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(Not for submission under 37 CFR 1.99)

Application Number	16325653
Filing Date	2017-08-14
First Named Inventor	XIAOYING JIN
Art Unit	1797
Examiner Name	Xu, Xiaolin
Attorney Docket Number	15979-20141.00

U.S.PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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	1	2007046703	WO	A2	2007-04-26	AMSTERDAM MOLECULAR THERAPEUTICS B.V.		☐
	2	2010093784	WO	A2	2010-08-19	THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL		☐
	3	2015114365	WO	A1	2015-08-06	THE SECRETARY OF STATE FOR HEALTH		☐

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	4	2015137802	WO	A1	2015-09-17	UNIQURE IP B.V.		☐

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	1	"RIPA Buffer" (2021). Brochure for product data, located at https://www.sigmaaldrich.com/GB/en/product/sigma/r0278, last visited on November 9, 2021, 9 pages.	☐
	2	BENEVENTO, M. et al. (April 18, 2014). "Adenovirus Composition, Proteolysis, And Disassembly Studied By In-Depth Qualitative And Quantitative Proteomics," Journal of Biological Chemistry 289(16):11421-11430.	☐
	3	CALDERARO, A. et al (October 30, 2014). "Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) 5 Mass Spectrometry Applied To Virus Identification", Scientific Reports 4(6803):1-10.	☐
	4	CHENG, K. et al. (February 21, 2013). "MS-H: A Novel Proteomic Approach to Isolate and Type the E.coli H Antigen Using Membrane Filtration and Liquid Chromatography-Tandem Mass Spectrometry(LCMS/MS)", PLoS One 8(2): e57339, 12 pages.	☐
	5	DONG, B. et al (February 3, 2014). "Proteomics Analysis of Co-Purifying Cellular Proteins Associated with rAAV Vectors", PLoS One 9(2):e86453, 7 pages.	☐
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	7	MICROMASS UK Limited. (not dated). "Quattro LC User's Guide", PDF located at https://www.waters.com/webassets/cms/support/docs/quattro_lc_guide_issue2.pdf, last visited on November 9, 2021, 164 pages.	☐
	8	Notice of Opposition for European Patent No. 3497207, dated January 06, 2021, Proprietor Genzyme Corporation, Opponent Definition IP Limited, 13 pages.	☐

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	9	Notice of Opposition for European Patent No. 3497207, dated January 06, 2021, Proprietor Genzyme Corporation, Opponent Definition IP Limited, 39 pages.	☐
	10	PIERSON, E.E. et al. (July 5, 2016). "Resolving Adeno-Associated Viral Particle Diversity With Charge Detection Mass Spectrometry," Analytical Chemistry 88:6718-6725.	☐
	11	SHE, Y-M. et al. (June 8, 2001). "Determination Of The Complete Amino Acid Sequence For The CoatProtein Of Brome Mosaic Virus By Time-Of-Flight Mass Spectrometry", Journal of BiologicalChemistry 276(23):20039-20047.	☐
	12	STROBEL, B. et al. (July 28, 2015). "Comparative Analysis of Cesium Chloride- and Iodixanol-Based Purification of Recombinant Adeno-Associated Viral Vectors for Preclinical Applications", Human Gene Therapy Methods 26 (4):147-157.	☐
	13	TAN, S. et al (2000). "Rapid Simultaneous Detection Of Two Orchid Viruses Using LC- And/Or MALDI-mass Spectrometry", Journal of Virological Methods 85:93-99.	☐
	14	Team TFS (July 22, 2016). "Liquid Chromatography as the Future Replacement for Gel Electrophoresis", a website article available at URL: https://www.analyteguru.com/t5/Blog/Liquid-Chromatography-as-10 the-Future-Replacement-for-Gel/ba-p/3171, last visited on November 9, 2021, 9 pages.	☐
	15	WATERS (The Science of What's Possible) (Copyright Date 2021). "Beginner's Guide to UPLC", Introduction, a website article located at:https://www.waters.com/waters/en US/UPLC---Ultra-Performance-Liguid-Chromatography-Beginner's-Guide/nav.htm cid=134803622&1ocale=en US, last visited on November 9, 2021, 2 pages.	☐

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Signature	/Brian A. Donahue/	Date (YYYY-MM-DD)	2021-11-09
Name/Print	Brian Donahue	Registration Number	58,206

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(54) **Title:** IMPROVED AAV VECTORS PRODUCED IN INSECT CELLS

(57) **Abstract:** The present invention relates to the production of adeno-associated viral vectors in insect cells. The insect cells therefore comprise a first nucleotide sequence encoding the adeno-associated virus (AAV) capsid proteins, whereby the initiation codon for translation of the AAV VP1 capsid protein is a non-ATG, suboptimal initiation codon. The insect cell further comprises a second nucleotide sequence comprising at least one AAV inverted terminal repeat (ITR) nucleotide sequence; a third nucleotide sequence comprising a Rep52 or a Rep40 coding sequence operably linked to expression control sequences for expression in an insect cell; and, a fourth nucleotide sequence comprising a Rep78 or a Rep68 coding sequence operably linked to expression control sequences for expression in an insect cell. The invention further relates to adeno-associated viral vectors with an altered ratio of the viral capsid proteins that provides improved infectivity o f the viral particles.

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Improved AAV vectors produced in insect cells

Field of the invention

The present invention relates to the production of adeno-associated virus in insect cells and to adeno-associated virus with an altered ratio of the viral capsid proteins that provides improved infectivity.

Background of the invention

Adeno-associated virus (AAV) may be considered as one of the most promising viral vectors for human gene therapy. AAV has the ability to efficiently infect dividing as well as non-dividing human cells, the AAV viral genome integrates into a single chromosomal site in the host cell's genome, and most importantly, even though AAV is present in many humans it has never been associated with any disease. In view of these advantages, recombinant adeno-associated virus (rAAV) is being evaluated in gene therapy clinical trials for hemophilia B, malignant melanoma, cystic fibrosis, and other diseases.

Host cells that sustain AAV replication *in vitro* are all derived from mammalian cell types. Therefore, rAAV for use in gene therapy has thus far mainly been produced on mammalian cell lines such as e.g. 293 cells, COS cells, HeLa cells, KB cells, and other mammalian cell lines (see e.g. US 6,156,303, US 5,387,484, US 5,741,683, US 5,691,176, US 5,688,676, US 20020081721, WO 00/47757, WO 00/24916, and WO 96/17947). rAAV vectors are typically produced in such mammalian cell culture systems by providing DNA plasmids that contain the therapeutic gene flanked by the origin of AAV replication (inverted terminal repeats or ITRs), genes for AAV replication proteins Rep78, Rep68, Rep52, and Rep40, and genes for virion or structural proteins VP1, VP2, and VP3. In addition, a plasmid containing early genes from adenovirus (E2A, E4ORF6, VARNA) is provided to enhance the expression of the AAV genes and improve vector yield (see e.g. Grimm et al., 1998, Hum. Gene Ther. 9: 2745–2760). However, in most of these mammalian cell culture systems, the number of AAV particles generated per cell is on the order of 10^4 particles (reviewed in Clark, 2002, Kidney Int. 61(Suppl. 1): 9–15). For a clinical study, more than 10^{15} particles of rAAV may be required. To produce this number of rAAV particles,

transfection and culture with approximately 10^{11} cultured human 293 cells, the equivalent of 5,000 175-cm^2 flasks of cells, would be required., which means transfecting up to 10^{11} 293 cells. Therefore, large scale production of rAAV using mammalian cell culture systems to obtain material for clinical trials has already proven to be problematic, production at commercial scale may not even be feasible. Furthermore there is always the risk, that a vector for clinical use that is produced in a mammalian cell culture will be contaminated with undesirable, perhaps pathogenic, material present in the mammalian host cell.

To overcome these problems of mammalian productions systems, recently, an AAV production system has been developed using insect cells (Urabe et al., 2002, Hum. Gene Ther. 13: 1935–1943; US 20030148506 and US 20040197895). For production of AAV in insect cells from the baculovirus expression system some modifications were necessary because in mammalian cells expression of the three AAV capsid proteins (VP1, VP2 and VP3) in the correct stoichiometry relies on a combination of alternate usage of two splice acceptor sites and the suboptimal utilization of a ACG initiation codon for VP2 that will not be accurately reproduced by insect cells. Correct stoichiometry of the three capsid proteins is important for infectivity of the AAV particles. It is known that AAV particles containing reduced amounts of VP1 are less infectious and that VP1 contains phospholipase A2 activity which has a function in infectivity (Girod et al., 2002 J. Gen. Virol. 83: 973-8).

Therefore, for the expression of the three capsid proteins Urabe et al. (2002, *supra*) use a construct that is transcribed into a single polycistronic messenger that is able to express all three VP proteins without requiring splicing. To aim for production of the three capsid proteins in the correct stoichiometry, the VP1 reading frame, the first initiator codon that is seen by the scanning ribosome, has been endowed with the suboptimal initiator codon ACG and sequences surrounding this codon have been optimized. Urabe et al. (2002, *supra*) report that ribosome scanning through in insects cells leads to a stoichiometry of the three viral capsid proteins that is close to wild-type AAV.

The present inventors have, however, found that in AAV vectors produced in the baculovirus system VP1 is still expressed at a suboptimal level relative to VP2 and that this results in reduced infectivity in *in vitro* and *in vivo* studies in mice as compared to e.g. conventional AAV vectors produced on mammalian 293 cells. Hence, there is still

a need for a baculovirus-based production system for rAAV vectors with improved infectivity.

Description of the invention

Definitions

As used herein, the term "operably linked" refers to a linkage of polynucleotide (or polypeptide) elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a transcription regulatory sequence is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein encoding regions, contiguous and in reading frame.

"Expression control sequence" refers to a nucleic acid sequence that regulates the expression of a nucleotide sequence to which it is operably linked. An expression control sequence is "operably linked" to a nucleotide sequence when the expression control sequence controls and regulates the transcription and/or the translation of the nucleotide sequence. Thus, an expression control sequence can include promoters, enhancers, internal ribosome entry sites (IRES), transcription terminators, a start codon in front of a protein-encoding gene, splicing signal for introns, and stop codons. The term "expression control sequence" is intended to include, at a minimum, a sequence whose presence are designed to influence expression, and can also include additional advantageous components. For example, leader sequences and fusion partner sequences are expression control sequences. The term can also include the design of the nucleic acid sequence such that undesirable, potential initiation codons in and out of frame, are removed from the sequence. It can also include the design of the nucleic acid sequence such that undesirable potential splice sites are removed. It includes sequences or polyadenylation sequences (pA) which direct the addition of a polyA tail, i.e., a string of adenine residues at the 3'-end of a mRNA, sequences referred to as polyA sequences. It also can be designed to enhance mRNA stability. Expression control sequences which affect the transcription and translation stability, e.g., promoters, as well as sequences which effect the translation, e.g., Kozak sequences, are known in insect cells. Expression control sequences can be of such nature as to modulate the nucleotide

sequence to which it is operably linked such that lower expression levels or higher expression levels are achieved.

As used herein, the term "promoter" or "transcription regulatory sequence" refers to a nucleic acid fragment that functions to control the transcription of one or more coding sequences, and is located upstream with respect to the direction of transcription of the transcription initiation site of the coding sequence, and is structurally identified by the presence of a binding site for DNA-dependent RNA polymerase, transcription initiation sites and any other DNA sequences, including, but not limited to transcription factor binding sites, repressor and activator protein binding sites, and any other sequences of nucleotides known to one of skill in the art to act directly or indirectly to regulate the amount of transcription from the promoter. A "constitutive" promoter is a promoter that is active in most tissues under most physiological and developmental conditions. An "inducible" promoter is a promoter that is physiologically or developmentally regulated, e.g. by the application of a chemical inducer. A "tissue specific" promoter is only active in specific types of tissues or cells.

The terms "substantially identical", "substantial identity" or "essentially similar" or "essential similarity" means that two peptide or two nucleotide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default parameters, share at least a certain percentage of sequence identity as defined elsewhere herein. GAP uses the Needleman and Wunsch global alignment algorithm to align two sequences over their entire length, maximizing the number of matches and minimizes the number of gaps. Generally, the GAP default parameters are used, with a gap creation penalty = 50 (nucleotides) / 8 (proteins) and gap extension penalty = 3 (nucleotides) / 2 (proteins). For nucleotides the default scoring matrix used is nwsgapdna and for proteins the default scoring matrix is Blosum62 (Henikoff & Henikoff, 1992, PNAS 89, 915-919). It is clear than when RNA sequences are said to be essentially similar or have a certain degree of sequence identity with DNA sequences, thymine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence. Sequence alignments and scores for percentage sequence identity may be determined using computer programs, such as the GCG Wisconsin Package, Version 10.3, available from Accelrys Inc., 9685 Scranton Road, San Diego, CA 92121-3752 USA or the open-source software Emboss for Windows (current version 2.7.1-07).

Alternatively percent similarity or identity may be determined by searching against databases such as FASTA, BLAST, etc.

Detailed description of the invention

The present invention relates the use animal parvoviruses, in particular dependoviruses such as infectious human or simian AAV, and the components thereof (e.g., an animal parvovirus genome) for use as vectors for introduction and/or expression of nucleic acids in mammalian cells. In particular, the invention relates to improvements in infectivity of such parvoviral vectors when produced in insect cells.

Viruses of the Parvoviridae family are small DNA animal viruses. The family Parvoviridae may be divided between two subfamilies: the Parvovirinae, which infect vertebrates, and the Densovirinae, which infect insects. Members of the subfamily Parvovirinae are herein referred to as the parvoviruses and include the genus Dependovirus. As may be deduced from the name of their genus, members of the Dependovirus are unique in that they usually require coinfection with a helper virus such as adenovirus or herpes virus for productive infection in cell culture. The genus Dependovirus includes AAV, which normally infects humans (e.g., serotypes 1, 2, 3A, 3B, 4, 5, and 6) or primates (e.g., serotypes 1 and 4), and related viruses that infect other warm-blooded animals (e.g., bovine, canine, equine, and ovine adeno-associated viruses). Further information on parvoviruses and other members of the Parvoviridae is described in Kenneth I. Berns, "Parvoviridae: The Viruses and Their Replication," Chapter 69 in Fields Virology (3d Ed. 1996). For convenience the present invention is further exemplified and described herein by reference to AAV. It is however understood that the invention is not limited to AAV but may equally be applied to other parvoviruses.

The genomic organization of all known AAV serotypes is very similar. The genome of AAV is a linear, single-stranded DNA molecule that is less than about 5,000 nucleotides (nt) in length. Inverted terminal repeats (ITRs) flank the unique coding nucleotide sequences for the non-structural replication (Rep) proteins and the structural (VP) proteins. The VP proteins form the capsid. The terminal 145 nt are self-complementary and are organized so that an energetically stable intramolecular duplex forming a T-shaped hairpin may be formed. These hairpin structures function as an origin for viral DNA replication, serving as primers for the cellular DNA polymerase

complex. The Rep genes encode the Rep proteins, Rep78, Rep68, Rep52, and Rep40. Rep78 and Rep68 are transcribed from the p5 promoter, and Rep 52 and Rep40 are transcribed from the p19 promoter. The cap genes encode the VP proteins, VP1, VP2, and VP3. The cap genes are transcribed from the p40 promoter.

In a first aspect the invention relates to a nucleotide sequence comprising an open reading frame comprising nucleotide sequences encoding animal parvoviruses VP1, VP2, and VP3 capsid proteins, wherein the initiation codon for translation of the AAV VP1 capsid protein is a suboptimal initiation codon that is not ATG and that is not ACG. Suboptimal is herein understood to mean that the codon is less efficient in the initiation of translation in an otherwise identical context as compared to the normal ATG codon. Preferably the initiation codon for translation of the AAV VP1 capsid protein is selected from ACG, TTG, GTG, and CTG, more preferably the initiation codon for translation of the AAV VP1 capsid protein is selected from TTG, GTG, and CTG and most preferably the initiation codon for translation of the AAV VP1 capsid protein is CTG. The animal parvovirus preferably is a dependovirus, more preferably a human or simian adeno-associated virus (AAV).

In a nucleotide sequence comprising an open reading frame comprising nucleotide sequences encoding animal parvoviruses VP1, VP2, and VP3 capsid proteins, wherein the initiation codon for translation of the AAV VP1 capsid protein is a TTG or GTG initiation codon, the ratio of the amounts of VP1 : VP2 proteins in the virion is about equal, i.e. equimolar, whereas if the initiation codon is CTG, the amount of VP1 protein in the virion is higher than the amount of VP2. If the initiation codon is ACG, the amount of VP1 protein in the virion is lower than the amount of VP2. The infectivity of the virions increases with the ratio of VP1 over VP2 in the virions.

A preferred nucleotide sequence of the invention for the expression of the AAV capsid proteins is a nucleotide sequence comprising an expression control sequence comprising a nine nucleotide sequence of SEQ. ID NO: 7 or a nucleotide sequence substantially homologous to SEQ. ID NO: 7, upstream of the initiation codon of the nucleotide sequence encoding the AAV VP1 capsid protein. A sequence with substantial identity to the nucleotide sequence of SEQ. ID NO: 7 and that will help increase expression of VP1 is e.g. a sequence which has at least 60%, 70%, 80% or 90% identity to the nine nucleotide sequence of SEQ ID NO: 7.

A further preferred nucleotide sequence of the invention for the expression of the AAV capsid proteins is a nucleotide sequence comprising an expression control sequence comprising a Kozak consensus sequence around the initiation codon of the nucleotide sequence encoding the AAV VP1 capsid protein. The Kozak consensus sequence is herein defined as GCCRCC(NNN)G (SEQ. ID NO: 8), wherein R is a purine (i.e. A or G) and wherein (NNN) stands for any of the suboptimal initiation codons as defined herein above. Preferably, in the Kozak consensus sequence in the nucleotide sequence of the invention, the R is a G. The nucleotide sequence of the invention for the expression of the AAV capsid proteins comprising a Kozak consensus sequence is thus preferably selected from GCCACC(ACG)G, GCCGCC(ACG)G, GCCACC(TTG)G, GCCGCC(TTG)G, GCCACC(GTG)G, GCCGCC(GTG)G, GCCACC(CTG)G and GCCGCC(CTG)G, more preferably the nucleotide sequence comprising the Kozak consensus sequence is selected from GCCACC(CTG)G and GCCGCC(CTG)G, most preferably, the nucleotide sequence comprising the Kozak consensus sequence is GCCGCC(CTG)G. The nucleotides in brackets herein indicate the position of the initiation codon of the VP1 protein.

The nucleotide sequence of the invention for expression of the AAV capsid proteins further preferably comprises at least one modification of the nucleotide sequence encoding AAV VP1 capsid protein selected from among a G at nucleotide position 12, an A at nucleotide position 21, and a C at nucleotide position 24. Elimination of possible false start sites for translation of VP1 of other serotypes will be well understood by an artisan of skill in the art, as will be the elimination of putative splice sites that may be recognized in insect cells. The various modifications of the wild-type AAV sequences for proper expression in insect cells is achieved by application of well-known genetic engineering techniques such as described e.g. in Sambrook and Russell (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, New York. Various further modifications of VP coding regions are known to the skilled artisan which could either increase yield of VP and virion or have other desired effects, such as altered tropism or reduce antigenicity of the virion. These modifications are within the scope of the present invention.

Preferably the nucleotide sequence of the invention encoding the AAV capsid proteins is operably linked to expression control sequences for expression in an insect

cell. These expression control sequences will at least include a promoter that is active in insect cells. Techniques known to one skilled in the art for expressing foreign genes in insect host cells can be used to practice the invention. Methodology for molecular engineering and expression of polypeptides in insect cells is described, for example, in Summers and Smith. 1986. A Manual of Methods for Baculovirus Vectors and Insect Culture Procedures, Texas Agricultural Experimental Station Bull. No. 7555, College Station, Tex.; Luckow. 1991. In Prokop et al., Cloning and Expression of Heterologous Genes in Insect Cells with Baculovirus Vectors' Recombinant DNA Technology and Applications, 97-152; King, L. A. and R. D. Possee, 1992, The baculovirus expression system, Chapman and Hall, United Kingdom; O'Reilly, D. R., L. K. Miller, V. A. Luckow, 1992, Baculovirus Expression Vectors: A Laboratory Manual, New York; W. H. Freeman and Richardson, C. D., 1995, Baculovirus Expression Protocols, Methods in Molecular Biology, volume 39; US 4,745,051; US2003148506; and WO 03/074714. A particularly suitable promoter for transcription of the nucleotide sequence of the invention encoding of the AAV capsid proteins is e.g. the polyhedron promoter. However, other promoters that are active in insect cells are known in the art, e.g. the p10, p35 or IE-1 promoters and further promoters described in the above references.

Preferably the nucleic acid construct for expression of the AAV capsid proteins in insect cells is an insect cell-compatible vector. An "insect cell-compatible vector" or "vector" is understood to a nucleic acid molecule capable of productive transformation or transfection of an insect or insect cell. Exemplary biological vectors include plasmids, linear nucleic acid molecules, and recombinant viruses. Any vector can be employed as long as it is insect cell-compatible. The vector may integrate into the insect cells genome but the presence of the vector in the insect cell need not be permanent and transient episomal vectors are also included. The vectors can be introduced by any means known, for example by chemical treatment of the cells, electroporation, or infection. In a preferred embodiment, the vector is a baculovirus, a viral vector, or a plasmid. In a more preferred embodiment, the vector is a baculovirus, i.e. the construct is a baculoviral vector. Baculoviral vectors and methods for their use are described in the above cited references on molecular engineering of insect cells.

In another aspect the invention relates to an insect cell comprising a nucleic acid construct of the invention as defined above. Any insect cell which allows for replication of AAV and which can be maintained in culture can be used in accordance

with the present invention. For example, the cell line used can be from Spodoptera frugiperda, drosophila cell lines, or mosquito cell lines, e.g., Aedes albopictus derived cell lines. Preferred insect cells or cell lines are cells from the insect species which are susceptible to baculovirus infection, including e.g. Se301, SeIZD2109, SeUCR1, Sf9, Sf900+, Sf21, BTI-TN-5B1-4, MG-1, Tn368, HzAm1, Ha2302, Hz2E5 and High Five from Invitrogen.

A preferred insect cell according to the invention further comprises: (a) a second nucleotide sequence comprising at least one AAV inverted terminal repeat (ITR) nucleotide sequence; (b) a third nucleotide sequence comprising a Rep52 or a Rep40 coding sequence operably linked to expression control sequences for expression in an insect cell; and, (c) a fourth nucleotide sequence comprising a Rep78 or a Rep68 coding sequence operably linked to expression control sequences for expression in an insect cell.

