggtct gggcgttagg atacagcgcc tgcatgaaag ccttgatctg 480

cttaaaagcc acctgagcct ttgcgccttc agagaagaac atgccgcaag acttgccgga 540

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caacgcgcag cccgtgggct cgtggtgctt gtaggttacc tctgcaaacg actgcaggta 840

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caacccgcgg tgctcctcgt ttagccaggt cttgcatacg gccgccagag cttccacttg 960

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<210> 52
<211> 2446
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic

polynucleotide

<400> 52

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ccccgtcgag gcacccccgc ttgaggagga ggaagtgatt atcgagcagg acccaggttt 180

tgtaagcgaa gacgacgagg atcgctcagt accaacagag gataaaaagc aagaccagga 240

cgacgcagag gcaaacgagg aacaagtcgg gcgggggggac caaaggcatg gcgactacct 300

agatgtggga gacgacgtgc tgttgaagca tctgcagcgc cagtgcgcca ttatctgcga 360

cgcgttgcaa gagcgcagcg atgtgcccct cgccatagcg gatgtcagcc ttgcctacga 420

acgccacctg ttctcaccgc gcgtaccccc caaacgccaa gaaaacggca catgcgagcc 480

caacccgcgc ctcaacttct accccgtatt tgccgtgcca gaggtgcttg ccacctatca 540

catcttttc caaaactgca agataccct atcctgccgt gccaaccgca gccgagcgga 600

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gcgcctagcc gtgctgaaac gcagcatcga ggtcacccac tttgcctacc cggcacttaa 840

cctacccccc aaggttatga gcacagtcat gagcgagctg atcgtgcgcc gtgcacgacc 900

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tgagcagctg gcgcgctggc ttgagacgcg cgagcctgcc gacttggagg agcgacgcaa 1020

gctaatgatg gccgcagtgc ttgttaccgt ggagcttgag tgcatgcagc ggttctttgc 1080

tgacccggag atgcagcgca agctagagga aacgttgcac tacacctttc gccagggcta 1140

cgtgcgccag gcctgcaaaa tttccaacgt ggagctctgc aacctggtct cctaccttgg 1200

aattttgcac gaaaaccgcc tcgggcaaaa cgtgcttcat tccacgctca agggcgaggc 1260

gcgccgcgac tacgtccgcg actgcgttta cttatttctg tgctacacct ggcaaacggc 1320

catgggcgtg tggcagcaat gcctggagga gcgcaaccta aaggagctgc agaagctgct 1380

aaagcaaaac ttgaaggacc tatggacggc cttcaacgag cgctccgtgg ccgcgcacct 1440

ggcggacatt atcttccccg aacgcctgct taaaaccctg caacagggtc tgccagactt 1500

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<211> 375
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

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aggcgaccgg atagcaagac tctgacaaag cccaagaaat ccacagcggc ggcagcagca 120

ggaggaggag cgctgcgtct ggcgcccaac gaacccgtat cgacccgcga gcttagaaat 180

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aaaataaaaa acaggtctct gcgctccctc acccgcagct gcctgtatca caaaagcgaa 300

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actcttaagg actag 375

<210> 54
<211> 354
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

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gctggagctg cccaagacta ctcaacccga ataaactaca tgagcgcggg accccacatg 120

atatcccggg tcaacggaat ccgcgcccac cgaaaccgaa ttctcctcga acaggcggct 180

attaccacca cacctcgtaa taaccttaat ccccgtagtt ggcccgctgc cctggtgtac 240

caggaaagtc ccgctcccac cactgtggta cttcccagag acgcccaggc cgaagttcag 300

atgactaact caggggcgca gcttgcgggc ggctttcgtc acagggtgcg gtcg 354

<210> 55
<211> 1164
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

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cggcgcactc cgtacagtag ggatcgccta cctccttttg agacagagac ccgcgctacc 120

atactggagg atcatccgct gctgcccgaa tgtaacactt tgacaatgca caacgtgagt 180

tacgtgcgag gtcttccctg cagtgtggga tttacgctga ttcaggaatg ggttgttccc 240

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gctctccact gtcattgttc cagtcccggt tccctgcagt gcatagccgg cgggcaggtt 420

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taccgggagg tggtgaatta caacatgcca aaagaggtaa tgtttatgtc cagcgtgttt 540

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gacatgaact taagcgagct gcccggggag tttattaata tcactgatga gcgtttggct 1020

cgacaggaaa ccgtgtggaa tataacacct aagaatatgt ctgttaccca tgatatgatg 1080

ctttttaagg ccagccgggg agaaaggact gtgtactctg tgtgttggga gggaggtggc 1140

aggttgaata ctagggttct gtga 1164

<210> 56
<211> 345
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic polynucleotide