In the context of the invention "at least one AAV ITR nucleotide sequence" is understood to mean a palindromic sequence, comprising mostly complementary, symmetrically arranged sequences also referred to as "A," "B," and "C" regions. The ITR functions as an origin of replication, a site having a "cis" role in replication, i.e., being a recognition site for trans acting replication proteins (e.g., Rep 78 or Rep68) which recognize the palindrome and specific sequences internal to the palindrome. One exception to the symmetry of the ITR sequence is the "D" region of the ITR. It is unique (not having a complement within one ITR). Nicking of single-stranded DNA occurs at the junction between the A and D regions. It is the region where new DNA synthesis initiates. The D region normally sits to one side of the palindrome and provides directionality to the nucleic acid replication step. An AAV replicating in a mammalian cell typically has two ITR sequences. It is, however, possible to engineer an ITR so that binding sites are on both strands of the A regions and D regions are located symmetrically, one on each side of the palindrome. On a double-stranded circular DNA template (e.g., a plasmid), the Rep78- or Rep68-assisted nucleic acid replication then proceeds in both directions and a single ITR suffices for AAV replication of a circular vector. Thus, one ITR nucleotide sequence can be used in the context of the present invention. Preferably, however, two or another even number of regular ITRs are used. Most preferably, two ITR sequences are used. In view of the safety of viral vectors it may be desirable to construct a viral vector that is unable to

further propagate after initial introduction into a cell. Such a safety mechanism for limiting undesirable vector propagation in a recipient may be provided by using rAAV with a chimeric ITR as described in US2003148506.

The number of vectors or nucleic acid constructs employed is not limiting of the invention. For example, one, two, three, four, five, six, or more vectors can be employed to produce AAV in insect cells in accordance with the present inventive method. If six vectors are employed, one vector encodes AAV VP 1, another vector encodes AAV VP2, yet another vector encodes AAV VP3, still yet another vector encodes Rep52 or Rep40, while Rep78 or Rep 68 is encoded by another vector and a final vector comprises at least one AAV ITR. Additional vectors might be employed to express, for example, Rep52 and Rep40, and Rep78 and Rep 68. If fewer than six vectors are used, the vectors can comprise various combinations of the at least one AAV ITR and the VP1, VP2, VP3, Rep52/Rep40, and Rep78/Rep68 coding sequences. Preferably, two vectors or three vectors are used, with two vectors being more preferred as described above. If two vectors are used, preferably the insect cell comprises: (a) a first nucleic acid construct for expression of the AAV capsid proteins as defined above, which construct further comprises the third and fourth nucleotide sequences as defined in (b) and (c) above, the third nucleotide sequence comprising a Rep52 or a Rep40 coding sequence operably linked to at least one expression control sequence for expression in an insect cell, and the fourth nucleotide sequence comprising a Rep78 or a Rep68 coding sequence operably linked to at least one expression control sequence for expression in an insect cell; and (b) a second nucleic acid construct comprising the second nucleotide sequence as defined in (a) above, comprising at least one AAV ITR nucleotide sequence. If three vectors are used, preferably the same configuration as used for two vectors is used except that separate vectors are used for expression of the capsid proteins and for expression of the Rep52, Rep40 Rep78 and Rep68 proteins. The sequences on each vector can be in any order relative to each other. For example, if one vector comprises ITRs and an ORF comprising nucleotide sequences encoding VP capsid proteins, the VP ORF can be located on the vector such that, upon replication of the DNA between ITR sequences, the VP ORF is replicated or not replicated. For another example, the Rep coding sequences and/or the ORF comprising nucleotide sequences encoding VP capsid proteins can be in any order on a vector. In is understood that also the second, third and further nucleic acid construct(s) preferably

are an insect cell-compatible vectors, preferably a baculoviral vectors as described above. Alternatively, in the insect cell of the invention, one or more of the first nucleotide sequence, second nucleotide sequence, third nucleotide sequence, and fourth nucleotide sequence and optional further nucleotide sequences may be stably integrated in the genome of the insect cell. One of ordinary skill in the art knows how to stably introduce a nucleotide sequence into the insect genome and how to identify a cell having such a nucleotide sequence in the genome. The incorporation into the genome may be aided by, for example, the use of a vector comprising nucleotide sequences highly homologous to regions of the insect genome. The use of specific sequences, such as transposons, is another way to introduce a nucleotide sequence into a genome.

The a preferred embodiment of the invention, the second nucleotide sequence present in the insect cells of the invention, i.e. the sequence comprising at least one AAV ITR, further comprises at least one nucleotide sequence encoding a gene product of interest, whereby preferably the at least one nucleotide sequence encoding a gene product of interest becomes incorporated into the genome of an AAV produced in the insect cell. Preferably, at least one nucleotide sequence encoding a gene product of interest is a sequence for expression in a mammalian cell. Preferably, the second nucleotide sequence comprises two AAV ITR nucleotide sequences and wherein the at least one nucleotide sequence encoding a gene product of interest is located between the two AAV ITR nucleotide sequences. Preferably, the nucleotide sequence encoding a gene product of interest (for expression in the mammalian cell) will be incorporated into the AAV genome produced in the insect cell if it is located between two regular ITRs, or is located on either side of an ITR engineered with two D regions.

The second nucleotide sequence defined herein above may thus comprise a nucleotide sequence encoding at least one "gene product of interest" for expression in a mammalian cell, located such that it will be incorporated into an AAV genome replicated in the insect cell. Any nucleotide sequence can be incorporated for later expression in a mammalian cell transfected with the AAV produced in accordance with the present invention. The nucleotide sequence may e.g. encode a protein it may express an RNAi agent, i.e. an RNA molecule that is capable of RNA interference such as e.g. a shRNA (short hairpinRNA) or an siRNA (short interfering RNA). "siRNA" means a small interfering RNA that is a short-length double-stranded RNA that are not toxic in mammalian cells (Elbashir et al., 2001, Nature 411: 494-98; Caplen et al.,

2001, Proc. Natl. Acad. Sci. USA 98: 9742-47). In a preferred embodiment, the second nucleotide sequence may comprise two nucleotide sequences and each encodes one gene product of interest for expression in a mammalian cell. Each of the two nucleotide sequences encoding a product of interest is located such that it will be incorporated into a rAAV genome replicated in the insect cell.

The product of interest for expression in a mammalian cell may be a therapeutic gene product. A therapeutic gene product can be a polypeptide, or an RNA molecule (siRNA), or other gene product that, when expressed in a target cell, provides a desired therapeutic effect such as e.g. ablation of an undesired activity, e.g. the ablation of an infected cell, or the complementation of a genetic defect, e.g. causing a deficiency in an enzymatic activity. Examples of therapeutic polypeptide gene products include CFTR, Factor IX, Lipoprotein lipase (LPL, preferably LPL S447X; see WO 01/00220), Apolipoprotein A1, Uridine Diphosphate Glucuronosyltransferase (UGT), Retinitis Pigmentosa GTPase Regulator Interacting Protein (RP-GRIP), and cytokines or interleukins like e.g. IL-10.

Alternatively, or in addition as a second gene product, second nucleotide sequence defined herein above may comprise a nucleotide sequence encoding a polypeptide that serve as marker proteins to assess cell transformation and expression. Suitable marker proteins for this purpose are e.g. the fluorescent protein GFP, and the selectable marker genes HSV thymidine kinase (for selection on HAT medium), bacterial hygromycin B phosphotransferase (for selection on hygromycin B), Tn5 aminoglycoside phosphotransferase (for selection on G418), and dihydrofolate reductase (DHFR) (for selection on methotrexate), CD20, the low affinity nerve growth factor gene. Sources for obtaining these marker genes and methods for their use are provided in Sambrook and Russel (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, New York. Furthermore, second nucleotide sequence defined herein above may comprise a nucleotide sequence encoding a polypeptide that may serve as a fail-safe mechanism that allows to cure a subject from cells transduced with the rAAV of the invention, if deemed necessary. Such a nucleotide sequence, often referred to as a suicide gene, encodes a protein that is capable of converting a prodrug into a toxic substance that is capable of killing the transgenic cells in which the protein is expressed. Suitable examples of such suicide genes include e.g. the *E.coli* cytosine

deaminase gene or one of the thymidine kinase genes from Herpes Simplex Virus, Cytomegalovirus and Varicella-Zoster virus, in which case ganciclovir may be used as prodrug to kill the transgenic cells in the subject (see e.g. Clair et al., 1987, Antimicrob. Agents Chemother. 31: 844-849).

In another embodiment the gene product of interest can be an AAV protein. In particular, a Rep protein, such as Rep78 or Rep68, or a functional fragment thereof. A nucleotide sequence encoding a Rep78 and/or a Rep68, if present on the rAAV genome of the invention and expressed in a mammalian cell transduced with the rAAV of the invention, allows for integration of the rAAV into the genome of the transduced mammalian cell. Expression of Rep78 and/or Rep68 in an rAAV-transduced or infected mammalian cell can provide an advantage for certain uses of the rAAV, by allowing long term or permanent expression of any other gene product of interest introduced in the cell by the rAAV.

In the rAAV vectors of the invention the at least one nucleotide sequence(s) encoding a gene product of interest for expression in a mammalian cell, preferably is/are operably linked to at least one mammalian cell-compatible expression control sequence, e.g., a promoter. Many such promoters are known in the art (see Sambrook and Russel, 2001, *supra*). Constitutive promoters that are broadly expressed in many cell-types, such as the CMV promoter may be used. However, more preferred will be promoters that are inducible, tissue-specific, cell-type-specific, or cell cycle-specific. For example, for liver-specific expression a promoter may be selected from an α1-anti-trypsin promoter, a thyroid hormone-binding globulin promoter, an albumin promoter, LPS (thyroxine-binding globlin) promoter, HCR-ApoCII hybrid promoter, HCR-hAAT hybrid promoter and an apolipoprotein E promoter. Other examples include the E2F promoter for tumor-selective, and, in particular, neurological cell tumor-selective expression (Parr et al., 1997, Nat. Med. 3:1145-9) or the IL-2 promoter for use in mononuclear blood cells (Hagenbaugh et al., 1997, J Exp Med; 185: 2101-10).

AAV is able to infect a number of mammalian cells. See, e.g., Tratschin et al., Mol. Cell Biol., 5(11):3251-3260 (1985) and Grimm et al., Hum. Gene Ther., 10(15):2445-2450 (1999). However, AAV transduction of human synovial fibroblasts is significantly more efficient than in similar murine cells, Jennings et al., Arthritis Res, 3:1 (2001), and the cellular tropicity of AAV differs among serotypes. See, e.g., Davidson et al., Proc. Natl. Acad. Sci. USA, 97(7):3428-3432 (2000) (discussing

differences among AAV2, AAV4, and AAV5 with respect to mammalian CNS cell tropism and transduction efficiency).

AAV sequences that may be used in the present invention for the production of AAV in insect cells can be derived from the genome of any AAV serotype. Generally, the AAV serotypes have genomic sequences of significant homology at the amino acid and the nucleic acid levels, provide an identical set of genetic functions, produce virions which are essentially physically and functionally equivalent, and replicate and assemble by practically identical mechanisms. For the genomic sequence of the various AAV serotypes and an overview of the genomic similarities see e.g. GenBank Accession number U89790; GenBank Accession number J01901; GenBank Accession number AF043303; GenBank Accession number AF085716; Chlorini et al. (1997, J. Vir. 71: 6823-33); Srivastava et al. (1983, J. Vir. 45:555-64); Chlorini et al. (1999, J. Vir. 73:1309-1319); Rutledge et al. (1998, J. Vir. 72:309-319); and Wu et al. (2000, J. Vir. 74: 8635-47). Human or simian adeno-associated virus (AAV) serotypes are preferred sources of AAV nucleotide sequences for use in the context of the present invention, more preferably AAV serotypes which normally infects humans (e.g., serotypes 1, 2, 3A, 3B, 4, 5, and 6) or primates (e.g., serotypes 1 and 4).

Preferably the AAV ITR sequences for use in the context of the present invention are derived from AAV1, AAV2, and/or AAV4. Likewise, the Rep52, Rep40, Rep78 and/or Rep68 coding sequences are preferably derived from AAV1, AAV2, and/or AAV4. The sequences coding for the VP1, VP2, and VP3 capsid proteins for use in the context of the present invention may however be taken from any of the known 42 serotypes, more preferably from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8 or AAV9 or newly developed AAV-like particles obtained by e.g. capsid shuffling techniques and AAV capsid libraries.

AAV Rep and ITR sequences are particularly conserved among most serotypes. The Rep78 proteins of various AAV serotypes are e.g. more than 89% identical and the total nucleotide sequence identity at the genome level between AAV2, AAV3A, AAV3B, and AAV6 is around 82% (Bantel-Schaal et al., 1999, J. Virol., 73(2):939-947). Moreover, the Rep sequences and ITRs of many AAV serotypes are known to efficiently cross-complement (i.e., functionally substitute) corresponding sequences from other serotypes in production of AAV particles in mammalian cells.

US2003148506 reports that AAV Rep and ITR sequences also efficiently cross-complement other AAV Rep and ITR sequences in insect cells.

The AAV VP proteins are known to determine the cellular tropicity of the AAV virion. The VP protein-encoding sequences are significantly less conserved than Rep proteins and genes among different AAV serotypes. The ability Rep and ITR sequences to cross-complement corresponding sequences of other serotypes allows for the production of pseudotyped AAV particles comprising the capsid proteins of a serotype (e.g., AAV3) and the Rep and/or ITR sequences of another AAV serotype (e.g., AAV2). Such pseudotyped AAV particles are a part of the present invention.

Modified "AAV" sequences also can be used in the context of the present invention, e.g. for the production of rAAV vectors in insect cells. Such modified sequences e.g. include sequences having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more nucleotide and/or amino acid sequence identity (e.g., a sequence having about 75-99% nucleotide sequence identity) to an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8 or AAV9 ITR, Rep, or VP can be used in place of wild-type AAV ITR, Rep, or VP sequences.

Although similar to other AAV serotypes in many respects, AAV5 differs from other human and simian AAV serotypes more than other known human and simian serotypes. In view thereof, the production of AAV5 can differ from production of other serotypes in insect cells. Where methods of the invention are employed produce rAAV5, it is preferred that one or more vectors comprising, collectively in the case of more than one vector, a nucleotide sequence comprising an AAV5 ITR, a nucleotide sequence comprises an AAV5 Rep52 and/or Rep40 coding sequence, and a nucleotide sequence comprises an AAV5 Rep78 and/or Rep68 coding sequence. Such ITR and Rep sequences can be modified as desired to obtain efficient production of rAAV5 or pseudotyped rAAV5 vectors in insect cells. E.g., the start codon of the Rep sequences can be modified, VP splice sites can be modified or eliminated, and/or the VP1 start codon and nearby nucleotides can be modified to improve the production of rAAV5 in the insect cell.

In a further aspect the invention relates to an AAV virions. Preferably the AAV virion comprising in its genome at least one nucleotide sequence encoding a gene product of interest, whereby the at least one nucleotide sequence is not a native AAV

nucleotide sequence, and whereby in the stoichiometry of the AAV VP1, VP2, and VP3 capsid proteins the amount of VP1: (a) is at least 100, 105, 110, 120, 150, 200 or 400% of the amount of VP2; or (b) is at least 8, 10, 10.5, 11, 12, 15, 20 or 40% of the amount of VP3; or (c) is at least as defined in both (a) and (b). Preferably, the amount of VP1, VP2 and VP3 is determined using an antibody recognizing an epitope that is common to each of VP1, VP2 and VP3. Various immunoassays are available in the art that will allow quantify the relative amounts of VP1, VP2 and/or VP3 (see e.g. Using Antibodies, E. Harlow and D. Lane, 1999, Cold Spring Harbor Laboratory Press, New York). An suitable antibody recognizing an epitope that is common to each of the three capsid proteins is e.g. the mouse anti-Cap B1 antibody (as is commercially available from Progen, Germany).

A preferred AAV according to the invention is a virion comprising in its genome at least one nucleotide sequence encoding a gene product of interest, whereby the at least one nucleotide sequence is not a native AAV nucleotide sequence, and whereby the AAV virion comprises a VP1 capsid protein that comprises a leucine or a valine at amino acid position 1. A more preferred AAV virion according to the invention has the ratio's of capsid proteins as defined above and comprises a VP1 capsid protein comprises a leucine or a valine at amino acid position 1. Even more preferred is an AAV virion that is obtainable from an insect cell as defined above in e.g. a method as defined herein below.

An advantage of the AAV virions of the invention that have the above defined ratio's of capsid proteins is their improved infectivity. In particular the infectivity increases with an increase of the amount of VP1 protein in the capsid in relation to the amounts of VP2 and/or VP3 in the capsid. The infectivity of an AAV virion is herein understood to mean the efficiency of transduction of the transgene comprised in the virion, as may be deduced from the expression rate of the transgene and the amount or activity of the product expressed from the transgene.

In another aspect the invention thus relates to a method for producing an AAV in an insect cell. Preferably the method comprises the steps of: (a) culturing an insect cell as defined in herein above under conditions such that AAV is produced; and, (b) recovery of the AAV. Growing conditions for insect cells in culture, and production of heterologous products in insect cells in culture are well-known in the art and described e.g. in the above cited references on molecular engineering of insects cells.

Preferably the method further comprises the step of affinity-purification of the AAV using an anti-AAV antibody, preferably an immobilized antibody. The anti-AAV antibody preferably is an monoclonal antibody. A particularly suitable antibody is a single chain camelid antibody or a fragment thereof as e.g. obtainable from camels or llamas (see e.g. Muyldermans, 2001, Biotechnol. 74: 277-302). The antibody for affinity-purification of AAV preferably is an antibody that specifically binds an epitope on a AAV capsid protein, whereby preferably the epitope is an epitope that is present on capsid protein of more than one AAV serotype. E.g. the antibody may be raised or selected on the basis of specific binding to AAV2 capsid but at the same time also it may also specifically bind to AAV1, AAV3 and AAV5 capsids.

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

Description of the figures

Figure 1: Western-blot analysis of rAAV produced with a recombinant baculovirus containing a TTG or an ACG initiation codon for the VP1 capsid protein.

Figure 2: LPL-mass assay: HEK293 cells in a microtiter plate were infected with rAAV produced with the baculovirus-production system and the plasmid-production system at and moi between 100 and 25,000. At two days after infection LPL^{S447X} protein was measured by LPL^{S447X} -mass ELISA. “DKFZ/Llama” (A-079-024 and A-093-130/137) represent rAAV-LPL^{S447X} batches generated with the plasmid production system. “Control” represents a rAAV-LacZ virus batch not expressing LPL^{S447X} but the bacterial protein LacZ. Baculo/Llama represents a rAAV- LPL^{S447X} batch generated with the baculovirus production system using the rBac-Cap virus with the TTG-initiation codon.

Figure 3: Tryglycerides (TG) level in blood plasma was determined following injection of AAV vector batches. Mice (n=2) were injected with rAAV produced with the baculovirus-production system (DJ17jun05) and (n=2) the mammalian-production

system (Clin). At 3, 7 and 14 days post-administration of the vector blood samples were taken and assayed for TG fasting.

Figure 4: LPL^{S447X} –activity was measured in plasma samples: mice were injected with rAAV produced with the baculovirus-production system (DJ17jun05) and the mammalian-production system (C-0045). At 14 days post-administration of the vector blood samples were taken and LPL^{S447X} –activity was determined.

Figure 5 Fig.1: Four AAV1-batches produced in insect cells with the baculovirus expression system were loaded onto a Nupage gel to evaluate the VP1,2,3 stoichiometry. Lanes: AAV1-batch prepared with a baculovirus-Cap construct containing VP1-initiation codon CTG (lane 1), GTG (lane 2), ACG (lane 3), TTG (lane4). As a control a plasmid-produced AAV1-batch (FSB-02) was loaded (lane5). The stoichiometry of AAV1 using a CTG-initiation codon is comparable with the FSB-02 batch.

Figure 6 Batches of AAV1-LPL were injected in muscle of LPL-deficient (-/-) mice with $3x10^{12}$ gc/kg. The AAV1-LPL batch prepared with the rBac-Cap construct containing the CTG-initiation codon performs significantly better in terms of Triglyceride (TG) lowering compared to the other batches.

Examples

1. Materials &Methods

1.1 Baculovirus plasmid construction

In order to express VP1,2,3 from a sole polycistronic messenger RNA, the baculovirus-AAV-Cap construct was designed as described by (Urabe et al., 2002, *supra*). Briefly, the ATG initiation codon of VP1 was mutated to ACG. A potential ATG initiation codon at position 11 has been changed to ACG. The splice acceptor site downstream of the VP1 initiation codon was destroyed (mutation at position 21 and 24). The mutated Cap expression cassette was cloned into a baculovirus expression construct; pFastBacDual (pFBDAAV1VPm11) with BamH1/StuI restriction sites. This plasmid (pFBDAAV1VPm11) was the starting material for introduction of alternate initiation codons for VP1. The forward primer used by Urabe et al. (2002, *supra*) in order to introduce the mentioned mutations was:

BamHI 1 11 21 24

5'-cgcggatcctgttaacACGCTGCCCGACGCTTATCTACCCGATTGCCTC-3'

(SEQ ID NO. 1)

The following forward primers were used to make the expression constructs using pFBDAAV1VPm11 (Urabe et al., 2002, *supra*) as starting material:

5'-cgcggatcctgttaacTTGCCTGCCCGACGCTTATCTACCCGATTGCCTC-3'

(SEQ ID NO.2)

5'-cgcggatcctgttaacATTGCTGCCCGACGCTTATCTACCCGATTGCCTC-3'

(SEQ ID NO.3)

5'-cgcggatcctgttaacGTGCCTGCCCGACGCTTATCTACCCGATTGCCTC-3'

(SEQ ID NO. 4)

5'-cgcggatcctgttaacCTGCCTGCCCGACGCTTATCTACCCGATTGCCTC-3'

(SEQ ID NO. 5)

In addition, two forward primers were used to prepare constructs wherein the ACG and CTG codons, respectively are preceded with a preferred form of the Kozak sequence "GCCGCC(NNN)G" (see SEQ ID NO: 8; and wherein (NNN) stands for the suboptimal initiation codon).

5'-ccatcgccgccgccatcctgcgcgcgACGCCTGCCCGACGCTTATCTAC-3'
(SEQ ID NO. 9)

5'-ccatcgccgccgccatcctgcgcgcCTGCCTGCCCGACGCTTATCTAC-3'
(SEQ ID NO. 10)

The backward-primer that was used in the PCR reactions with the above forward primers was directed to position ~ 230 bp downstream of the VP1 initiation codon and contains a unique Stu I site (AGGCCT).

5'-GTCGTAGCGCTTGTCGTGCTCGAGGCGGCGC-3'
(SEQ ID NO. 6)

Fragments were amplified with the above-mentioned sets of forward and backward primer pairs by PCR. Following digestion of PCR products with Bam*HI* and Stu*I* the PCR products were subcloned into the Bam*HI* / Stu*I* sites of pFBDAAV1vpm11 resulting in the various to be tested baculovirus-AAV-Cap constructs. DNA constructs were verified by sequence analysis at Baseclear, Leiden, the Netherlands.

1.2 Recombinant baculovirus production

Recombinant baculoviruses derived from the Autographa californica nuclear polyhydrosis virus (AcNPV) were produced using the Bac-to-Bac baculovirus expression system (Invitrogen). rBac-Cap was amplified by infecting $2x10^6$ Sf9 cells per ml at an moi of 0.1. Three days after infection the cells were spun down and the supernatant containing the virus recovered.

rAAV batches were produced using three recombinant baculoviruses according to Urabe et al., 2002. However, for this study one baculovirus harbored an expression construct for the LPL^{S447X} –transgene. The therapeutically active agent expressed from the transgene is a naturally occurring variant of human lipoprotein lipase, a single chain polypeptide of 448 amino acids. The LPL^{S447X} variant has a deletion of two amino acids at the C-terminus of the protein. The second baculovirus harboured an expression construct for the AAV replication genes, Rep 78 and Rep 52. The third baculovirus harboured the AAV1 capsid sequence with either an ACG or a TTG, CTG, GTG initiation codon for VP1.