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tggctgggtg tggcttattc tgcggtggtg gatgttatca gggcagcggc gcatgaagga 120

gtttacatag aacccgaagc caggggggcgc ctggatgctt tgagagagtg gatatactac 180

aactactaca cagagcgagc taagcgacga gaccggagac gcagatctgt ttgtcacgcc 240

cgcacctggt tttgcttcag gaaatatgac tacgtccggc gttccatttg gcatgacact 300

acgaccaaca cgatctcggt tgtctcggcg cactccgtac agtag 345

<210> 57
<211> 319
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic polynucleotide

<400> 57
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ggacttaata ttcgggattt gcttagagac atattgataa ggtggcgaga tgaaaattat 120

ttgggcatgg ttgaaggtgc tggaatgttt atagaggaga ttcaccctga agggtttagc 180

ctttacgtcc acttggacgt gagggcagtt tgccttttgg aagccattgt gcaacatctt 240

acaaatgcca ttatctgttc tttggctgta gagtttgacc acgccaccgg aggggagcgc 300

gttcacttaa tagatcttc 319

<210> 58
<211> 411
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

<400> 58
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gaacttacct gcctttatct gcatgagcat gactacgatg tgcttgcttt tttgcgcgag 120

gctttgcctg atttttgag cagcaccttg cattttatat cgccgcccat gcaacaagct 180

tacatagggg ctacgctggt tagcatagct ccgagtatgc gtgtcataat cagtgtgggt 240

tcttttgtca tggttcctgg cggggaagtg gccgcgctgg tccgtgcaga cctgcacgat 300

tatgttcagc tggccctgcg aagggaccta cgggatcgcg gtatttttgt taatgttccg 360

cttttgaatc ttatacaggt ctgtgaggaa cctgaatttt tgcaatcatg a 411

<210> 59
<211> 7327
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

<400> 59
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gatcagaagt tcctattccg aagttcctat tctctagaaa gtataggaac ttctgatctg 120

cgcagccgcc atgccggggt tttacgagat tgtgattaag gtccccagcg accttgacga 180

gcatctgccc ggcatttctg acagctttgt gaactgggtg gccgagaagg aatgggagtt 240

gccgccagat tctgacatgg atctgaatct gattgagcag gcacccctga ccgtggccga 300

gaagctgcag cgcgactttc tgacggaatg gcgccgtgtg agtaaggccc cggaggccct 360

tttctttgtg caatttgaga agggagagag ctacttccac atgcacgtgc tcgtggaaac 420

caccggggtg aaatccatgg ttttgggacg tttcctgagt cagattcgcg aaaaactgat 480

tcagagaatt taccgcggga tcgagccgac tttgccaaac tggttcgcgg tcacaaagac 540

cagaaatggc gccggaggcg ggaacaaggt ggtggatgag tgctacatcc ccaattactt 600

gctccccaaa acccagcctg agctccagtg ggcgtggact aatatggaac agtatttaag 660

cgcctgtttg aatctcacgg agcgtaaacg gttggtggcg cagcatctga cgcacgtgtc 720

gcagacgcag gagcagaaca aagagaatca gaatcccaat tctgatgcgc cggtgatcag 780

atcaaaaact tcagccaggt acatggagct ggtcgggtgg ctcgtggaca aggggattac 840

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cgtcacctcc aacaccaaca tgtgcgccgt gattgacggg aactcaacga ccttcgaaca 1440

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acgccaa 7327

<210> 60
<211> 6902
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

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gagcgcggtg gcaatgccct gcctccggtg ctcgccggag actgcgagat ttaagatata 180

gatctcacta cgcggctgct caaacttggg cagaacgtaa gccgcgagag cgccaacaac 240

cgcttcttgg tcgaaggcag caagcgcgat gaatgtctta ctacggagca agttcccgag 300

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gttgctgctc ttaaatta 498

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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide

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<222> (3714)..(3813)
<223> a, c, t, g, unknown or other

<220>
<221> misc_feature
<222> (3714)..(3813)
<223> This region may encompass 60-100 nucleotides

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catatcacca gctcaccgtc tttcattgcc atacggaact ccggatgagc attcatcagg 180

cgggcaagaa tgtgaataaa ggccggataa aacttgtgct tatttttctt tacggtcttt 240

aaaaaggccg taatatccag ctgaacggtc tggttatagg tacattgagc aactgactga 300

aatgcctcaa aatgttcttt acgatgccat tgggatatat caacggtggt atatccagtg 360

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<220>
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Peptide

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<223> This region may or may not be present

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1 5 10 15

Glu Asn Pro Gly Pro
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Glu Glu Asn Pro Gly Pro
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Gly Asp Val Glu Ser Asn Pro Gly Pro
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