Mammalian-rAAV batches produced with the plasmid-transfection system were produced according to Grimm et al., 1998 (Novel tools for production and purification of recombinant adeno-associated virus vectors. Hum Gene Ther. 1998 Dec 10;9(18):2745-60).

1.3 Western blot analysis

Insect cells were infected with baculovirus-Cap. At three days post-infection cells were centrifuged (3,000 g; 15 min). The supernatant was filtered through a 0.22um Millex filter. NuPAGE LDS sample buffer (Invitrogen) was added to a sample of the supernatant and was loaded onto a 4-12% Bis-Tris gel. The gel was run at 100V. Proteins were blotted onto a nitrocellulose membrane (BioRad) for 1 hr, 100V, 350mA. Western immunochemistry was performed by blocking the membrane with 1% marvel, dried skimmed milk and subsequently incubation with mouse anti-Cap (B1 from Progen, Germany; dilution 1:50) and rabbit anti-mouse—HRP (DAKO, dilution 1:100). VP1 ,2 and 3 were visualized by chemoluminescent staining with lumi-light plus Western-blotting substrate (Roche).

1.4 Biochemical measurements

Human LPL^{S447X} activity was assayed as previously described using a radioactive trioleoylglycerol emulsion substrate (Nilsson-Ehle and Scholtz, 1976). Human LPL^{S447X} immunoreactive mass was assayed using a sandwich ELISA with chicken IgY and mouse 5D2 anti-hLPL antibodies (Liu et al., 2000). Plasma triglyceride levels were measured by using commercial kits following manufacturer protocols (Boehringer Mannheim, #450032).

2. Results

2.1 Construction of recombinant baculovirus

In order to introduce different alternate initiation codons for VP1 expression in the baculovirus plasmid designed by Urabe et al. (2002, *supra*) a series of upstream primers were designed containing a BamHI restriction site and either a TTG, ATT, GTG or CTG codon in place of the ACG initiation codon of VP1. PCR using these primers in combination with a downstream primer containing a StuI site resulted in amplified fragments that were subcloned into the BamHI/StuI site of pFBDVPm11 (Bac-Cap). The resulting baculovirus plasmids were used for the preparation of recombinant baculoviruses using the Bac-to-Bac baculovirus expression system. The prepared recombinant baculoviruses were infected on insect cells in order to produce AAV capsids. At three days following infection viral protein expression of the different baculovirus batches were determined on Western blots. From the Western blots it became clear that the baculovirus construct containing the TTG initiation codon for VP1 expressed this protein to a higher level compared to the previously used ACG initiation codon. The ratio between VP1 and VP2 using the TTG codon was found to be 1:1 which is similar to what is reported for wild type AAV (Figure 1).

2.2 Infection of rAAV batches on cells in culture

In order to investigate the infectivity of the AAV capsids derived from recombinant baculoviruses with the TTG initiation codon rAAV was generated. Also a rAAV batch was generated by plasmid transfection on mammalian HEK293 cells. A vector genome titer of both rAAV batches was determined by qPCR. This titer was used to infect HEK 293 cells in a microtiter plate at an increasing moi. At two days following infection an quantitative assay (LPL^{S447X} -mass assay) for the transgene product (LPL^{S447X}) was performed on the medium of the infected cells. The assay showed that the amount of LPL^{S447X} produced by baculovirus-produced rAAV was similar to the LPL produced by the plasmid-produced rAAV (Figure 2).

2.3 Injection of rAAV batches in mice

The rAAV batches produced with the baculovirus-production system and with the conventional mammalian plasmid-production system were injected intramuscularly in

mice to follow LPL^{S447X}-protein activity and triglyceride fasting *in vivo*. At 3 days, 7 days and at 2 weeks following injection blood samples were taken and evaluated. Between 3 and 7 days post virus administration blood-plasma sampled from both mice injected with mammalian-rAAV and one mouse injected with baculo-rAAV was turned from milky to completely clear. Blood plasma derived from one baculo-rAAV-injected mouse remained relatively milky however fat level was clearly reduced. Triglyceride levels were lowered respectively of all treated mice (Figure 3). On day 14 TG levels in both mammalian-AAV and baculovirus-(TTG)-AAV treated mice TG levels were reduced for 96%. Plasma samples taken at two weeks after virus administration showed that the LPL^{S447X} -activity of the mice treated with baculovirus-AAV and mammalian-AAV was similar (Figure 4).

2.4 Production of recombinant AAV batches with different VP1-initiation codons

Recombinant baculoviruses containing a AAV-Cap type 1 expression unit with different VP1-initiation codons (CTG, GTG, TTG, ACG) were used to prepare rAAV1-LPL batches in insect cells. The viruses batches were purified and loaded onto a NuPage gel for evaluation (Figure 5). The gel shows the stoichiometry between the VP1, VP2 and VP3 proteins of the various viruses. First we point to the previously produced rAAV1-LPL using a conventional plasmid-transfection system (pPD1, DKFZ, Heidelberg, Germany) on HEK293 cells. In our hands this system produces an unusual stoichiometry between the VP1, VP2 and VP3 proteins whereby VP1 is present in a higher amount than VP2. This stoichiometry is significantly different from the 1:1:10 reported stoichiometry for the VP1, VP2 and VP3 proteins as is seen in wtAAV or other mammalian AAV vector production platforms.

In the Baculovirus system, the rAAV construct with the CTG codon produces virus that has a significantly higher amount of VP1 compared to the amount of VP2 in the capsid. In this particular experiment (Figure 5) the stoichiometry of the VP1, VP2 and VP3 capsid proteins produced with BAC-Cap GTG or ACG constructs show similar VP1 to VP2-ratio's on gel and the TTG construct appears to express slightly less VP1 than VP2. However, we have repeatedly found that TTG and GTG constructs show a similar stoichiometry whereby the VP1 and VP2 proteins are present in about equal amounts, whereas the ACG construct usually produces a stoichiometry whereby there is less VP1 than VP2 protein. The CTG constructs consistently produce a stoichiometry whereby there is more VP1 than VP2 protein.

To further improve production rates of rAAV we prepared constructs wherein the ACG and CTG codons, respectively are present in a Kozak consensus sequence "GCCGCC(NNN)G" (see SEQ. ID NO: 8), wherein "(NNN)" identifies the position of the ACG and CTG codons, respectively. AAV production in insect cells from the construct wherein the CTG codon is preceded with the Kozak sequence resulted in significantly higher AAV production than the construct without the Kozak sequence (data not shown). In contrast, AAV production in insect cells from the construct wherein an ACG codon is preceded with the Kozak sequence did not result in higher AAV production (data not shown).

2.5 Injection of rAAV batches in mice

In order to verify that the content of VP1 in the viral capsid is linked to vector infectivity the baculovirus-produced AAV1 batches and the plasmid-produced AAV1 batch were injected in the muscle of homozygous LPL (-/-) mice and Triglyceride (TG) lowering as a result of active LPL-expression was monitored in time (Figure 6). The results show that the AAV1-LPL batch produced with the CTG-initiation codon as expected lowers TG-levels faster and more profound compared to the other AAV1 batches. Remarkable however is that, although stoichiometry is similar, the TG-lowering with the baculo-CTG mutant is even more profound compared to the plasmid-produced AAV1 (produced with pDP1).

We have repeatedly seen that baculovirus-produced AAV made with the CTG construct performed better, i.e. showed a higher infectivity, both in vitro and in vivo compared to AAV made with the other suboptimal initiation codon constructs, especially when compared with the construct with the ACG codon. We have also repeatedly seen that the VP1 content in AAV virions made with the CTG construct contains more VP1 compared to VP2 and that this is not the case with the other constructs, especially ACG.

Claims

1. A nucleotide sequence comprising an open reading frame comprising nucleotide sequences encoding adeno-associated virus (AAV) VP1, VP2, and VP3 capsid proteins, wherein the initiation codon for translation of the AAV VP1 capsid protein is selected from TTG, CTG, and GTG.

2. A nucleotide sequence according to claim 1, wherein the nucleotide sequence comprises an expression control sequence comprising a nine nucleotide sequence of SEQ. ID NO: 7 or a nucleotide sequence substantially homologous to SEQ. ID NO: 7, upstream of the initiation codon of the nucleotide sequence encoding the AAV VP1 capsid protein.

3. A nucleotide sequence according to claim 1, wherein the nucleotide sequence comprises an expression control sequence comprising a Kozak consensus sequence around the initiation codon of the nucleotide sequence encoding the AAV VP1 capsid protein, wherein the Kozak consensus sequence is GCCRCC NNN G (SEQ. ID NO: 8), wherein R is a purine and wherein NNN stands for a suboptimal initiation codon selected from TTG, CTG, and GTG.

4. A nucleotide sequence according to any one of claims 1 - 3, wherein the nucleotide sequence further comprises at least one modification of the nucleotide sequence encoding AAV VP1 capsid protein selected from among a G at nucleotide position 12, an A at nucleotide position 21, and a C at nucleotide position 24.

5. A nucleic acid construct comprising a nucleotide sequence according to any one of claims 1 - 4, wherein the nucleotide sequence is operably linked to expression control sequences for expression in an insect cell.

6. A nucleic acid construct according to claim 5, wherein the nucleotide sequence is operably linked to a polyhedron promoter.

7. A nucleic acid construct according to claims 5 or 6, wherein the construct is an insect cell-compatible vector, preferably a baculoviral vector.

8. An insect cell comprising a nucleic acid construct according to any one of claims 5 - 7.

9. An insect cell according to claim 8, wherein the insect cell further comprises:

(a) a second nucleotide sequence comprising at least one AAV inverted terminal repeat (ITR) nucleotide sequence;

(b) a third nucleotide sequence comprising a Rep52 or a Rep40 coding sequence operably linked to expression control sequences for expression in an insect cell; and,

(c) a fourth nucleotide sequence comprising a Rep78 or a Rep68 coding sequence operably linked to expression control sequences for expression in an insect cell.

10. A insect cell according to claim 9, wherein the insect cell comprises:

(a) a first nucleic acid construct according to any of claims 5 - 7, whereby the first nucleic acid construct further comprises the third and fourth nucleotide sequences as defined in (b) and (c) of claim 9; and,

(b) a second nucleic acid construct comprising the second nucleotide sequence as defined in (a) of claim 9.

11. An insect cell according to claim 10, wherein the second nucleic acid construct is an insect cell-compatible vector, preferably a baculoviral vector.

12. An insect cell according to any one of claims 9 - 11, wherein the second nucleotide sequence further comprises at least one nucleotide sequence encoding a gene product of interest (for expression in a mammalian cell) and whereby the at least one nucleotide sequence encoding a gene product of interest becomes incorporated into the genome of an AAV produced in the insect cell.

13. An insect cell according to claim 12, wherein the second nucleotide sequence comprises two AAV ITR nucleotide sequences and wherein the at least one nucleotide

sequence encoding a gene product of interest is located between the two AAV ITR nucleotide sequences.

14. The insect cell according to claim 9, wherein the first nucleotide sequence, second nucleotide sequence, third nucleotide sequence, and fourth nucleotide sequence are stably integrated in the genome of the insect cell.

15. An AAV virion comprising in its genome at least one nucleotide sequence encoding a gene product of interest, whereby the at least one nucleotide sequence is not a native AAV nucleotide sequence, and whereby in the stoichiometry of the AAV VP1, VP2, and VP3 capsid proteins the amount of VP1:
(a) is at least 110% of the amount of VP2; or
(b) is at least 11% of the amount of VP3; or
(c) is at least 110% of the amount of VP2 and at least 11% of the amount of VP3; and, whereby the amount of VP1, VP2 and VP3 is determined using an antibody recognizing an epitope that is common to each of VP1, VP2 and VP3.

16. An AAV virion comprising in its genome at least one nucleotide sequence encoding a gene product of interest, whereby the at least one nucleotide sequence is not a native AAV nucleotide sequence, and whereby the AAV virion comprises a VP1 capsid protein comprises a leucine or a valine at amino acid position 1.

17. An AAV virion according to claim 15, whereby the AAV virion comprises a VP1 capsid protein comprises a leucine or a valine at amino acid position 1.

18. A method for producing an AAV in an insect cell, comprising the steps of: (a) culturing an insect cell as defined in any one of claims 9 - 14 under conditions such that AAV is produced; and, (b) recovery of the AAV.

19. A method according to claim 18, further comprising the step of affinity-purification of the AAV using an anti-AAV antibody, preferably an immobilized antibody.

20. A method according to claim 19, wherein the anti-AAV antibody is a single chain cameloid antibody or a fragment thereof.

Fig 1

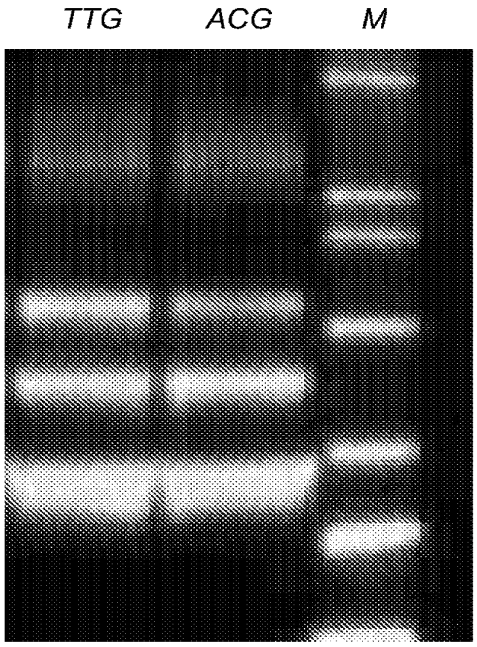

TTG
ACG
M

Fig 2

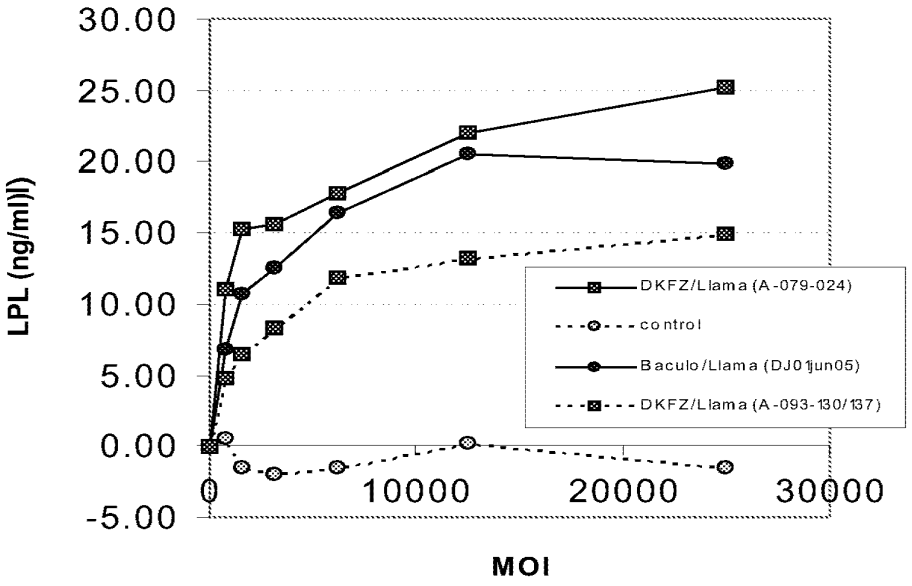
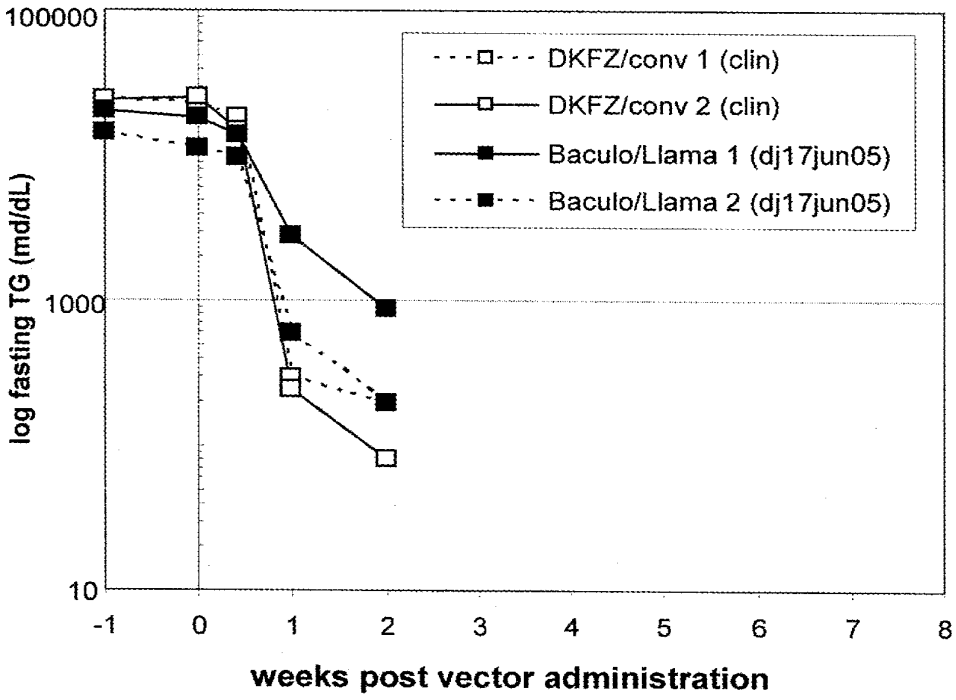

30.00
25.00
20.00
15.00
10.00
5.00
0.00
-5.00
LPL (ng/ml)l)
0
10000
20000
30000
MOI
DKFZ/Llama (A-079-024)
control
Baculo/Llama (DJ01jun05)
DKFZ/Llama (A-093-130/137)

Fig 3


100000
1000
10
log fasting TG (md/dL)
-1
0
1
2
3
4
5
6
7
8
weeks post vector administration
DKFZ/conv 1 (clin)
DKFZ/conv 2 (clin)
Baculo/Llama 1 (dj17jun05)
Baculo/Llama 2 (dj17jun05)

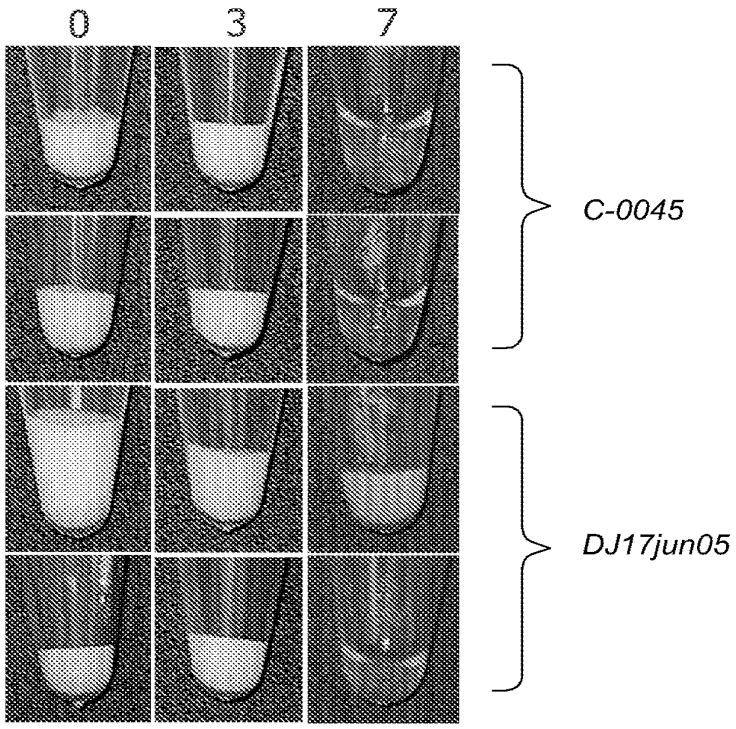

0
3
7
C-0045
DJ17jun05

Fig 4

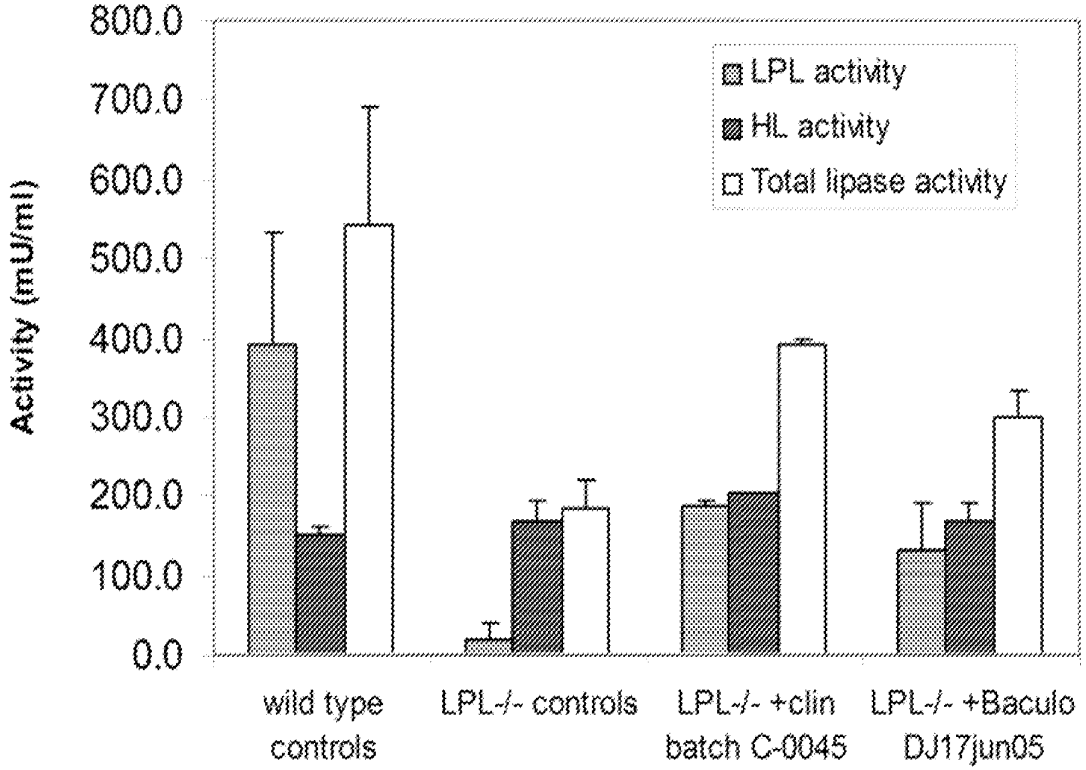

800.0
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
Activity (mU/ml)
LPL activity
HL activity
Total lipase activity
wild type controls
LPL-/- controls
LPL-/- +clin batch C-0045
LPL-/- +Baculo DJ17jun05

Fig 5

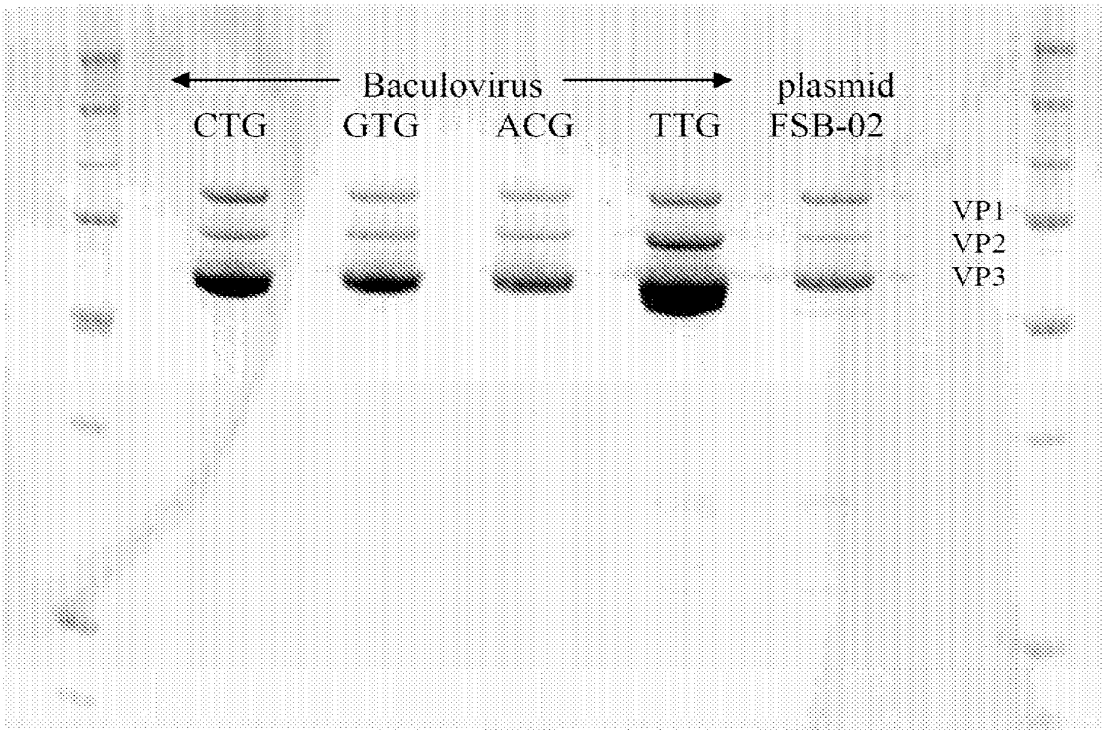
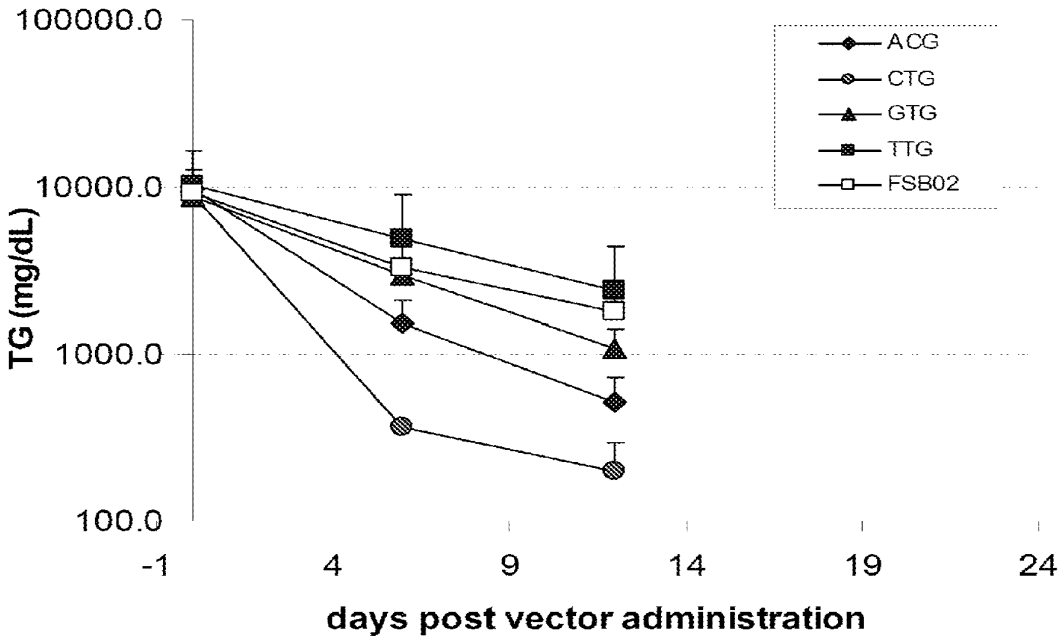

Baculovirus
plasmid
CTG
GTG
ACG
TTG
FSB-02
VP1
VP2
VP3

Fig 6


100000.0
10000.0
1000.0
100.0
TG (mg/dL)
-1
4
9
14
19
24
days post vector administration
ACG
CTG
GTG
TTG
FSB02

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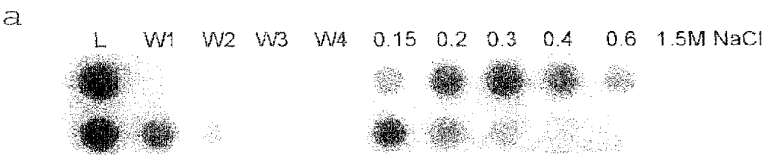
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(54) **Title**: MODIFIED VIRUS VECTORS AND METHODS OF MAKING AND USING THE SAME

Figure 1



(57) **Abstract**: The present invention provides AAV capsid proteins (VP1, VP2 and/or VP3) comprising a modification in the amino acid sequence in the three-fold axis loop 4 and virus capsids and virus vectors comprising the modified AAV capsid protein. In particular embodiments, the modification comprises a substitution of one or more amino acids at amino acid positions 585 to 590 (inclusive) of the native AAV2 capsid protein sequence or the corresponding positions of other AAV capsid proteins. The invention also provides methods of administering the virus vectors and virus capsids of the invention to a cell or to a subject in vivo.

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Modified Virus Vectors and Methods of Making and Using the Same

RELATED APPLICATION INFORMATION

This application claims the benefit of U.S. Provisional Application No. 61/151,736; Filed February 11, 2009, the disclosure of which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

The present invention relates to modified capsid proteins from adeno-associated virus (AAV) and virus capsids and virus vectors comprising the same. In particular, the invention relates to modified AAV capsid proteins and capsids comprising the same that can be incorporated into virus vectors to confer a desirable transduction profile with respect to a target tissue(s) of interest.

STATEMENT OF GOVERNMENT SUPPORT

This invention was supported in part by funding provided under Grant Nos. 1 U54 AR056953 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases, Grant No. 1RO1AI072176 from the National Institute of Allergy and Infectious Diseases, Grant No. 1RO1HL089221 from the National Heart, Lung, and Blood Institute, Grant No. 1 R21 AR055712 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases, and Grant No. 1U24-NS059696 from the National Institute of Neurological Disorders and Stroke. The United States government has certain rights in this invention.

BACKGROUND OF THE INVENTION

New adeno-associated virus (AAV) strains isolated from animal tissues and adenoviral stocks have expanded the panel of AAV vectors available for therapeutic gene transfer applications. Comprehensive efforts to map tissue tropisms of these AAV isolates in animal models are currently underway. For instance, recent studies with AAV serotypes 1-9 indicate a broad tissue tropism in mice following intravenous administration. The AAV serotypes 8 and 9 are particularly notable for their ability to transduce multiple organs including heart, liver and skeletal muscle following intravenous administration. While the latter serotypes are well-suited for systemic gene transfer modalities, the ability to direct homing of AAV vectors to selective

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organs is useful for gene therapy. The development of tissue-specific promoters and miRNA-based gene regulation strategies to sharply segregate gene expression patterns among different tissue types is noteworthy in this regard. However, such regulatory components do not preclude sequestration of AAV vector genomes in off-target organs following systemic administration.

A particularly striking aspect of tissue tropisms displayed by AAV serotypes and variants is their propensity to ubiquitously accumulate within and transduce the liver, albeit with varying efficiency. The molecular basis of this preferential liver tropism has been mapped, in the case of AAV2 and AAV6, to a continuous basic footprint that appears to be involved in the interaction of either serotype with heparin. Specifically, it has previously been demonstrated that a single lysine residue on AAV6 (K531) dictates heparin binding ability and consequently, liver tropism. In corollary, substitutional mutagenesis of the corresponding glutamate/aspartate residue on other serotypes with a lysine residue confers heparin binding, possibly by forming a minimum continuous basic footprint on the capsid surface.

The present inventor addresses a need in the art for nucleic acid delivery vectors with desirable features.

SUMMARY OF THE INVENTION

A comprehensive mutagenesis approach yielded three groups of modified AAV capsid proteins conferring overlapping phenotypes: (a) AAV capsid protein mutants that confer systemic spread; (b) AAV capsid protein mutants that confer detargeting from the liver; (c) AAV capsid protein mutants that confer low efficiency transduction in one or more tissues (*e.g.*, brain). Thus, the invention provides an array of synthetic viral vectors displaying a range of transduction profiles that are suitable for different *in vitro* and *in vivo* applications.

Accordingly, as one aspect the invention provides an adeno-associated virus (AAV) capsid protein, wherein the capsid protein comprises a modification resulting in the amino acid sequence

X^1-X^2-X^3-X^4-X^5-X^6 (SEQ ID NO:1)

at the amino acids corresponding to amino acid positions 585 to 590 (VP1 numbering) of the native AAV2 capsid protein,

wherein X^1 is Q, N, S, P, A or G;

wherein X^2 is any amino acid;

wherein X^3 is any amino acid;

wherein X^4 is T, A, G or N;

wherein X^5 is any amino acid; and

wherein X^6 is P or A.

In particular embodiments, X^1-X^2-X^3-X^4-X^5-X^6 (SEQ ID NO:1) is not AGNAQA.

As a further aspect, the invention also provides an AAV capsid comprising an AAV capsid protein of the invention.

As yet another aspect, the invention provides a virus vector comprising:

(a) an AAV capsid of the invention; and

(b) a nucleic acid comprising at least one terminal repeat sequence,

wherein the nucleic acid is encapsidated by the AAV capsid.

Further provided are pharmaceutical formulations comprising a virus capsid and/or virus vector of the invention in a pharmaceutically acceptable carrier.

As still a further aspect, the invention provides a method of administering a nucleic acid to a cell, the method comprising contacting the cell with a virus vector or pharmaceutical formulation of the invention.

As another aspect, the invention provides a method of delivering a nucleic acid to a subject, the method comprising administering to the subject a virus vector or pharmaceutical formulation of the invention.

These and other aspects of the invention are addressed in more detail in the description of the invention set forth below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1.

(a) Elution profile of parental AAV2 capsids (top) and AAV2i8 (bottom) from a heparin affinity column. Samples were loaded (L) onto heparin-agarose affinity columns in 1xPBS, washed multiple times with 0.1xPBS (W1-4) and eluted at different salt concentrations (0.15M to 1.5M NaCl). Collected fractions were loaded onto a nitrocellulose membrane using a dot blot manifold and detected using the A20 monoclonal antibody, specific for intact AAV2 capsids. AAV2 peak fractions elute at ~300mM NaCl, while AAV2i8 capsids elute under physiological conditions (pH 7.4; 0.15M NaCl).

(b) Analysis of *in vitro* transduction of AAV2i mutants in HEK 293 cells untreated (black bars) or treated with 50mU/mL sialidase (white bars). Luciferase transgene expression levels were determined in cell lysates harvested 24 hours post-infection with AAV2 or an AAV2i mutant (MOI 1000 vg/cell). AAV4 was included as a

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positive control. All experiments were performed in triplicate and standard deviation is shown.

(c) Analysis of *in vitro* transduction of AAV2i mutants in heparin sulfate-negative CHOpgsD cells. Luciferase transgene expression levels were determined in cell lysates harvested 24 hours post-infection with AAV2 or an AAV2i mutant (MOI 1000). All experiments were performed in triplicate and standard deviation is shown.

Figure 2.

(a) Analysis of *in vivo* transduction of AAV2i mutants via intramuscular administration. BALB/c mice (n=3) were injected intramuscularly with AAV2i CMV-Luc vectors (dose 1×10^{10} vg in 200 µl PBS). Representative photographs and live animal bioluminescent images of luciferase transgene expression were obtained at 1 week after injection. Bioluminescence scale ranges from $0–4 \times 10^6$ relative light units (photons/sec/cm^2).

(b) Analysis of *in vivo* transduction of AAV2i mutants via intravenous administration. BALB/c mice (n=3) were injected intravenously (tail vein) with AAV2i CMV-Luc vectors (dose 1×10^{10} vg in 200 µl PBS). Representative photographs and live animal bioluminescent images of luciferase transgene expression were obtained at 1 week after injection. Bioluminescence scale ranges from $0–4 \times 10^5$ relative light units (photons/sec/cm^2).

Figure 3.

Comparison of the *in vivo* transduction profiles of AAV2 and AAV2i8 administered via different intravenous injection routes. BALB/c mice were injected with AAV2 or AAV2i8 CMV-Luc vector (dose 4×10^{10} vg in 200 µl PBS) through either the tail or portal vein. Representative photographs and live animal bioluminescent images of luciferase transgene expression were obtained at 1 week after injection. Bioluminescence scale ranges from $0–2 \times 10^5$ relative light units (photons/sec/cm^2).

Figure 4.

Comparison of *in vivo* transduction via intravenous administration of AAV2i vectors with AAV2 and AAV8 vectors. BALB/c mice (n = 3) were injected intravenously (tail vein) with AAV2, AAV8, AAV2i8 and structurally related AAV2i mutants (dose 1×10^{11} vg in 200 µl PBS) packaging the chicken beta actin (CBA)-Luc cassette. Representative photographs and live animal bioluminescent images of

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luciferase transgene expression were obtained. Bioluminescence scale ranges from 0–3 × 10^6 relative light units (photons/sec/cm^2).

Figure 5.

Comparison of the *in vivo* transduction profiles of AAV1i8 and AAV3i8. BALB/c mice were injected intravenously via the tail vein with AAV1, AAV3, AAV1i8 or AAV3i8 vectors (dose 1 x 10^{11} vg in 200 µl PBS) packaging the CBA-Luc cassette. Representative photographs and live animal bioluminescent images of luciferase transgene expression were obtained at 1 week after injection. Bioluminescence scale ranges from 1–3 × 10^6 relative light units (photons/sec/cm^2).

Figure 6.

(a) Quantitation of transduction efficiency of AAV2i8 vectors compared to AAV2 and AAV8 in cardiac, skeletal muscle (pooled hind limb and abdominal muscles) and liver tissues as measured by luciferase expression. Tissue lysates were obtained from BALB/c mice (n = 3) at 2 weeks after administration of AAV2, AAV2i8 and AAV8 (dose 1 × 10^{11} vg, tail vein) and subjected to luminometric analysis. Luciferase levels are shown as relative light units normalized to protein levels determined using a Bradford assay. Error bars indicate standard deviation.

(b) Quantitation of transduction efficiency of AAV2i8 vectors compared to AAV2 and AAV8 in cardiac, skeletal muscle (pooled hind limb and abdominal muscles) and liver tissues as measured by luciferase genome copy number via Q-PCR. Host genomic as well vector DNA was extracted from tissue lysates obtained from BALB/c mice (n = 3) at 2 weeks after administration of AAV2, AAV2i8 and AAV8 (dose 1 × 10^{11} vg, tail vein). Host and vector genome copy number were determined by Q-PCR with specific primer sets against the lamin gene and luciferase transgene, respectively.

Figure 7.

In vivo transduction efficiency of AAV2i8 of various muscle groups following intravenous administration. Luciferase transgene expression in major muscle sub-groups obtained from BALB/c mice (n = 3) at 2 weeks after intravenous administration of AAV2i8 (dose 1 × 10^{11} vg, tail vein) packaging the CBA-Luc cassette. Tissue lysates from five different muscle groups from the hind limb skeletal muscle (alternating black and white bars), three groups from the forelimb (alternating black and white bars), intercostals, cardiac, facial, diaphragm, tongue, abdominal and vertebral muscle types (black bars) were subjected to luminometric analysis.

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Luciferase levels are shown as relative light units normalized to protein levels determined by a Bradford assay. Error bars indicate standard deviation.

Figure 8.

Comparison of the biodistribution of AAV2i8 and related vectors. BALB/c mice were injected with AAV2i8 or related vectors having a Q/NxxTxP motif (dose 1 x 10^{11} vg in 200 µl PBS) packaging the CBA-Luc cassette. Host and vector genome copy numbers were determined by Q-PCR with specific primer sets against the lamin gene and luciferase transgene, respectively.

Figure 9.

(a) Luciferase transgene expression in pooled skeletal muscle subgroups from right and left hind limb of BALB/c mice (n = 4) after isolated perfusion of AAV2i8 (black bars) or AAV8 (gray bars) into each saphenous vein. Tissue lysates prepared after administration of three different doses (1 × 10^{9}, 1 × 101^{0}, 1 × 10^{11} vg) in low (200 µl), medium (500 µl) or high (1 ml) volume of injection were subjected to luminometric analysis. Luciferase levels are shown as relative light units normalized to protein levels determined using a Bradford assay.

(b) Vector genome copy numbers recovered from blood at different time intervals after administration through the tail vein (n = 3). Whole blood DNA was extracted and analyzed by Q-PCR with primers against the luciferase transgene. AAV2i8 shows prolonged circulation compared with AAV8. Error bars indicate standard deviation.

Figure 10.

Kinetics of luciferase transgene expression in mice following intravenous injection of AAV2i8 vector (dose 1 × 10^{11} vg in 200 µl PBS) packaging the (CBA)-Luc cassette. Representative photographs and live animal bioluminescent images of luciferase transgene expression were obtained at different time intervals after injection (3 days, 1 week, 4 weeks or 12 weeks).

Figure 11.

Comparison of the *in vivo* transduction profiles of 2i8D and 2i8E. BALB/c mice were injected intravenously via the tail vein with AAV2, AAV8, AAV9, 2i8D or 2i8E vectors (dose 1 x 10^{11} vg in 200 µl PBS) packaging the CBA-Luc cassette. Representative photographs and live animal bioluminescent images of luciferase transgene expression were obtained at 4 days after injection.

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DETAILED DESCRIPTION OF THE INVENTION

The present invention will now be described with reference to the accompanying drawings, in which representative embodiments of the invention are shown. This invention may, however, be embodied in different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

The designation of all amino acid positions in the AAV capsid proteins in the description of the invention and the appended claims is with respect to VP1 capsid subunit numbering (native AAV2 VP1 capsid protein: GenBank Accession No. AAC03780 or YP680426). It will be understood by those skilled in the art that the modifications described herein if inserted into the AAV *cap* gene may result in modifications in the VP1, VP2 and/or VP3 capsid subunits. Alternatively, the capsid subunits can be expressed independently to achieve modification in only one or two of the capsid subunits (VP1, VP2, VP3, VP1 + VP2, VP1+VP3, or VP2 +VP3).

Definitions.

The following terms are used in the description herein and the appended claims:

The singular forms "a," "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

Furthermore, the term "about," as used herein when referring to a measurable value such as an amount of the length of a polynucleotide or polypeptide sequence, dose, time, temperature, and the like, is meant to encompass variations of ± 20%, ± 10%, ± 5%, ± 1%, ± 0.5%, or even ± 0.1% of the specified amount.

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Also as used herein, "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative ("or").

Unless the context indicates otherwise, it is specifically intended that the various features of the invention described herein can be used in any combination.

Moreover, the present invention also contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted.

To illustrate further, if, for example, the specification indicates that a particular amino acid can be selected from A, G, I, L and/or V, this language also indicates that the amino acid can be selected from any subset of these amino acid(s) for example A, G, I or L; A, G, I or V; A or G; only L; etc. as if each such subcombination is expressly set forth herein. Moreover, such language also indicates that one or more of the specified amino acids can be disclaimed. For example, in particular embodiments the amino acid is not A, G or I; is not A; is not G or V; etc. as if each such possible disclaimer is expressly set forth herein.

As used herein, the terms "reduce," "reduces," "reduction" and similar terms mean a decrease of at least about 25%, 35%, 50%, 75%, 80%, 85%, 90%, 95%, 97% or more.

As used herein, the terms "enhance," "enhances," "enhancement" and similar terms indicate an increase of at least about 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400%, 500% or more.

The term "parvovirus" as used herein encompasses the family *Parvoviridae*, including autonomously replicating parvoviruses and dependoviruses. The autonomous parvoviruses include members of the genera *Parvovirus*, *Erythrovirus*, *Densovirus*, *Iteravirus*, and *Contravirus*. Exemplary autonomous parvoviruses include, but are not limited to, minute virus of mouse, bovine parvovirus, canine parvovirus, chicken parvovirus, feline panleukopenia virus, feline parvovirus, goose parvovirus, H1 parvovirus, muscovy duck parvovirus, B19 virus, and any other autonomous parvovirus now known or later discovered. Other autonomous parvoviruses are known to those skilled in the art. *See, e.g.*, BERNARD N. FIELDS *et al.*, VIROLOGY, volume 2, chapter 69 (4th ed., Lippincott-Raven Publishers).

As used herein, the term "adeno-associated virus" (AAV), includes but is not limited to, AAV type 1, AAV type 2, AAV type 3 (including types 3A and 3B), AAV type 4, AAV type 5, AAV type 6, AAV type 7, AAV type 8, AAV type 9, AAV type 10, AAV type 11, avian AAV, bovine AAV, canine AAV, equine AAV, ovine AAV, and any other AAV now known or later discovered. *See, e.g.*, BERNARD N. FIELDS *et al.*,

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VIROLOGY, volume 2, chapter 69 (4th ed., Lippincott-Raven Publishers). A number of relatively new AAV serotypes and clades have been identified (*see, e.g.*, Gao et al., (2004) *J. Virology* 78:6381-6388; Moris et al., (2004) *Virology* 33-:375-383; and **Table 1**).

The genomic sequences of various serotypes of AAV and the autonomous parvoviruses, as well as the sequences of the native terminal repeats (TRs), Rep proteins, and capsid subunits are known in the art. Such sequences may be found in the literature or in public databases such as GenBank. *See, e.g.*, GenBank Accession Numbers NC_002077, NC_001401, NC_001729, NC_001863, NC_001829, NC_001862, NC_000883, NC_001701, NC_001510, NC_006152, NC_006261, AF063497, U89790, AF043303, AF028705, AF028704, J02275, J01901, J02275, X01457, AF288061, AH009962, AY028226, AY028223, NC_001358, NC_001540, AF513851, AF513852, AY530579; the disclosures of which are incorporated by reference herein for teaching parvovirus and AAV nucleic acid and amino acid sequences. *See also, e.g.*, Srivistava et al., (1983) *J. Virology* 45:555; Chiorini et al., (1998) *J. Virology* 71:6823; Chiorini *et al.*, (1999) *J. Virology* 73:1309; Bantel-Schaal et al., (1999) *J. Virology* 73:939; Xiao et al., (1999) *J. Virology* 73:3994; Muramatsu et al., (1996) *Virology* 221:208; Shade et al., (1986) *J. Virol.* 58:921; Gao et al., (2002) *Proc. Nat. Acad. Sci. USA* 99:11854; Moris et al., (2004) *Virology* 33-:375-383; international patent publications WO 00/28061, WO 99/61601, WO 98/11244; and U.S. Patent No. 6,156,303; the disclosures of which are incorporated by reference herein for teaching parvovirus and AAV nucleic acid and amino acid sequences. *See also* **Table 1**.

The capsid structures of autonomous parvoviruses and AAV are described in more detail in BERNARD N. FIELDS *et al.*, VIROLOGY, volume 2, chapters 69 & 70 (4th ed., Lippincott-Raven Publishers). *See also*, description of the crystal structure of AAV2 (Xie et al., (2002) *Proc. Nat. Acad. Sci.* 99:10405-10), AAV4 (Padron et al., (2005) *J. Virol.* 79: 5047-58), AAV5 (Walters et al., (2004) *J. Virol.* 78: 3361-71) and CPV (Xie et al., (1996) *J. Mol. Biol.* 6:497-520 and Tsao et al., (1991) *Science* 251: 1456-64).

The term "tropism" as used herein refers to preferential entry of the virus into certain cells or tissues, optionally followed by expression (*e.g.*, transcription and, optionally, translation) of a sequence(s) carried by the viral genome in the cell, *e.g.*, for a recombinant virus, expression of a heterologous nucleic acid(s) of interest. Those skilled in the art will appreciate that transcription of a heterologous nucleic acid sequence from the viral genome may not be initiated in the absence of trans-

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Table 1

Complete Genomes	GenBank Accession Number			GenBank Accession Number			GenBank Accession Number
Adeno-associated virus 1	NC_002077,AF063497		Hu T88	AY695375		Clade E	
Adeno-associated virus 2	NC_001401		Hu T71	AY695374		Rh38	AY530558
Adeno-associated virus 3	NC_001729		Hu T70	AY695373		Hu66	AY530626
Adeno-associated virus 3B	NC_001863		Hu T40	AY695372		Hu42	AY530605
Adeno-associated virus 4	NC_001829		Hu T32	AY695371		Hu67	AY530627
Adeno-associated virus 5	Y18065, AF085716		Hu T17	AY695370		Hu40	AY530603
Adeno-associated virus 6	NC_001862		Hu LG15	AY695377		Hu41	AY530604
Avian AAV ATCC VR-865	AY186198, AY629583, NC_004828					Hu37	AY530600
Avian AAV strain DA-1	NC_006263, AY629583		Clade C			Rh40	AY530559
Bovine AAV	NC_005889, AY388617		Hu9	AY530629		Rh2	AY243007
			Hu10	AY530576		Bb1	AY243023
Clade A			Hu11	AY530577		Bb2	AY243022
AAV1	NC_002077,AF063497		Hu53	AY530615		Rh10	AY243015
AAV6	NC_001862		Hu55	AY530617		Hu17	AY530582
Hu 48	AY530611		Hu54	AY530616		Hu6	AY530621
Hu 43	AY530606		Hu7	AY530628		Rh25	AY530557
Hu 44	AY530607		Hu18	AY530583		Pi2	AY530554
Hu 46	AY530609		Hu15	AY530580		Pi1	AY530553
			Hu16	AY530581		Pi3	AY530555
Clade B			Hu25	AY530591		Rh57	AY530569
Hu. 19	AY530584		Hu60	AY530622		Rh50	AY530563
Hu. 20	AY530586		Ch5	AY243021		Rh49	AY530562
Hu 23	AY530589		Hu3	AY530595		Hu39	AY530601
Hu22	AY530588		Hu1	AY530575		Rh58	AY530570
Hu24	AY530590		Hu4	AY530602		Rh61	AY530572
Hu21	AY530587		Hu2	AY530585		Rh52	AY530565
Hu27	AY530592		Hu61	AY530623		Rh53	AY530566
Hu28	AY530593					Rh51	AY530564
Hu 29	AY530594		Clade D			Rh64	AY530574
Hu63	AY530624		Rh62	AY530573		Rh43	AY530560
Hu64	AY530625		Rh48	AY530561		AAV8	AF513852
Hu13	AY530578		Rh54	AY530567		Rh8	AY242997
Hu56	AY530618		Rh55	AY530568		Rh1	AY530556
Hu57	AY530619		Cy2	AY243020			
Hu49	AY530612		AAV7	AF513851		Clade F	
Hu58	AY530620		Rh35	AY243000		Hu14 (AAV9)	AY530579
Hu34	AY530598		Rh37	AY242998		Hu31	AY530596
Hu35	AY530599		Rh36	AY242999		Hu32	AY530597
AAV2	NC_001401		Cy6	AY243016			
Hu45	AY530608		Cy4	AY243018		Clonal Isolate	
Hu47	AY530610		Cy3	AY243019		AAV5	Y18065, AF085716
Hu51	AY530613		Cy5	AY243017		AAV 3	NC_001729
Hu52	AY530614		Rh13	AY243013		AAV 3B	NC_001863
Hu T41	AY695378					AAV4	NC_001829
Hu S17	AY695376					Rh34	AY243001
						Rh33	AY243002
						Rh32	AY243003

acting factors, *e.g.*, for an inducible promoter or otherwise regulated nucleic acid sequence. In the case of a rAAV genome, gene expression from the viral genome may be from a stably integrated provirus, from a non-integrated episome, as well as any other form in which the virus may take within the cell.

As used here, "systemic tropism" and "systemic transduction" (and equivalent terms) indicate that the virus capsid or virus vector of the invention exhibits tropism for or transduces, respectively, tissues throughout the body (*e.g.*, brain, lung, skeletal muscle, heart, liver, kidney and/or pancreas). In embodiments of the invention, systemic transduction of muscle tissues (*e.g.*, skeletal muscle, diaphragm and cardiac muscle) is observed. In other embodiments, systemic transduction of skeletal muscle tissues achieved. For example, in particular embodiments, essentially all skeletal muscles throughout the body are transduced (although the efficiency of transduction may vary by muscle type). In particular embodiments, systemic transduction of limb muscles, cardiac muscle and diaphragm muscle is achieved. Optionally, the virus capsid or virus vector is administered via a systemic route (*e.g.*, systemic route such as intravenously, intra-articularly or intra-lymphatically). Alternatively, in other embodiments, the capsid or virus vector is delivered locally (*e.g.*, to the footpad, intramuscularly, intradermally, subcutaneously, topically). Examples of modified virus vectors according to the present invention are provided in Table 5 (*see also*, Figure 4).

Unless indicated otherwise, "efficient transduction" or "efficient tropism," or similar terms, can be determined by reference to a suitable control (*e.g.*, at least about 50%, 60%, 70%, 80%, 85%, 90%, 95% or more of the transduction or tropism, respectively, of the control). In particular embodiments, the virus vector efficiently transduces or has efficient tropism for skeletal muscle, cardiac muscle, diaphragm muscle, pancreas (including β-islet cells), spleen, the gastrointestinal tract (*e.g.*, epithelium and/or smooth muscle), cells of the central nervous system, lung, joint cells, and/or kidney. Suitable controls will depend on a variety of factors including the desired tropism profile. For example, AAV8 and AAV9 are highly efficient in transducing skeletal muscle, cardiac muscle and diaphragm muscle, but have the disadvantage of also transducing liver with high efficiency. Thus, the invention can be practiced to identify viral vectors of the invention that demonstrate the efficient transduction of skeletal, cardiac and/or diaphragm muscle of AAV8 or AAV9, but with a much lower transduction efficiency for liver. Further, because the tropism profile of interest may reflect tropism toward multiple target tissues, it will be appreciated that a suitable vector may represent some tradeoffs. To illustrate, a virus vector of the invention may be less efficient than AAV8 or AAV9 in transducing skeletal muscle,

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cardiac muscle and/or diaphragm muscle, but because of low level transduction of liver, may nonetheless be very desirable.

Similarly, it can be determined if a virus "does not efficiently transduce" or "does not have efficient tropism" for a target tissue, or similar terms, by reference to a suitable control. In particular embodiments, the virus vector does not efficiently transduce (*i.e.*, has does not have efficient tropism) for liver, kidney, gonads and/or germ cells. In particular embodiments, undesirable transduction of tissue(s) (*e.g.*, liver) is 20% or less, 10% or less, 5% or less, 1% or less, 0.1% or less of the level of transduction of the desired target tissue(s) (*e.g.*, skeletal muscle, diaphragm muscle, cardiac muscle and/or cells of the central nervous system).

As used herein, the term "polypeptide" encompasses both peptides and proteins, unless indicated otherwise.

A "polynucleotide" is a sequence of nucleotide bases, and may be RNA, DNA or DNA-RNA hybrid sequences (including both naturally occurring and non-naturally occurring nucleotide), but in representative embodiments are either single or double stranded DNA sequences.

As used herein, an "isolated" polynucleotide (e.g., an "isolated DNA" or an "isolated RNA") means a polynucleotide at least partially separated from at least some of the other components of the naturally occurring organism or virus, for example, the cell or viral structural components or other polypeptides or nucleic acids commonly found associated with the polynucleotide. In representative embodiments an "isolated" nucleotide is enriched by at least about 10-fold, 100-fold, 1000-fold, 10,000-fold or more as compared with the starting material.

Likewise, an "isolated" polypeptide means a polypeptide that is at least partially separated from at least some of the other components of the naturally occurring organism or virus, for example, the cell or viral structural components or other polypeptides or nucleic acids commonly found associated with the polypeptide. In representative embodiments an "isolated" polypeptide is enriched by at least about 10-fold, 100-fold, 1000-fold, 10,000-fold or more as compared with the starting material.

As used herein, by "isolate" or "purify" (or grammatical equivalents) a virus vector, it is meant that the virus vector is at least partially separated from at least some of the other components in the starting material. In representative embodiments an "isolated" or "purified" virus vector is enriched by at least about 10-fold, 100-fold, 1000-fold, 10,000-fold or more as compared with the starting material.

A "therapeutic polypeptide" is a polypeptide that can alleviate, reduce, prevent, delay and/or stabilize symptoms that result from an absence or defect in a

protein in a cell or subject and/or is a polypeptide that otherwise confers a benefit to a subject, *e.g.*, anti-cancer effects or improvement in transplant survivability.

By the terms "treat," "treating" or "treatment of" (and grammatical variations thereof) it is meant that the severity of the subject's condition is reduced, at least partially improved or stabilized and/or that some alleviation, mitigation, decrease or stabilization in at least one clinical symptom is achieved and/or there is a delay in the progression of the disease or disorder.

The terms "prevent," "preventing" and "prevention" (and grammatical variations thereof) refer to prevention and/or delay of the onset of a disease, disorder and/or a clinical symptom(s) in a subject and/or a reduction in the severity of the onset of the disease, disorder and/or clinical symptom(s) relative to what would occur in the absence of the methods of the invention. The prevention can be complete, *e.g.*, the total absence of the disease, disorder and/or clinical symptom(s). The prevention can also be partial, such that the occurrence of the disease, disorder and/or clinical symptom(s) in the subject and/or the severity of onset is less than what would occur in the absence of the present invention.

A "treatment effective" amount as used herein is an amount that is sufficient to provide some improvement or benefit to the subject. Alternatively stated, a "treatment effective" amount is an amount that will provide some alleviation, mitigation, decrease or stabilization in at least one clinical symptom in the subject. Those skilled in the art will appreciate that the therapeutic effects need not be complete or curative, as long as some benefit is provided to the subject.

A "prevention effective" amount as used herein is an amount that is sufficient to prevent and/or delay the onset of a disease, disorder and/or clinical symptoms in a subject and/or to reduce and/or delay the severity of the onset of a disease, disorder and/or clinical symptoms in a subject relative to what would occur in the absence of the methods of the invention. Those skilled in the art will appreciate that the level of prevention need not be complete, as long as some benefit is provided to the subject.

The terms "heterologous nucleotide sequence" and "heterologous nucleic acid" are used interchangeably herein and refer to a sequence that is not naturally occurring in the virus. Generally, the heterologous nucleic acid comprises an open reading frame that encodes a polypeptide or nontranslated RNA of interest (*e.g.*, for delivery to a cell or subject).

As used herein, the terms "virus vector," "vector" or "gene delivery vector" refer to a virus (*e.g.*, AAV) particle that functions as a nucleic acid delivery vehicle, and which comprises the vector genome (*e.g.*, viral DNA [vDNA]) packaged within a

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virion. Alternatively, in some contexts, the term "vector" may be used to refer to the vector genome/vDNA alone.

A "rAAV vector genome" or "rAAV genome" is an AAV genome (*i.e.*, vDNA) that comprises one or more heterologous nucleic acid sequences. rAAV vectors generally require only the terminal repeat(s) (TR(s)) in *cis* to generate virus. All other viral sequences are dispensable and may be supplied in *trans* (Muzyczka, (1992) *Curr. Topics Microbiol. Immunol.* 158:97). Typically, the rAAV vector genome will only retain the one or more TR sequence so as to maximize the size of the transgene that can be efficiently packaged by the vector. The structural and non-structural protein coding sequences may be provided in *trans* (*e.g.*, from a vector, such as a plasmid, or by stably integrating the sequences into a packaging cell). In embodiments of the invention the rAAV vector genome comprises at least one TR sequence (e.g., AAV TR sequence), optionally two TRs (*e.g.*, two AAV TRs), which typically will be at the 5' and 3' ends of the vector genome and flank the heterologous nucleic acid, but need not be contiguous thereto. The TRs can be the same or different from each other.

The term "terminal repeat" or "TR" includes any viral terminal repeat or synthetic sequence that forms a hairpin structure and functions as an inverted terminal repeat (*i.e.*, mediates the desired functions such as replication, virus packaging, integration and/or provirus rescue, and the like). The TR can be an AAV TR or a non-AAV TR. For example, a non-AAV TR sequence such as those of other parvoviruses (e.g., canine parvovirus (CPV), mouse parvovirus (MVM), human parvovirus B-19) or any other suitable virus sequence (*e.g.*, the SV40 hairpin that serves as the origin of SV40 replication) can be used as a TR, which can further be modified by truncation, substitution, deletion, insertion and/or addition. Further, the TR can be partially or completely synthetic, such as the "double-D sequence" as described in United States Patent No. 5,478,745 to Samulski *et al.*

An "AAV terminal repeat" or "AAV TR" may be from any AAV, including but not limited to serotypes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 or any other AAV now known or later discovered (*see, e.g.*, **Table 1**). An AAV terminal repeat need not have the native terminal repeat sequence (*e.g.*, a native AAV TR sequence may be altered by insertion, deletion, truncation and/or missense mutations), as long as the terminal repeat mediates the desired functions, *e.g.*, replication, virus packaging, integration, and/or provirus rescue, and the like.

The virus vectors of the invention can further be "targeted" virus vectors (*e.g.*, having a directed tropism) and/or a "hybrid" parvovirus (*i.e.*, in which the viral TRs

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and viral capsid are from different parvoviruses) as described in international patent publication WO 00/28004 and Chao et al., (2000) *Molecular Therapy* 2:619.

The virus vectors of the invention can further be duplexed parvovirus particles as described in international patent publication WO 01/92551 (the disclosure of which is incorporated herein by reference in its entirety). Thus, in some embodiments, double stranded (duplex) genomes can be packaged into the virus capsids of the invention.

Further, the viral capsid or genomic elements can contain other modifications, including insertions, deletions and/or substitutions.

As used herein, the term "amino acid" encompasses any naturally occurring amino acid, modified forms thereof, and synthetic amino acids.

Naturally occurring, levorotatory (L-) amino acids are shown in **Table 2**).

TABLE 2

Amino Acid Residue	Abbreviation	
	Three-Letter Code	One-Letter Code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid (Aspartate)	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid (Glutamate)	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Alternatively, the amino acid can be a modified amino acid residue (nonlimiting examples are shown in **Table 3**) and/or can be an amino acid that is modified by post-translation modification (*e.g.*, acetylation, amidation, formylation, hydroxylation, methylation, phosphorylation or sulfatation).

TABLE 3

Modified Amino Acid Residue	Abbreviation
Amino Acid Residue Derivatives	
2-Aminoadipic acid	Aad
3-Aminoadipic acid	bAad
beta-Alanine, beta-Aminoproprionic acid	bAla
2-Aminobutyric acid	Abu
4-Aminobutyric acid, Piperidinic acid	4Abu
6-Aminocaproic acid	Acp
2-Aminoheptanoic acid	Ahe
2-Aminoisobutyric acid	Aib
3-Aminoisobutyric acid	bAib
2-Aminopimelic acid	Apm
t-butylalanine	t-BuA
Citrulline	Cit
Cyclohexylalanine	Cha
2,4-Diaminobutyric acid	Dbu
Desmosine	Des
2,2'-Diaminopimelic acid	Dpm
2,3-Diaminoproprionic acid	Dpr
N-Ethylglycine	EtGly
N-Ethylasparagine	EtAsn
Homoarginine	hArg
Homocysteine	hCys
Homoserine	hSer
Hydroxylysine	Hyl
Allo-Hydroxylysine	aHyl
3-Hydroxyproline	3Hyp
4-Hydroxyproline	4Hyp
Isodesmosine	Ide
allo-Isoleucine	aIle
Methionine sulfoxide	MSO
N-Methylglycine, sarcosine	MeGly
N-Methylisoleucine	MeIle
6-N-Methyllysine	MeLys
N-Methylvaline	MeVal
2-Naphthylalanine	2-Nal
Norvaline	Nva
Norleucine	Nle
Ornithine	Orn
4-Chlorophenylalanine	Phe(4-Cl)
2-Fluorophenylalanine	Phe(2-F)
3-Fluorophenylalanine	Phe(3-F)
4-Fluorophenylalanine	Phe(4-F)
Phenylglycine	Phg
Beta-2-thienylalanine	Thi

Further, the non-naturally occurring amino acid can be an "unnatural" amino acid as described by Wang et al., *Annu Rev Biophys Biomol Struct.* 35:225-49 (2006)). These unnatural amino acids can advantageously be used to chemically link molecules of interest to the AAV capsid protein.

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Modified AAV Capsid Proteins and Virus Capsids and Virus Vectors Comprising the Same.

The present invention provides AAV capsid proteins (VP1, VP2 and/or VP3) comprising a modification in the amino acid sequence in the three-fold axis loop 4 (Opie et al., *J. Virol.* 77: 6995-7006 (2003)) and virus capsids and virus vectors comprising the modified AAV capsid protein. The inventors have discovered that modifications in this loop can confer one or more desirable properties to virus vectors comprising the modified AAV capsid protein including without limitation (i) reduced transduction of liver, (ii) enhanced movement across endothelial cells, (iii) systemic transduction; (iv) enhanced transduction of muscle tissue (*e.g.*, skeletal muscle, cardiac muscle and/or diaphragm muscle), and/or (v) reduced transduction of brain tissues (*e.g.*, neurons). Thus, the present invention addresses some of the limitations associated with conventional AAV vectors. For example, vectors based on AAV8 and rAAV9 vectors are attractive for systemic nucleic acid delivery because they readily cross the endothelial cell barrier; however, systemic administration of rAAV8 or rAAV9 results in most of the vector being delivered to the liver, thereby reducing transduction of other important target tissues such as skeletal muscle.

In embodiments of the invention, transduction of cardiac muscle and/or skeletal muscle (determined on the basis of an individual skeletal muscle, multiple skeletal muscles, or the whole range of skeletal muscles) is at least about five-fold, ten-fold, 50-fold, 100-fold, 1000-fold or higher than transduction levels in liver.

In particular embodiments, the modified AAV capsid protein of the invention comprises one or more modifications in the amino acid sequence of the three-fold axis loop 4 (*e.g.*, amino acid positions 575 to 600 [inclusive] of the native AAV2 VP1 capsid protein or the corresponding region of a capsid protein from another AAV). As used herein, a "modification" in an amino acid sequence includes substitutions, insertions and/or deletions, each of which can involve one, two, three, four, five, six, seven, eight, nine, ten or more amino acids. In particular embodiments, the modification is a substitution. For example, in particular embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more amino acids from the three-fold axis loop 4 from one AAV can be substituted into amino acid positions 575-600 of the native AAV2 capsid protein or the corresponding positions of the capsid protein from another AAV. However, the modified virus capsids of the invention are not limited to AAV capsids in which amino acids from one AAV capsid are substituted into another AAV capsid, and the substituted and/or inserted amino

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acids can be from any source, and can further be naturally occurring or partially or completely synthetic.

As described herein, the nucleic acid and amino acid sequences of the capsid proteins from a number of AAV are known in the art. Thus, the amino acids "corresponding" to amino acid positions 575 to 600 (inclusive) or amino acid positions 585 to 590 (inclusive) of the native AAV2 capsid protein can be readily determined for any other AAV (*e.g.*, by using sequence alignments).

The invention contemplates that the modified capsid proteins of the invention can be produced by modifying the capsid protein of any AAV now known or later discovered. Further, the AAV capsid protein that is to be modified can be a naturally occurring AAV capsid protein (*e.g.*, an AAV2, AAV3a or 3b, AAV4, AAV5, AAV8, AAV9, AAV10 or AAV11 capsid protein or any of the AAV shown in **Table 1**) but is not so limited. Those skilled in the art will understand that a variety of manipulations to the AAV capsid proteins are known in the art and the invention is not limited to modifications of naturally occurring AAV capsid proteins. For example, the capsid protein to be modified may already have alterations as compared with naturally occurring AAV (*e.g.*, is derived from a naturally occurring AAV capsid protein, *e.g.*, AAV2, AAV3a, AAV3b, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10 and/or AAV11 or any other AAV now known or later discovered). Such AAV capsid proteins are also within the scope of the present invention.

For example, the AAV capsid protein to be modified can comprise an amino acid insertion directly following amino acid 264 of the native AAV2 capsid protein sequence (*see, e.g.*, WO 2006/066066) and/or can be an AAV with an altered HI loop as described in WO 2009/108274 and/or can be an AAV that is modified to contain a poly-His sequence to facilitate purification. As another illustrative example, the AAV capsid protein can have a peptide targeting sequence incorporated therein as an insertion or substitution. Further, the AAV capsid protein can comprise a large domain from another AAV that has been substituted and/or inserted into the capsid protein.

Thus, in particular embodiments, the AAV capsid protein to be modified can be derived from a naturally occurring AAV but further comprise one or more foreign sequences (*e.g.*, that are exogenous to the native virus) that are inserted and/or substituted into the capsid protein and/or has been altered by deletion of one or more amino acids.

Accordingly, when referring herein to a specific AAV capsid protein (*e.g.*, an AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10 or AAV11 capsid protein or a capsid protein from any of the AAV shown in **Table 1**, *etc.*), it is intended

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to encompass the native capsid protein as well as capsid proteins that have alterations other than the modifications of the invention. Such alterations include substitutions, insertions and/or deletions. In particular embodiments, the capsid protein comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20, less than 20, less than 30, less than 40 less than 50, less than 60, or less than 70 amino acids inserted therein (other than the insertions of the present invention) as compared with the native AAV capsid protein sequence. In embodiments of the invention, the capsid protein comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20, less than 20, less than 30, less than 40 less than 50, less than 60, or less than 70 amino acid substitutions (other than the amino acid substitutions according to the present invention) as compared with the native AAV capsid protein sequence. In embodiments of the invention, the capsid protein comprises a deletion of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20, less than 20, less than 30, less than 40 less than 50, less than 60, or less than 70 amino acids (other than the amino acid deletions of the invention) as compared with the native AAV capsid protein sequence.

Thus, for example, the term "AAV2 capsid protein" includes AAV capsid proteins having the native AAV2 capsid protein sequence (*see* GenBank Accession No. AAC03780) as well as those comprising substitutions, insertions and/or deletions (as described in the preceding paragraph) in the native AAV2 capsid protein sequence.

In particular embodiments, the AAV capsid protein has the native AAV capsid protein sequence or has an amino acid sequence that is at least about 90%, 95%, 97%, 98% or 99% similar or identical to a native AAV capsid protein sequence. For example, in particular embodiments, an "AAV2" capsid protein encompasses the native AAV2 capsid protein sequence as well as sequences that are at least about 90%, 95%, 97%, 98% or 99% similar or identical to the native AAV2 capsid protein sequence.

Methods of determining sequence similarity or identity between two or more amino acid sequences are known in the art. Sequence similarity or identity may be 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. USA* 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, WI), the

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Best Fit sequence program described by Devereux *et al.*, *Nucl. Acid Res.* 12, 387-395 (1984), or by inspection.

Another suitable algorithm is the BLAST algorithm, described in Altschul et al., *J. Mol. Biol.* 215, 403-410, (1990) and Karlin et al., *Proc. Natl. Acad. Sci. USA* 90, 5873-5787 (1993). A particularly useful BLAST program is the WU-BLAST-2 program which was obtained from Altschul et al., *Methods in Enzymology*, 266, 460-480 (1996); http://blast.wustl/edu/blast/ README.html. WU-BLAST-2 uses several search parameters, which are optionally set to the default values. The parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched; however, the values may be adjusted to increase sensitivity.

Further, an additional useful algorithm is gapped BLAST as reported by Altschul *et al.*, (1997) *Nucleic Acids Res.* 25, 3389-3402.

In embodiments of the invention, the AAV capsid protein sequence is not an AAV1, AAV3a and/or AAV3b capsid protein sequence.

In embodiments of the invention, the AAV capsid protein sequence is not the native AAV1, AAV3a and/or AAV3b capsid protein sequence.

In representative embodiments of the invention, a modification is made in the region of amino acid positions 585 to 590 (inclusive) of the native AAV2 capsid protein (using VP1 numbering) or the corresponding positions of other AAV (native AAV2 VP1 capsid protein: GenBank Accession No. AAC03780 or YP680426), *i.e.*, at the amino acids corresponding to amino acid positions 585 to 590 (VP1 numbering) of the native AAV2 capsid protein. The amino acid positions in other AAV serotypes or modified AAV capsids that "correspond to" positions 585 to 590 of the native AAV2 capsid protein will be apparent to those skilled in the art and can be readily determined using sequence alignment techniques (*see, e.g.*, Figure 7 of WO 2006/066066) and/or crystal structure analysis (Padron et al., (2005) J. Virol. 79:5047-58).

To illustrate, the modification can be introduced into an AAV capsid protein that already contains insertions and/or deletions such that the position of all downstream sequences is shifted. In this situation, the amino acid positions corresponding to amino acid positions 585 to 590 in the AAV2 capsid protein would still be readily identifiable to those skilled in the art. To illustrate, the capsid protein can be an AAV2 capsid protein that contains an insertion following amino acid position 264 (*see, e.g.*, WO 2006/066066). The amino acids found at positions 585

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through 590 (*e.g.*, RGNRQA in the native AAV2 capsid protein) would now be at positions 586 through 591 but would still be identifiable to those skilled in the art.

In representative embodiments, the one or more modifications of the invention are incorporated into the AAV capsid at or directly adjacent to one or more amino acids in the following sequences:

(a) SSSTDP;

(b) RGNRQA;

(c) SSNTAP;

(d) SNSNLP;

(e) SSTTAP;

(f) AANTAA;

(g) QQNTAP;

(h) SAQAQA;

(i) QANTGP; or

(j) NATTAP.

In other representative embodiments of the invention, one or more amino acid substitutions (*e.g.*, 1, 2, 3, 4, 5 or 6) are incorporated in the region of amino acid 585 to 590 (inclusive) of the amino acid sequence of the AAV2 capsid protein or the corresponding positions of other AAV.

The invention also contemplates a modified AAV capsid protein (*e.g.*, VP1, VP2 and/or VP3) comprising the sequence X^1-X^2-X^3-X^4-X^5-X^6 (SEQ ID NO:1) at the amino acid positions corresponding to 585 to 590 (inclusive) of the native AAV2 capsid protein or the corresponding positions of other AAV.

In embodiments of the invention, the amino acid sequence X^1-X^2-X^3-X^4-X^5-X^6 (SEQ ID NO:1) is substituted for the amino acid sequence:

(a) SSSTDP;

(b) RGNRQA;

(c) SSNTAP;

(d) SNSNLP;

(e) SSTTAP;

(f) AANTAA;

(g) QQNTAP;

(h) SAQAQA;

(i) QANTGP; or

(j) NATTAP

in the native amino acid sequence of the capsid protein.

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A library of sequences can be generated for X^1-X^2-X^3-X^4-X^5-X^6 (SEQ ID NO:1) using methods well-known to those skilled in the art. The library sequences can be incorporated into AAV capsid subunits and screened for desirable characteristics using known techniques.

In embodiments of the invention, X^1 can be any naturally occurring and/or non-naturally occurring amino acid. In embodiments of the invention, X^1 is an amino acid selected from: A, N, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y and/or V.

In embodiments of the invention, X^1 is not a basic amino acid.

In embodiments of the invention, X^1 is not selected from R, K and/or H.

In embodiments of the invention, X^1 is not an amino acid comprising a cyclic side chain (for example, is not selected from H, F, P, W and/or Y).

In embodiments of the invention, X^1 is not selected from C, E, A and/or M.

In embodiments of the invention, X^1 is not P.

In embodiments of the invention, X^1 is not S and/or N.

In embodiments of the invention, X^1 is a neutral amino acid (for example, is selected from A, N, C, Q, G, I, L, M, F, P, S, T, W, Y and/or V).

In embodiments of the invention, X^1 is a hydrophilic amino acid (for example, is selected from R, N, D, E, Q, H, K, S, T and/or Y).

In embodiments of the invention, X^1 is a hydrophilic and neutral amino acid (for example, is selected from Q, N, S and T).

In embodiments of the invention, X^1 is selected from Q, N, S, P, A and/or G.

In embodiments of the invention, X^1 is selected from Q, N, S, A, D and/or E.

In embodiments of the invention, X^1 is selected from Q, S, N and/or A.

In embodiments of the invention, X^1 is selected from Q and/or N.

In embodiments of the invention, X^1 is Q.

In embodiments of the invention, X^1 is N.

In embodiments of the invention, X^1 is A.

In embodiments of the invention, X^1 is S.

X^2 can be any naturally occurring and/or non-naturally occurring amino acid. In representative embodiments, X^2 is an amino acid selected from: A, R, N, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y and/or V.

In embodiments of the invention, X^2 is not a basic amino acid.

In embodiments of the invention, X^2 is not selected from R, K and/or H.

In embodiments of the invention, X^2 is not an amino acid comprising a cyclic side chain (for example, is not selected from H, F, P, W and/or Y).

In embodiments of the invention, X^2 is not selected from C and/or M.

In embodiments of the invention, X^2 is not P.

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In embodiments of the invention, X^2 is not S.

In embodiments of the invention, X^2 is a neutral amino acid (for example, is selected from A, N, C, Q, G, I, L, M, F, P, S, T, W, Y and/or V).

In embodiments of the invention, X^2 is a hydrophilic amino acid (for example, is selected from R, N, D, E, Q, H, K, S, T and/or Y).

In embodiments of the invention, X^2 is selected from G, A, I, L, V, Q, N, D and/or E.

In embodiments of the invention, X^2 is selected from Q, S, N, G and/or A.

In embodiments of the invention, X^2 is not selected from G and/or A.

In embodiments of the invention, X^2 is Q.

In embodiments of the invention, X^2 is A.

X^3 can be any naturally occurring or non-naturally occurring amino acid. In embodiments of the invention X^3 is an amino acid selected from: A, R, N, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y and/or V.

In embodiments of the invention, X^3 is not a basic amino acid.

In embodiments of the invention, X^3 is not selected from R, K and/or H.

In embodiments of the invention, X^3 is not an amino acid comprising a cyclic side chain (for example, is not selected from H, F, P, W and/or Y).

In embodiments of the invention, X^3 is not selected from C and/or M.

In embodiments of the invention, X^3 is not N.

In embodiments of the invention, X^3 is not I.

In embodiments of the invention, X^3 is not A.

In embodiments of the invention, X^3 is not P.

In embodiments of the invention, X^3 is a neutral amino acid (for example, is selected from A, N, C, Q, G, I, L, M, F, P, S, T, W, Y and/or V).

In embodiments of the invention, X^3 is a hydrophilic amino acid (for example, is selected from R, N, D, E, Q, H, K, S, T and/or Y).

In embodiments of the invention, X^3 is selected from Q, N, D and/or E.

In embodiments of the invention, X^3 is selected from Q, N, T and/or S.

In embodiments of the invention, X^3 is N.

In embodiments of the invention, X^3 is Q.

X^4 can be any naturally occurring and/or non-naturally occurring amino acid. In embodiments of the invention, X^4 is an amino acid selected from: A, R, N, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y and/or V.

In embodiments of the invention, X^4 is not a basic amino acid.

In embodiments of the invention, X^4 is not selected from R, K and/or H.

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In embodiments of the invention, X^4 is not an amino acid comprising a cyclic side chain (for example, is not selected from H, F, P, W and/or Y).

In embodiments of the invention, X^4 is not selected from C, A, E and/or M.

In embodiments of the invention, X^4 is not P.

In embodiments of the invention, X^4 is a neutral amino acid (for example, is selected from A, N, C, Q, G, I, L, M, F, P, S, T, W, Y and/or V).

In embodiments of the invention, X^4 is a hydrophilic amino acid (for example, is selected from R, N, D, E, Q, H, K, S, T and/or Y).

In embodiments of the invention, X^4 is a hydrophilic and neutral amino acid (for example, is selected from Q, N, S and T).

In embodiments of the invention, X^4 is selected from T, S, A, G, I, L and/or V.

In embodiments of the invention, X^4 is selected from T, A, G and/or N.

In embodiments of the invention, X^4 is T.

In embodiments of the invention, X^4 is A.

X^5 can be any naturally occurring and/or non-naturally occurring amino acid. In embodiments of the invention X^5 is an amino acid selected from: A, R, N, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y and/or V.

In embodiments of the invention, X^5 is not a basic amino acid.

In embodiments of the invention, X^5 is not selected from R, K and/or H.

In embodiments of the invention, X^5 is not an amino acid comprising a cyclic side chain (for example, is not selected from H, F, P, W and/or Y).

In embodiments of the invention, X^5 is not selected from C and/or M.

In embodiments of the invention, X^5 is not P.

In embodiments of the invention, X^5 is a neutral amino acid (for example, is selected from A, N, C, Q, G, I, L, M, F, P, S, T, W, Y and/or V).

In embodiments of the invention, X^5 is a hydrophilic amino acid (for example, is selected from R, N, D, E, Q, H, K, S, T and/or Y).

In embodiments of the invention, X^5 is selected from Q, N, T, S, A, G, I, L and/or V.

In embodiments of the invention, X^5 is selected from Q, N, T, S, A, G, L and/or D.

In embodiments of the invention, X^5 is Q.

In embodiments of the invention, X^5 is A.

In embodiments of the invention, X^5 is not Q.

X^6 can be any naturally occurring and/or non-naturally occurring amino acid. In embodiments of the invention, X^6 is an amino acid selected from: A, R, N, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y and/or V.

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In embodiments of the invention, X^5 is not a basic amino acid.

In embodiments of the invention, X^6 is not selected from R, K and/or H.

In embodiments of the invention, X^6 is not selected from C and/or M.

In embodiments of the invention, X^6 is a neutral amino acid (for example, is selected from A, N, C, Q, G, I, L, M, F, P, S, T, W, Y and/or V).

In embodiments of the invention, X^6 is a hydrophilic amino acid (for example, is selected from R, N, D, E, Q, H, K, S, T and/or Y).

In embodiments of the invention, X^6 is selected from P, A, G, I, L and/or V.

In embodiments of the invention, X^6 is P.

In embodiments of the invention, X^6 is A.

In embodiments of the invention, X^6 is not A.

In the modified AAV capsid proteins of the invention, the amino acid sequence X^1-X^2-X^3-X^4-X^5-X^6 (SEQ ID NO:1) can comprise any combination of the features described individually for each of X^1, X^2, X^3, X^4, X^5 and X^6.

In representative embodiments, the modified AAV capsid protein comprises the amino acid sequence X^1-X^2-X^3-X^4-X^5-X^6 (SEQ ID NO:1) at the amino acids corresponding to amino acids positions 585 to 590 (inclusive; VP1 numbering) of the native AAV2 capsid protein or the corresponding amino acid positions of other AAV capsid proteins,

wherein X^1 is selected from Q, N, S, P, A and/or G;

wherein X^2 is selected from any amino acid;

wherein X^3 is selected from any amino acid;

wherein X^4 is selected from T, A, G and/or N;

wherein X^5 is selected from any amino acid; and

wherein X^6 is selected from P and/or A.

In embodiments of the invention, X^1 is selected from Q, S, N and/or A.

In embodiments of the invention, X^2 is selected from Q, S, N, A and/or G.

In embodiments of the invention, X^3 is selected from S, N, T and/or Q.

In embodiments of the invention, X^5 is selected from S, N, T and/or Q.

In embodiments of the invention, X^6 is P.

In embodiments of the invention:

X^1 is selected from Q and/or N;

X^4 is T; and/or

X^6 is P.

In embodiments of the invention:

X^2 is Q;

X^3 is N; and/or

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X^5 is A.

In other exemplary embodiments of the invention, X^1-X^2-X^3-X^4-X^5-X^6 (SEQ ID NO:1) has the sequence:

(a) QQNTAP
(b) AANTAA
(c) SSTAGP
(d) QQNTAA
(e) PSTAGP
(f) SSSTDP
(g) SNSNLP
(h) SSTTAP
(i) SAQAQA
(j) QANTGP
(k) NATTAP
(l) NQNTAP
(m) QAANAP
(n) SIVGLP
(o) AASTAA
(p) SSNTAP
(q) SSTAGP
(r) SQNTTA
(s) QQDTAP
(t) QTNTGP
(u) QTNGAP
(v) QQNAAP
(w) AANTQA; or
(x) AASTAA.

In some embodiments of the invention X^1-X^2-X^3-X^4-X^5-X^6 (SEQ ID NO:1) is not selected from AGNAQA and/or AGAAQA.

In embodiments of the invention, X^1-X^2-X^3-X^4-X^5-X^6 (SEQ ID NO:1) is not SGNTQA, SSNTQA and/or NSNTAP.

In embodiments of the invention, X^1-X^2-X^3-X^4-X^5-X^6 (SEQ ID NO:1) is not AXXAXA.

In embodiments of the invention, X^1-X^2-X^3-X^4-X^5-X^6 (SEQ ID NO:1) does not comprise RGD.

In embodiments of the invention, X^5 is not G when X^6 is D.

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In embodiments of the invention, X^1-X^2-X^3-X^4-X^5-X^6 (SEQ ID NO:1) is RGNRQA (*e.g.*, when the AAV capsid subunit is not an AAV2 capsid subunit or an AAV capsid subunit derived from AAV2).

The invention also provides a virus capsid comprising, consisting essentially of, or consisting of the modified AAV capsid protein of the invention. In particular embodiments, the virus capsid is a parvovirus capsid, which may further be an autonomous parvovirus capsid or a dependovirus capsid. Optionally, the virus capsid is an AAV capsid. In particular embodiments, the AAV capsid is an AAV1, AAV2, AAV3a, AAV3b, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11 or any other AAV shown in **Table 1** or is derived from any of the foregoing by one or more insertions, substitutions and/or deletions.

The modified virus capsids can be used as "capsid vehicles," as has been described, for example, in U.S. Patent No. 5,863,541. Molecules that can be packaged by the modified virus capsid and transferred into a cell include heterologous DNA, RNA, polypeptides, small organic molecules, metals, or combinations of the same.

Heterologous molecules are defined as those that are not naturally found in an AAV infection, *e.g.*, those not encoded by a wild-type AAV genome. Further, therapeutically useful molecules can be associated with the outside of the chimeric virus capsid for transfer of the molecules into host target cells. Such associated molecules can include DNA, RNA, small organic molecules, metals, carbohydrates, lipids and/or polypeptides. In one embodiment of the invention the therapeutically useful molecule is covalently linked (*i.e.*, conjugated or chemically coupled) to the capsid proteins. Methods of covalently linking molecules are known by those skilled in the art.

The modified virus capsids of the invention also find use in raising antibodies against the novel capsid structures. As a further alternative, an exogenous amino acid sequence may be inserted into the modified virus capsid for antigen presentation to a cell, *e.g.*, for administration to a subject to produce an immune response to the exogenous amino acid sequence.

In other embodiments, the virus capsids can be administered to block certain cellular sites prior to and/or concurrently with (*e.g.*, within minutes or hours of each other) administration of a virus vector delivering a nucleic acid encoding a polypeptide or functional RNA of interest. For example, the inventive capsids can be delivered to block cellular receptors on liver cells and a delivery vector can be administered subsequently or concurrently, which may reduce transduction of liver

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cells, and enhance transduction of other targets (*e.g.*, skeletal, cardiac and/or diaphragm muscle).

According to representative embodiments, modified virus capsids can be administered to a subject prior to and/or concurrently with a modified virus vector according to the present invention. Further, the invention provides compositions and pharmaceutical formulations comprising the inventive modified virus capsids; optionally, the composition also comprises a modified virus vector of the invention.

The invention also provides nucleic acids (optionally, isolated nucleic acids) encoding the modified virus capsids and capsid proteins of the invention. Further provided are vectors comprising the nucleic acids, and cells (*in vivo* or in culture) comprising the nucleic acids and/or vectors of the invention. Suitable vectors include without limitation viral vectors (*e.g.*, adenovirus, AAV, herpesvirus, vaccinia, poxviruses, baculoviruses, and the like), plasmids, phage, YACs, BACs, and the like. Such nucleic acids, vectors and cells can be used, for example, as reagents (*e.g.*, helper packaging constructs or packaging cells) for the production of modified virus capsids or virus vectors as described herein.

Virus capsids according to the invention can be produced using any method known in the art, *e.g.*, by expression from a baculovirus (Brown et al., (1994) *Virology* 198:477-488).

The modifications to the AAV capsid protein according to the present invention are "selective" modifications. This approach is in contrast to previous work with whole subunit or large domain swaps between AAV serotypes (*see, e.g.*, international patent publication WO 00/28004 and Hauck et al., (2003) *J. Virology* 77:2768-2774). In particular embodiments, a "selective" modification results in the insertion and/or substitution and/or deletion of less than about 20, 18, 15, 12, 10, 9, 8, 7, 6, 5, 4 or 3 contiguous amino acids.

The modified capsid proteins and capsids of the invention can further comprise any other modification, now known or later identified.

For example, the AAV capsid proteins and virus capsids of the invention can be chimeric in that they can comprise all or a portion of a capsid subunit from another virus, optionally another parvovirus or AAV, *e.g.*, as described in international patent publication WO 00/28004.

The virus capsid can be a targeted virus capsid comprising a targeting sequence (*e.g.*, substituted or inserted in the viral capsid) that directs the virus capsid to interact with cell-surface molecules present on a desired target tissue(s) (*see, e.g.*, international patent publication WO 00/28004 and Hauck et al., (2003) *J. Virology* 77:2768-2774); Shi et al., *Human Gene Therapy* 17:353-361 (2006) [describing

insertion of the integrin receptor binding motif RGD at positions 520 and/or 584 of the AAV capsid subunit]; and U.S. Patent No. 7,314,912 [describing insertion of the P1 peptide containing an RGD motif following amino acid positions 447, 534, 573 and 587 of the AAV2 capsid subunit]). Other positions within the AAV capsid subunit that tolerate insertions are known in the art (*e.g.*, positions 449 and 588 described by Grifman et al., *Molecular Therapy* 3:964-975 (2001)).

For example, some of the virus capsids of the invention have relatively inefficient tropism toward most target tissues of interest (*e.g.*, liver, skeletal muscle, heart, diaphragm muscle, kidney, brain, stomach, intestines, skin, endothelial cells, and/or lungs). A targeting sequence can advantageously be incorporated into these low-transduction vectors to thereby confer to the virus capsid a desired tropism and, optionally, selective tropism for particular tissue(s). AAV capsid proteins, capsids and vectors comprising targeting sequences are described, for example in international patent publication WO 00/28004. As another possibility one or more non-naturally occurring amino acids as described by Wang et al., *Annu Rev Biophys Biomol Struct.* 35:225-49 (2006)) can be incorporated into the AAV capsid subunit at an orthogonal site as a means of redirecting a low-transduction vector to a desired target tissue(s). These unnatural amino acids can advantageously be used to chemically link molecules of interest to the AAV capsid protein including without limitation: glycans (mannose – dendritic cell targeting); RGD, bombesin or a neuropeptide for targeted delivery to specific cancer cell types; RNA aptamers or peptides selected from phage display targeted to specific cell surface receptors such as growth factor receptors, integrins, and the like. Methods of chemically modifying amino acids are known in the art (*see, e.g.*, Greg T. Hermanson, Bioconjugate Techniques, 1st edition, Academic Press, 1996).

In representative embodiments, the targeting sequence may be a virus capsid sequence (*e.g.*, an autonomous parvovirus capsid sequence, AAV capsid sequence, or any other viral capsid sequence) that directs infection to a particular cell type(s).

As another nonlimiting example, a heparin binding domain (*e.g.*, the respiratory syncytial virus heparin binding domain) may be inserted or substituted into a capsid subunit that does not typically bind HS receptors (*e.g.*, AAV 4, AAV5) to confer heparin binding to the resulting mutant.

B19 infects primary erythroid progenitor cells using globoside as its receptor (Brown *et al.*, (1993) Science 262:114). The structure of B19 has been determined to 8 Å resolution (Agbandje-McKenna *et al.*, (1994) *Virology* 203:106). The region of the B19 capsid that binds to globoside has been mapped between amino acids 399-406 (Chapman *et al.*, (1993) *Virology* 194:419), a looped out region between β-barrel

structures E and F (Chipman *et al.*, (1996) *Proc. Nat. Acad. Sci. USA* 93:7502). Accordingly, the globoside receptor binding domain of the B19 capsid may be substituted into the AAV capsid protein to target a virus capsid or virus vector comprising the same to erythroid cells.

In representative embodiments, the exogenous targeting sequence may be any amino acid sequence encoding a peptide that alters the tropism of a virus capsid or virus vector comprising the modified AAV capsid protein. In particular embodiments, the targeting peptide or protein may be naturally occurring or, alternately, completely or partially synthetic. Exemplary targeting sequences include ligands and other peptides that bind to cell surface receptors and glycoproteins, such as RGD peptide sequences, bradykinin, hormones, peptide growth factors (*e.g.*, epidermal growth factor, nerve growth factor, fibroblast growth factor, platelet-derived growth factor, insulin-like growth factors I and II, *etc.*), cytokines, melanocyte stimulating hormone (*e.g.*, α, β or γ), neuropeptides and endorphins, and the like, and fragments thereof that retain the ability to target cells to their cognate receptors. Other illustrative peptides and proteins include substance P, keratinocyte growth factor, neuropeptide Y, gastrin releasing peptide, interleukin 2, hen egg white lysozyme, erythropoietin, gonadoliberin, corticostatin, β-endorphin, leu-enkephalin, rimorphin, α-neo-enkephalin, angiotensin, pneumadin, vasoactive intestinal peptide, neurotensin, motilin, and fragments thereof as described above. As yet a further alternative, the binding domain from a toxin (*e.g.*, tetanus toxin or snake toxins, such as α-bungarotoxin, and the like) can be substituted into the capsid protein as a targeting sequence. In a yet further representative embodiment, the AAV capsid protein can be modified by substitution of a "nonclassical" import/export signal peptide (*e.g.*, fibroblast growth factor-1 and –2, interleukin 1, HIV-1 Tat protein, herpes virus VP22 protein, and the like) as described by Cleves (*Current Biology* 7:R318 (1997)) into the AAV capsid protein. Also encompassed are peptide motifs that direct uptake by specific cells, *e.g.*, a FVFLP peptide motif triggers uptake by liver cells.

Phage display techniques, as well as other techniques known in the art, may be used to identify peptides that recognize any cell type of interest.

The targeting sequence may encode any peptide that targets to a cell surface binding site, including receptors (*e.g.*, protein, carbohydrate, glycoprotein or proteoglycan). Examples of cell surface binding sites include, but are not limited to, heparan sulfate, chondroitin sulfate, and other glycosaminoglycans, sialic acid moieties found on mucins, glycoproteins, and gangliosides, MHC I glycoproteins,

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carbohydrate components found on membrane glycoproteins, including, mannose, N-acetyl-galactosamine, N-acetyl-glucosamine, fucose, galactose, and the like.

In particular embodiments, a heparan sulfate (HS) or heparin binding domain is substituted into the virus capsid (for example, in an AAV that otherwise does not bind to HS or heparin). It is known in the art that HS/heparin binding is mediated by a "basic patch" that is rich in arginines and/or lysines. In exemplary embodiments, a sequence following the motif BXXB, where "B" is a basic residue and X is neutral and/or hydrophobic. As one nonlimiting example, BXXB is RGNR. In particular embodiments, BXXB is substituted for amino acid positions 262 through 265 in the native AAV2 capsid protein or the corresponding position in the capsid protein of another AAV.

Other nonlimiting examples of suitable targeting sequences include the peptides targeting coronary artery endothelial cells identified by Müller et al., *Nature Biotechnology* 21:1040-1046 (2003) (consensus sequences NSVRDLG/S, PRSVTVP, NSVSSXS/A; *see also* Table 2); tumor-targeting peptides as described by Grifman et al., *Molecular Therapy* 3:964-975 (2001) (*e.g.*, NGR, NGRAHA); lung or brain targeting sequences as described by Work et al., *Molecular Therapy* 13:683-693 (2006) (QPEHSST, VNTANST, HGPMQKS, PHKPPLA, IKNNEMW, RNLDTPM, VDSHRQS, YDSKTKT, SQLPHQK, STMQQNT, TERYMTQ, QPEHSST, DASLSTS, DLPNKKT, DLTAARL, EPHQFNY, EPQSNHT, MSSWPSQ, NPKHNAT, PDGMRTT, PNNNKTT, QSTTHDS, TGSKQKQ, SLKHQAL and SPIDGEQ, ; *see also* Table 1)); vascular targeting sequences described by Hajitou et al., *TCM* 16:80-88 (2006) (WIFPWIQL, CDCRGDCFC, CNGRC, CPRECES, GSL, CTTHWGFTLC, CGRRAGGSC, CKGGRAKDC, and CVPELGHEC); targeting peptides as described by Koivunen et al., *J. Nucl. Med.* 40:883-888 (1999) (CRRETAWAK, KGD, VSWFSHRYSPFAVS, GYRDGYAGPILYN, XXXY*XXX [where Y* is phospho-Tyr], Y*E/MNW, RPLPPLP, APPLPPR, DVFYPYPYASGS, MYWYPY, DITWDQLWDLMK, CWDDG/LWLC, EWCEYLGGYLRCYA, YXCXXGPXTWXCXP, IEGPTLRQWLAARA, LWXXY/W/F/H, XFXXYLW, SSIISHFRWGLCD, MSRPACPPNDKYE, CLRSGRGC, CHWMFSPWC, WXXF, CSSRLDAC, CLPVASC, CGFECVRQCPERC, CVALCREACGEGC, SWCEPGWCR, YSGKWGW, GLSGGRS, LMLPRAD, CSCFRDVCC, CRDVVSVIC, CNGRC, and GSL; *see also,* Tables 1, 2 and 3); and tumor targeting peptides as described by Newton & Deutscher, Phage Peptide Display *in* Handbook of Experimental Pharmacology, pages 145-163, Springer-Verlag, Berlin (2008) (MARSGL, MARAKE, MSRTMS, KCCYSL, WRR, WKR, WVR, WVK, WIK, WTR, WVL, WLL, WRT, WRG, WVS, WVA, MYWGDSHWLQYWYE, MQLPLAT, EWLS, SNEW, TNYL, WIFPWIQL, WDLAWMFRLPVG,

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CTVALPGGYVRVC, CVPELGHEC, CGRRAGGSC, CVAYCIEHHCWTC, CVFAHNYDYLVC, and CVFTSNYAFC, VHSPNKK, CDCRGDCFC, CRGDGWC, XRGCDX, PXXS/T, CTTHWGFTLC, SGKGPRQITAL, A9A/Q)(N/A)(L/Y)(T/V/M/R)(R/K), VYMSPF, MQLPLAT, ATWLPPR, HTMYYHHYQHHL, SEVGCRAGPLQWLCEKYFG, CGLLPVGRPDRNVWRWLC, CKGQCDRFKGLPWEC, SGRSA, WGFP, LWXXAr [Ar=Y, W, F, H), XFXXYLW, AEPMPHSLNFSQYLWYT, WAY(W/F)SP, IELLQAR, DITWDQLWDLMK, AYTKCSRQWRTCMTTH, PQNSKIPGPTFLDPH, SMEPALPDWWWKMFK, ANTPCGPYTHDCPVKR, TACHQHVRMVRP, VPWMEPAYQRFL, DPRATPGS, FRPNRAQDYNTN, CTKNSYLMC, C(R/Q)L/RT(G/N)XXG(A/V)GC, CPIEDRPMC, HEWSYLAPYPWF, MCPKHPLGC, RMWPSSTVNLSAGRR, SAKTAVSQRVWLPSHRGGEP, KSREHVNNSACPSKRITAAL, EGFR, RVS, AGS, AGLGVR, GGR, GGL, GSV, GVS, GTRQGHTMRLGVSDG, IAGLATPGWSHWLAL, SMSIARL, HTFEPGV, NTSLKRISNKRIRRK, LRIKRKRRKRKKTRK, GGG, GFS, LWS, EGG, LLV, LSP, LBS, AGG, GRR, GGH and GTV; *see also* Table 1).

As yet a further alternative, the targeting sequence may be a peptide that can be used for chemical coupling (*e.g.*, can comprise arginine and/or lysine residues that can be chemically coupled through their R groups) to another molecule that targets entry into a cell.

As another option, the AAV capsid protein or virus capsid of the invention can comprise a mutation as described in WO 2006/066066. For example, the capsid protein can comprise a selective amino acid substitution at amino acid position 263, 705, 708 and/or 716 of the native AAV2 capsid protein or a corresponding change(s) in a capsid protein from another AAV. Additionally, or alternatively, in representative embodiments, the capsid protein, virus capsid or vector comprises a selective amino acid insertion directly following amino acid position 264 of the AAV2 capsid protein or a corresponding change in the capsid protein from other AAV. By "directly following amino acid position X" it is intended that the insertion immediately follows the indicated amino acid position (for example, "following amino acid position 264" indicates a point insertion at position 265 or a larger insertion, e.g., from positions 265 to 268, etc.). The foregoing embodiments of the invention can be used to deliver a heterologous nucleic acid to a cell or subject as described herein. For example, the modified vector can be used to treat a lysosomal storage disorder such as a mucopolysaccharidosis disorder (*e.g.*, Sly syndrome [β-glucuronidase], Hurler Syndrome [α-L-iduronidase], Scheie Syndrome [α-L-iduronidase], Hurler-Scheie Syndrome [α-L-iduronidase], Hunter's Syndrome [iduronate sulfatase], Sanfilippo Syndrome A [heparan sulfamidase], B [N-acetylglucosaminidase], C [acetyl-CoA:α-

glucosaminide acetyltransferase], D [N-acetylglucosamine 6-sulfatase], Morquio Syndrome A [galactose-6-sulfate sulfatase], B [β-galactosidase], Maroteaux-Lamy Syndrome [N-acetylgalactosamine-4-sulfatase], *etc.*), Fabry disease (α-galactosidase), Gaucher's disease (glucocerebrosidase), or a glycogen storage disorder (*e.g.*, Pompe disease; lysosomal acid α-glucosidase) as described herein.

Those skilled in the art will appreciate that for some AAV capsid proteins the corresponding modification will be an insertion and/or a substitution, depending on whether the corresponding amino acid positions are partially or completely present in the virus or, alternatively, are completely absent. Likewise, when modifying AAV other than AAV2, the specific amino acid position(s) may be different than the position in AAV2 (*see, e.g.*, **Table 4**). As discussed elsewhere herein, the corresponding amino acid position(s) will be readily apparent to those skilled in the art using well-known techniques.

In representative embodiments, the insertion and/or substitution and/or deletion in the capsid protein(s) results in the insertion, substitution and/or repositioning of an amino acid that (i) maintains the hydrophilic loop structure in that region; (ii) an amino acid that alters the configuration of the loop structure; (iii) a charged amino acid; and/or (iv) an amino acid that can be phosphorylated or sulfated or otherwise acquire a charge by post-translational modification (*e.g.*, glycosylation) following 264 in an AAV2 capsid protein or a corresponding change in a capsid protein of another AAV. Suitable amino acids for insertion/substitution include aspartic acid, glutamic acid, valine, leucine, lysine, arginine, threonine, serine, tyrosine, glycine, alanine, proline, asparagine, phenylalanine, tyrosine or glutamine. In particular embodiments, a threonine is inserted or substituted into the capsid subunit. Nonlimiting examples of corresponding positions in a number of other AAV are shown in **Table 4** (Position 2). In particular embodiments, the amino acid insertion or substitution is a threonine, aspartic acid, glutamic acid or phenylalanine (excepting AAV that have a threonine, glutamic acid or phenylalanine, respectively, at this position).

According to this aspect of the invention, in particular embodiments the AAV capsid protein comprises an amino acid insertion following amino acid position 264 in an AAV2, AAV3a or AAV3b capsid protein(s) or in the corresponding position in an AAV2, AAV3a or AAV3b capsid protein that has been modified to comprise non-AAV2, AAV3a or AAV3b sequences, respectively, and/or has been modified by deletion of one or more amino acids (*i.e.*, is derived from AAV2, AAV3a or AAV3b). The amino acid corresponding to position 264 in an AAV2 (or AAV3a or AAV3b) capsid subunit(s) will be readily identifiable in the starting virus that has been derived

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from AAV2 (or AAV3a or AAV3b), which can then be further modified according to the present invention. Suitable amino acids for insertion include aspartic acid, glutamic acid, valine, leucine, lysine, arginine, threonine, serine, tyrosine, glycine, alanine, proline, asparagine, phenylalanine, tyrosine or glutamine.

In other embodiments, the AAV capsid protein comprises an amino acid substitution at amino acid position 265 in an AAV1 capsid protein(s), at amino acid position 266 in an AAV8 capsid protein, or an amino acid substitution at amino acid position 265 in an AAV9 capsid protein or in the corresponding position in an AAV1, AAV8 or AAV9 capsid protein that has been modified to comprise non-AAV1, non-AAV8 or non-AAV9 sequences, respectively, and/or has been modified by deletion of one or more amino acids (*i.e.*, is derived from AAV1, AAV8 or AAV9). The amino acid corresponding to position 265 in an AAV1 and AAV9 capsid subunit(s) and position 266 in the AAV8 capsid subunit(s) will be readily identifiable in the starting virus that has been derived from AAV1, AAV8 or AAV9, which can then be further modified according to the present invention. Suitable amino acids for insertion include aspartic acid, glutamic acid, valine, leucine, lysine, arginine, threonine, serine, tyrosine, glycine, alanine, proline, asparagine, phenylalanine, tyrosine or glutamine.

In representative embodiments of the invention, the capsid protein comprises a threonine, aspartic acid, glutamic acid, or phenylalanine following amino acid position 264 of the AAV2 capsid protein (*i.e.*, an insertion) or the corresponding position of another capsid protein.

In other representative embodiments, the modified capsid proteins or virus capsids of the invention further comprise one or more mutations as described in WO 2007/089632 (*e.g.*, an E→K mutation at amino acid position 531 of the AAV2 capsid protein or the corresponding position of the capsid protein from another AAV).

In further embodiments, the modified capsid protein or capsid can comprise a mutation as described in WO 2009/108274.

As another, possibility, the AAV capsid protein can comprise a mutation as described by Zhong et al. (*Virology* 381: 194-202 (2008); *Proc. Nat. Acad. Sci.* 105: 7827-32 (2008)). For example, the AAV capsid protein can comprise a Y→ F mutation at amino acid position 730.

The modifications described above can be incorporated into the capsid proteins or capsids of the invention in combination with each other and/or with any other modification now known or later discovered.

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Table 4

Serotype	Position 1	Position 2
AAV1	A263X	T265X
AAV2	Q263X	-265X
AAV3a	Q263X	-265X
AAV3b	Q263X	-265X
AAV4	S257X	-259X
AAV5	G253X	V255X
AAV6	A263X	T265X
AAV7	E264X	A266X
AAV8	G264X	S266X
AAV9	S263X	S265X
Where, (X) → mutation to any amino acid (-) → insertion of any amino acid Note: Position 2 inserts are indicated by the site of insertion		

The invention also encompasses virus vectors comprising the modified capsid proteins and capsids of the invention. In particular embodiments, the virus vector is a parvovirus vector (*e.g.*, comprising a parvovirus capsid and/or vector genome), for example, an AAV vector (*e.g.*, comprising an AAV capsid and/or vector genome). In representative embodiments, the virus vector comprises a modified AAV capsid comprising a modified capsid subunit of the invention and a vector genome.

For example, in representative embodiments, the virus vector comprises: (a) a modified virus capsid (*e.g.*, a modified AAV capsid) comprising a modified capsid protein of the invention; and (b) a nucleic acid comprising a terminal repeat sequence (*e.g.*, an AAV TR), wherein the nucleic acid comprising the terminal repeat sequence is encapsidated by the modified virus capsid. The nucleic acid can optionally comprise two terminal repeats (*e.g.*, two AAV TRs).

In representative embodiments, the virus vector is a recombinant virus vector comprising a heterologous nucleic acid encoding a polypeptide or functional RNA of interest. Recombinant virus vectors are described in more detail below.

In particular embodiments, the virus vectors of the invention (i) have reduced transduction of liver as compared with the level of transduction by a virus vector without the modified capsid protein; (ii) exhibit enhanced systemic transduction by

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the virus vector in an animal subject as compared with the level observed by a virus vector without the modified capsid protein; (iii) demonstrate enhanced movement across endothelial cells as compared with the level of movement by a virus vector without the modified capsid protein, and/or (iv) exhibit a selective enhancement in transduction of muscle tissue (*e.g.*, skeletal muscle, cardiac muscle and/or diaphragm muscle), and/or (v) reduced transduction of brain tissues (*e.g.*, neurons) as compared with the level of transduction by a virus vector without the modified capsid protein. In particular embodiments, the virus vector has systemic transduction toward muscle, *e.g.*, transduces multiple skeletal muscle groups throughout the body and optionally transduces cardiac muscle and/or diaphragm muscle.

Further, in embodiments of the invention, the modified virus vectors demonstrate efficient transduction of target tissues. In general, when X^1-X^2-X^3-X^4-X^5-X^6 (SEQ ID NO:1) is introduced into an AAV capsid protein, the order of transduction efficiency (*e.g.*, for muscle tissue including skeletal muscle, cardiac muscle and/or diaphragm muscle) appears to be:

QXXTXP > NXXTXP > SXXAXP > AXXAXA

when the modified capsid protein is incorporated into a virus vector (*e.g.*, an AAV vector comprising a modified AAV capsid comprising the modified AAV capsid protein of the invention).

Those skilled in the art will appreciate that some particular modifications may deviate from this general rule without departing from the scope of the present invention. For example, certain amino acids in the "X" positions may affect transduction efficiency. As one illustration, a proline (P) in one of the "X" positions may reduce transduction efficiency.

In particular embodiments, efficient muscle transduction (skeletal, cardiac and/or diaphragm) is achieved (*e.g.*, by an AAV vector comprising a modified AAV capsid comprising the modified AAV capsid protein of the invention) when X^1-X^2-X^3-X^4-X^5-X^6 (SEQ ID NO:1) is QXXTXP or NXXTXP. In particular embodiments, X is not selected from P, C and/or W.

It will be understood by those skilled in the art that the modified capsid proteins, virus capsids and virus vectors of the invention exclude those capsid proteins, capsids and virus vectors that have the indicated amino acids at the specified positions in their native state (i.e., are not mutants).

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Methods of Producing Virus Vectors.

The present invention further provides methods of producing the inventive virus vectors. In one representative embodiment, the present invention provides a method of producing a virus vector, the method comprising providing to a cell: (a) a nucleic acid template comprising at least one TR sequence (*e.g.*, AAV TR sequence), and (b) AAV sequences sufficient for replication of the nucleic acid template and encapsidation into AAV capsids (*e.g.*, AAV *rep* sequences and AAV *cap* sequences encoding the AAV capsids of the invention). Optionally, the nucleic acid template further comprises at least one heterologous nucleic acid sequence. In particular embodiments, the nucleic acid template comprises two AAV ITR sequences, which are located 5' and 3' to the heterologous nucleic acid sequence (if present), although they need not be directly contiguous thereto.

The nucleic acid template and AAV *rep* and *cap* sequences are provided under conditions such that virus vector comprising the nucleic acid template packaged within the AAV capsid is produced in the cell. The method can further comprise the step of collecting the virus vector from the cell. The virus vector can be collected from the medium and/or by lysing the cells.

The cell can be a cell that is permissive for AAV viral replication. Any suitable cell known in the art may be employed. In particular embodiments, the cell is a mammalian cell. As another option, the cell can be a trans-complementing packaging cell line that provides functions deleted from a replication-defective helper virus, *e.g.*, 293 cells or other E1a trans-complementing cells.

The AAV replication and capsid sequences may be provided by any method known in the art. Current protocols typically express the AAV *rep/cap* genes on a single plasmid. The AAV replication and packaging sequences need not be provided together, although it may be convenient to do so. The AAV *rep* and/or *cap* sequences may be provided by any viral or non-viral vector. For example, the *rep/cap* sequences may be provided by a hybrid adenovirus or herpesvirus vector (*e.g.*, inserted into the E1a or E3 regions of a deleted adenovirus vector). EBV vectors may also be employed to express the AAV *cap* and *rep* genes. One advantage of this method is that EBV vectors are episomal, yet will maintain a high copy number throughout successive cell divisions (*i.e.*, are stably integrated into the cell as extra-chromosomal elements, designated as an "EBV based nuclear episome," *see* Margolski, (1992) *Curr. Top. Microbiol. Immun.* 158:67).

As a further alternative, the *rep/cap* sequences may be stably incorporated into a cell.

Typically the AAV *rep/cap* sequences will not be flanked by the TRs, to prevent rescue and/or packaging of these sequences.

The nucleic acid template can be provided to the cell using any method known in the art. For example, the template can be supplied by a non-viral (*e.g.*, plasmid) or viral vector. In particular embodiments, the nucleic acid template is supplied by a herpesvirus or adenovirus vector (*e.g.*, inserted into the E1a or E3 regions of a deleted adenovirus). As another illustration, Palombo et al., (1998) *J. Virology* 72:5025, describes a baculovirus vector carrying a reporter gene flanked by the AAV TRs. EBV vectors may also be employed to deliver the template, as described above with respect to the *rep/cap* genes.

In another representative embodiment, the nucleic acid template is provided by a replicating rAAV virus. In still other embodiments, an AAV provirus comprising the nucleic acid template is stably integrated into the chromosome of the cell.

To enhance virus titers, helper virus functions (*e.g.*, adenovirus or herpesvirus) that promote a productive AAV infection can be provided to the cell. Helper virus sequences necessary for AAV replication are known in the art. Typically, these sequences will be provided by a helper adenovirus or herpesvirus vector. Alternatively, the adenovirus or herpesvirus sequences can be provided by another non-viral or viral vector, *e.g.*, as a non-infectious adenovirus miniplasmid that carries all of the helper genes that promote efficient AAV production as described by Ferrari et al., (1997) *Nature Med.* 3:1295, and U.S. Patent Nos. 6,040,183 and 6,093,570.

Further, the helper virus functions may be provided by a packaging cell with the helper sequences embedded in the chromosome or maintained as a stable extrachromosomal element. Generally, the helper virus sequences cannot be packaged into AAV virions, *e.g.*, are not flanked by TRs.

Those skilled in the art will appreciate that it may be advantageous to provide the AAV replication and capsid sequences and the helper virus sequences (*e.g.*, adenovirus sequences) on a single helper construct. This helper construct may be a non-viral or viral construct. As one nonlimiting illustration, the helper construct can be a hybrid adenovirus or hybrid herpesvirus comprising the AAV *rep/cap* genes.

In one particular embodiment, the AAV *rep/cap* sequences and the adenovirus helper sequences are supplied by a single adenovirus helper vector. This vector further can further comprise the nucleic acid template. The AAV *rep/cap* sequences and/or the rAAV template can be inserted into a deleted region (*e.g.*, the E1a or E3 regions) of the adenovirus.

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In a further embodiment, the AAV *rep/cap* sequences and the adenovirus helper sequences are supplied by a single adenovirus helper vector. According to this embodiment, the rAAV template can be provided as a plasmid template.

In another illustrative embodiment, the AAV *rep/cap* sequences and adenovirus helper sequences are provided by a single adenovirus helper vector, and the rAAV template is integrated into the cell as a provirus. Alternatively, the rAAV template is provided by an EBV vector that is maintained within the cell as an extrachromosomal element (*e.g.*, as an EBV based nuclear episome).

In a further exemplary embodiment, the AAV *rep/cap* sequences and adenovirus helper sequences are provided by a single adenovirus helper. The rAAV template can be provided as a separate replicating viral vector. For example, the rAAV template can be provided by a rAAV particle or a second recombinant adenovirus particle.

According to the foregoing methods, the hybrid adenovirus vector typically comprises the adenovirus 5' and 3' *cis* sequences sufficient for adenovirus replication and packaging (*i.e.*, the adenovirus terminal repeats and PAC sequence). The AAV *rep/cap* sequences and, if present, the rAAV template are embedded in the adenovirus backbone and are flanked by the 5' and 3' *cis* sequences, so that these sequences may be packaged into adenovirus capsids. As described above, the adenovirus helper sequences and the AAV *rep/cap* sequences are generally not flanked by TRs so that these sequences are not packaged into the AAV virions.

Zhang et al., ((2001) *Gene Ther.* 18:704-12) describe a chimeric helper comprising both adenovirus and the AAV *rep* and *cap* genes.

Herpesvirus may also be used as a helper virus in AAV packaging methods. Hybrid herpesviruses encoding the AAV Rep protein(s) may advantageously facilitate scalable AAV vector production schemes. A hybrid herpes simplex virus type I (HSV-1) vector expressing the AAV-2 *rep* and *cap* genes has been described (Conway et al., (1999) *Gene Therapy* 6:986 and WO 00/17377.

As a further alternative, the virus vectors of the invention can be produced in insect cells using baculovirus vectors to deliver the *rep/cap* genes and rAAV template as described, for example, by Urabe et al., (2002) *Human Gene Therapy* 13:1935-43.

AAV vector stocks free of contaminating helper virus may be obtained by any method known in the art. For example, AAV and helper virus may be readily differentiated based on size. AAV may also be separated away from helper virus based on affinity for a heparin substrate (Zolotukhin et al. (1999) *Gene Therapy* 6:973). Deleted replication-defective helper viruses can be used so that any contaminating helper virus is not replication competent. As a further alternative, an

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adenovirus helper lacking late gene expression may be employed, as only adenovirus early gene expression is required to mediate packaging of AAV virus. Adenovirus mutants defective for late gene expression are known in the art (*e.g.*, ts100K and ts149 adenovirus mutants).

Recombinant Virus Vectors.

The virus vectors of the present invention are useful for the delivery of nucleic acids to cells *in vitro, ex vivo*, and *in vivo*. In particular, the virus vectors can be advantageously employed to deliver or transfer nucleic acids to animal, including mammalian, cells.

Any heterologous nucleic acid sequence(s) of interest may be delivered in the virus vectors of the present invention. Nucleic acids of interest include nucleic acids encoding polypeptides, including therapeutic (*e.g.*, for medical or veterinary uses) or immunogenic (*e.g.*, for vaccines) polypeptides.

Therapeutic polypeptides include, but are not limited to, cystic fibrosis transmembrane regulator protein (CFTR), dystrophin (including mini- and micro-dystrophins, *see, e.g*, Vincent *et al.*, (1993) *Nature Genetics* 5:130; U.S. Patent Publication No. 2003/017131; International publication WO/2008/088895, Wang et al., *Proc. Natl. Acad. Sci. USA* 97:13714-13719 (2000); and Gregorevic et al., *Mol. Ther.* 16:657-64 (2008)), myostatin propeptide, follistatin, activin type II soluble receptor, IGF-1, anti-inflammatory polypeptides such as the Ikappa B dominant mutant, sarcospan, utrophin (Tinsley *et al.*, (1996) *Nature* 384:349), mini-utrophin, clotting factors (*e.g.*, Factor VIII, Factor IX, Factor X, *etc.),* erythropoietin, angiostatin, endostatin, catalase, tyrosine hydroxylase, superoxide dismutase, leptin, the LDL receptor, lipoprotein lipase, ornithine transcarbamylase, β-globin, α-globin, spectrin, α_1-antitrypsin, adenosine deaminase, hypoxanthine guanine phosphoribosyl transferase, β-glucocerebrosidase, sphingomyelinase, lysosomal hexosaminidase A, branched-chain keto acid dehydrogenase, RP65 protein, cytokines (*e.g.*, α-interferon, β-interferon, interferon-γ, interleukin-2, interleukin-4, granulocyte-macrophage colony stimulating factor, lymphotoxin, and the like), peptide growth factors, neurotrophic factors and hormones (*e.g.*, somatotropin, insulin, insulin-like growth factors 1 and 2, platelet derived growth factor, epidermal growth factor, fibroblast growth factor, nerve growth factor, neurotrophic factor –3 and –4, brain-derived neurotrophic factor, bone morphogenic proteins [including RANKL and VEGF], glial derived growth factor, transforming growth factor –α and –β, and the like), lysosomal acid α-glucosidase, α-galactosidase A, receptors (*e.g.*, the tumor necrosis growth factorα soluble receptor),

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S100A1, parvalbumin, adenylyl cyclase type 6, a molecule that modulates calcium handling (*e.g.*, $SERCA_{2A}$ Inhibitor 1 of PP1 and fragments thereof [*e.g.*, WO 2006/029319 and WO 2007/100465]), a molecule that effects G-protein coupled receptor kinase type 2 knockdown such as a truncated constitutively active bARKct, anti-inflammatory factors such as IRAP, anti-myostatin proteins, aspartoacylase, monoclonal antibodies (including single chain monoclonal antibodies; an exemplary Mab is the Herceptin® Mab), neuropeptides and fragments thereof (*e.g.*, galanin, Neuropeptide Y (*see*, U.S. 7,071,172), angiogenesis inhibitors such as Vasohibins and other VEGF inhibitors (*e.g.*, Vasohibin 2 [*see*, WO JP2006/073052]). Other illustrative heterologous nucleic acid sequences encode suicide gene products (*e.g.*, thymidine kinase, cytosine deaminase, diphtheria toxin, and tumor necrosis factor), proteins conferring resistance to a drug used in cancer therapy, tumor suppressor gene products (*e.g.*, p53, Rb, Wt-1), TRAIL, FAS-ligand, and any other polypeptide that has a therapeutic effect in a subject in need thereof. AAV vectors can also be used to deliver monoclonal antibodies and antibody fragments, for example, an antibody or antibody fragment directed against myostatin (*see, e.g.*, Fang et al., *Nature Biotechnology* 23:584-590 (2005)).

Heterologous nucleic acid sequences encoding polypeptides include those encoding reporter polypeptides (*e.g.*, an enzyme). Reporter polypeptides are known in the art and include, but are not limited to, Green Fluorescent Protein, β-galactosidase, alkaline phosphatase, luciferase, and chloramphenicol acetyltransferase gene.

Optionally, the heterologous nucleic acid encodes a secreted polypeptide (*e.g.*, a polypeptide that is a secreted polypeptide in its native state or that has been engineered to be secreted, for example, by operable association with a secretory signal sequence as is known in the art).

Alternatively, in particular embodiments of this invention, the heterologous nucleic acid may encode an antisense nucleic acid, a ribozyme (*e.g.*, as described in U.S. Patent No. 5,877,022), RNAs that effect spliceosome-mediated *trans*-splicing (*see*, Puttaraju *et al.*, (1999) *Nature Biotech.* 17:246; U.S. Patent No. 6,013,487; U.S. Patent No. 6,083,702), interfering RNAs (RNAi) including siRNA, shRNA or miRNA that mediate gene silencing (*see*, Sharp et al., (2000) *Science* 287:2431), and other non-translated RNAs, such as "guide" RNAs (Gorman *et al.*, (1998) *Proc. Nat. Acad. Sci. USA* 95:4929; U.S. Patent No. 5,869,248 to Yuan *et al.*), and the like. Exemplary untranslated RNAs include RNAi against a multiple drug resistance (MDR) gene product (*e.g.*, to treat and/or prevent tumors and/or for administration to the heart to prevent damage by chemotherapy), RNAi against myostatin (e.g., for

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Duchenne muscular dystrophy), RNAi against VEGF (*e.g.*, to treat and/or prevent tumors), RNAi against phospholamban (*e.g.*, to treat cardiovascular disease, *see, e.g.*, Andino et al., *J. Gene Med.* 10:132-142 (2008) and Li et al., *Acta Pharmacol Sin.* 26:51-55 (2005)); phospholamban inhibitory or dominant-negative molecules such as phospholamban S16E (*e.g.*, to treat cardiovascular disease, *see*, *e.g.*, Hoshijima et al. *Nat. Med.* 8:864-871 (2002)), RNAi to adenosine kinase (*e.g.*, for epilepsy), and RNAi directed against pathogenic organisms and viruses (*e.g.*, hepatitis B and/or C virus, human immunodeficiency virus, CMV, herpes simplex virus, human papilloma virus, *etc.*).

Further, a nucleic acid sequence that directs alternative splicing can be delivered. To illustrate, an antisense sequence (or other inhibitory sequence) complementary to the 5' and/or 3' splice site of dystrophin exon 51 can be delivered in conjunction with a U1 or U7 small nuclear (sn) RNA promoter to induce skipping of this exon. For example, a DNA sequence comprising a U1 or U7 snRNA promoter located 5' to the antisense/inhibitory sequence(s) can be packaged and delivered in a modified capsid of the invention.

The virus vector may also comprise a heterologous nucleic acid that shares homology with and recombines with a locus on a host chromosome. This approach can be utilized, for example, to correct a genetic defect in the host cell.

The present invention also provides virus vectors that express an immunogenic polypeptide, *e.g.*, for vaccination. The nucleic acid may encode any immunogen of interest known in the art including, but not limited to, immunogens from human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), influenza virus, HIV or SIV gag proteins, tumor antigens, cancer antigens, bacterial antigens, viral antigens, and the like.

The use of parvoviruses as vaccine vectors is known in the art (*see*, *e.g.*, Miyamura *et al.*, (1994) *Proc. Nat. Acad. Sci USA* 91:8507; U.S. Patent No. 5,916,563 to Young *et al.*, U.S. Patent No. 5,905,040 to Mazzara *et al.*, U.S. Patent No. 5,882,652, U.S. Patent No. 5,863,541 to Samulski *et al.*). The antigen may be presented in the parvovirus capsid. Alternatively, the antigen may be expressed from a heterologous nucleic acid introduced into a recombinant vector genome. Any immunogen of interest as described herein and/or as is known in the art can be provided by the virus vector of the present invention.

An immunogenic polypeptide can be any polypeptide suitable for eliciting an immune response and/or protecting the subject against an infection and/or disease, including, but not limited to, microbial, bacterial, protozoal, parasitic, fungal and/or viral infections and diseases. For example, the immunogenic polypeptide can be an

orthomyxovirus immunogen (*e.g.*, an influenza virus immunogen, such as the influenza virus hemagglutinin (HA) surface protein or the influenza virus nucleoprotein, or an equine influenza virus immunogen) or a lentivirus immunogen (*e.g.*, an equine infectious anemia virus immunogen, a Simian Immunodeficiency Virus (SIV) immunogen, or a Human Immunodeficiency Virus (HIV) immunogen, such as the HIV or SIV envelope GP160 protein, the HIV or SIV matrix/capsid proteins, and the HIV or SIV *gag*, *pol* and *env* genes products). The immunogenic polypeptide can also be an arenavirus immunogen (*e.g.*, Lassa fever virus immunogen, such as the Lassa fever virus nucleocapsid protein and the Lassa fever envelope glycoprotein), a poxvirus immunogen (*e.g.*, a vaccinia virus immunogen, such as the vaccinia L1 or L8 gene products), a flavivirus immunogen (*e.g.*, a yellow fever virus immunogen or a Japanese encephalitis virus immunogen), a filovirus immunogen (*e.g.*, an Ebola virus immunogen, or a Marburg virus immunogen, such as NP and GP gene products), a bunyavirus immunogen (*e.g.*, RVFV, CCHF, and/or SFS virus immunogens), or a coronavirus immunogen (*e.g.*, an infectious human coronavirus immunogen, such as the human coronavirus envelope glycoprotein, or a porcine transmissible gastroenteritis virus immunogen, or an avian infectious bronchitis virus immunogen). The immunogenic polypeptide can further be a polio immunogen, a herpes immunogen (*e.g.*, CMV, EBV, HSV immunogens) a mumps immunogen, a measles immunogen, a rubella immunogen, a diphtheria toxin or other diphtheria immunogen, a pertussis antigen, a hepatitis (*e.g.*, hepatitis A, hepatitis B, hepatitis C, etc.) immunogen, and/or any other vaccine immunogen now known in the art or later identified as an immunogen.

Alternatively, the immunogenic polypeptide can be any tumor or cancer cell antigen. Optionally, the tumor or cancer antigen is expressed on the surface of the cancer cell. Exemplary cancer and tumor cell antigens are described in S.A. Rosenberg (*Immunity* 10:281 (1991)). Other illustrative cancer and tumor antigens include, but are not limited to: BRCA1 gene product, BRCA2 gene product, gp100, tyrosinase, GAGE-1/2, BAGE, RAGE, LAGE, NY-ESO-1, CDK-4, β-catenin, MUM-1, Caspase-8, KIAA0205, HPVE, SART-1, PRAME, p15, melanoma tumor antigens (Kawakami et al., (1994) *Proc. Natl. Acad. Sci. USA* 91:3515; Kawakami et al., (1994) *J. Exp. Med.*, 180:347; Kawakami et al., (1994) *Cancer Res.* 54:3124), MART-1, gp100 MAGE-1, MAGE-2, MAGE-3, CEA, TRP-1, TRP-2, P-15, tyrosinase (Brichard et al., (1993) *J. Exp. Med.* 178:489); HER-2/neu gene product (U.S. Pat. No. 4,968,603), CA 125, LK26, FB5 (endosialin), TAG 72, AFP, CA19-9, NSE, DU-PAN-2, CA50, SPan-1, CA72-4, HCG, STN (sialyl Tn antigen), c-erbB-2 proteins, PSA, L-CanAg, estrogen receptor, milk fat globulin, p53 tumor suppressor protein

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(Levine, (1993) *Ann. Rev. Biochem.* 62:623); mucin antigens (International Patent Publication No. WO 90/05142); telomerases; nuclear matrix proteins; prostatic acid phosphatase; papilloma virus antigens; and/or antigens now known or later discovered to be associated with the following cancers: melanoma, adenocarcinoma, thymoma, lymphoma (*e.g.*, non-Hodgkin's lymphoma, Hodgkin's lymphoma), sarcoma, lung cancer, liver cancer, colon cancer, leukemia, uterine cancer, breast cancer, prostate cancer, ovarian cancer, cervical cancer, bladder cancer, kidney cancer, pancreatic cancer, brain cancer and any other cancer or malignant condition now known or later identified (*see, e.g.*, Rosenberg, (1996) *Ann. Rev. Med.* 47:481-91).

As a further alternative, the heterologous nucleic acid can encode any polypeptide that is desirably produced in a cell *in vitro*, *ex vivo*, or *in vivo*. For example, the virus vectors may be introduced into cultured cells and the expressed gene product isolated therefrom.

It will be understood by those skilled in the art that the heterologous nucleic acid(s) of interest can be operably associated with appropriate control sequences. For example, the heterologous nucleic acid can be operably associated with expression control elements, such as transcription/translation control signals, origins of replication, polyadenylation signals, internal ribosome entry sites (IRES), promoters, and/or enhancers, and the like.

Further, regulated expression of the heterologous nucleic acid(s) of interest can be achieved at the post-transcriptional level, *e.g.*, by regulating selective splicing of different introns by the presence or absence of an oligonucleotide, small molecule and/or other compound that selectively blocks splicing activity at specific sites (*e.g.*, as described in WO 2006/119137).

Those skilled in the art will appreciate that a variety of promoter/enhancer elements can be used depending on the level and tissue-specific expression desired. The promoter/enhancer can be constitutive or inducible, depending on the pattern of expression desired. The promoter/enhancer can be native or foreign and can be a natural or a synthetic sequence. By foreign, it is intended that the transcriptional initiation region is not found in the wild-type host into which the transcriptional initiation region is introduced.

In particular embodiments, the promoter/enhancer elements can be native to the target cell or subject to be treated. In representative embodiments, the promoters/enhancer element can be native to the heterologous nucleic acid sequence. The promoter/enhancer element is generally chosen so that it functions in the target cell(s) of interest. Further, in particular embodiments the

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promoter/enhancer element is a mammalian promoter/enhancer element. The promoter/enhancer element may be constitutive or inducible.

Inducible expression control elements are typically advantageous in those applications in which it is desirable to provide regulation over expression of the heterologous nucleic acid sequence(s). Inducible promoters/enhancer elements for gene delivery can be tissue-specific or –preferred promoter/enhancer elements, and include muscle specific or preferred (including cardiac, skeletal and/or smooth muscle specific or preferred), neural tissue specific or preferred (including brain-specific or preferred), eye specific or preferred (including retina-specific and cornea-specific), liver specific or preferred, bone marrow specific or preferred, pancreatic specific or preferred, spleen specific or preferred, and lung specific or preferred promoter/enhancer elements. Other inducible promoter/enhancer elements include hormone-inducible and metal-inducible elements. Exemplary inducible promoters/enhancer elements include, but are not limited to, a Tet on/off element, a RU486-inducible promoter, an ecdysone-inducible promoter, a rapamycin-inducible promoter, and a metallothionein promoter.

In embodiments wherein the heterologous nucleic acid sequence(s) is transcribed and then translated in the target cells, specific initiation signals are generally included for efficient translation of inserted protein coding sequences. These exogenous translational control sequences, which may include the ATG initiation codon and adjacent sequences, can be of a variety of origins, both natural and synthetic.

The virus vectors according to the present invention provide a means for delivering heterologous nucleic acids into a broad range of cells, including dividing and non-dividing cells. The virus vectors can be employed to deliver a nucleic acid of interest to a cell *in vitro*, *e.g.*, to produce a polypeptide *in vitro* or for *ex vivo* gene therapy. The virus vectors are additionally useful in a method of delivering a nucleic acid to a subject in need thereof, *e.g.*, to express an immunogenic or therapeutic polypeptide or a functional RNA. In this manner, the polypeptide or functional RNA can be produced *in vivo* in the subject. The subject can be in need of the polypeptide because the subject has a deficiency of the polypeptide. Further, the method can be practiced because the production of the polypeptide or functional RNA in the subject may impart some beneficial effect.

The virus vectors can also be used to produce a polypeptide of interest or functional RNA in cultured cells or in a subject (*e.g.*, using the subject as a bioreactor to produce the polypeptide or to observe the effects of the functional RNA on the subject, for example, in connection with screening methods).

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In general, the virus vectors of the present invention can be employed to deliver a heterologous nucleic acid encoding a polypeptide or functional RNA to treat and/or prevent any disease state for which it is beneficial to deliver a therapeutic polypeptide or functional RNA. Illustrative disease states include, but are not limited to: cystic fibrosis (cystic fibrosis transmembrane regulator protein) and other diseases of the lung, hemophilia A (Factor VIII), hemophilia B (Factor IX), thalassemia (ß-globin), anemia (erythropoietin) and other blood disorders, Alzheimer's disease (GDF; neprilysin), multiple sclerosis (ß-interferon), Parkinson's disease (glial-cell line derived neurotrophic factor [GDNF]), Huntington's disease (RNAi to remove repeats), amyotrophic lateral sclerosis, epilepsy (galanin, neurotrophic factors), and other neurological disorders, cancer (endostatin, angiostatin, TRAIL, FAS-ligand, cytokines including interferons; RNAi including RNAi against VEGF or the multiple drug resistance gene product, mir-26a [*e.g.*, for hepatocellular carcinoma]), diabetes mellitus (insulin), muscular dystrophies including Duchenne (dystrophin, mini-dystrophin, insulin-like growth factor I, a sarcoglycan [*e.g.*, α, β, γ], RNAi against myostatin, myostatin propeptide, follistatin, activin type II soluble receptor, anti-inflammatory polypeptides such as the Ikappa B dominant mutant, sarcospan, utrophin, mini-utrophin, antisense or RNAi against splice junctions in the dystrophin gene to induce exon skipping [*see, e.g.*, WO/2003/095647], antisense against U7 snRNAs to induce exon skipping [*see, e.g.*, WO/2006/021724], and antibodies or antibody fragments against myostatin or myostatin propeptide) and Becker, Gaucher disease (glucocerebrosidase), Hurler's disease (α-L-iduronidase), adenosine deaminase deficiency (adenosine deaminase), glycogen storage diseases (*e.g.*, Fabry disease [α-galactosidase] and Pompe disease [lysosomal acid α-glucosidase]) and other metabolic disorders, congenital emphysema (α1-antitrypsin), Lesch-Nyhan Syndrome (hypoxanthine guanine phosphoribosyl transferase), Niemann-Pick disease (sphingomyelinase), Tays Sachs disease (lysosomal hexosaminidase A), Maple Syrup Urine Disease (branched-chain keto acid dehydrogenase), retinal degenerative diseases (and other diseases of the eye and retina; *e.g.*, PDGF for macular degeneration and/or vasohibin or other inhibitors of VEGF or other angiogenesis inhibitors to treat/prevent retinal disorders, *e.g.*, in Type I diabetes), diseases of solid organs such as brain (including Parkinson's Disease [GDNF], astrocytomas [endostatin, angiostatin and/or RNAi against VEGF], glioblastomas [endostatin, angiostatin and/or RNAi against VEGF]), liver, kidney, heart including congestive heart failure or peripheral artery disease (PAD) (*e.g.*, by delivering protein phosphatase inhibitor I (I-1) and fragments thereof (*e.g.*, I1C), serca2a, zinc finger proteins that regulate the phospholamban gene, Barkct, β2-adrenergic receptor, β2-

adrenergic receptor kinase (BARK), phosphoinositide-3 kinase (PI3 kinase), S100A1, parvalbumin, adenylyl cyclase type 6, a molecule that effects G-protein coupled receptor kinase type 2 knockdown such as a truncated constitutively active bARKct; calsarcin, RNAi against phospholamban; phospholamban inhibitory or dominant-negative molecules such as phospholamban S16E, *etc.*), arthritis (insulin-like growth factors), joint disorders (insulin-like growth factor 1 and/or 2), intimal hyperplasia (*e.g.*, by delivering enos, inos), improve survival of heart transplants (superoxide dismutase), AIDS (soluble CD4), muscle wasting (insulin-like growth factor I), kidney deficiency (erythropoietin), anemia (erythropoietin), arthritis (anti-inflammatory factors such as IRAP and TNFα soluble receptor), hepatitis (α-interferon), LDL receptor deficiency (LDL receptor), hyperammonemia (ornithine transcarbamylase), Krabbe's disease (galactocerebrosidase), Batten's disease, spinal cerebral ataxias including SCA1, SCA2 and SCA3, phenylketonuria (phenylalanine hydroxylase), autoimmune diseases, and the like. The invention can further be used following organ transplantation to increase the success of the transplant and/or to reduce the negative side effects of organ transplantation or adjunct therapies (*e.g.*, by administering immunosuppressant agents or inhibitory nucleic acids to block cytokine production). As another example, bone morphogenic proteins (including BNP 2, 7, *etc.*, RANKL and/or VEGF) can be administered with a bone allograft, for example, following a break or surgical removal in a cancer patient.

The invention can also be used to produce induced pluripotent stem cells (iPS). For example, a virus vector of the invention can be used to deliver stem cell associated nucleic acid(s) into a non-pluripotent cell, such as adult fibroblasts, skin cells, liver cells, renal cells, adipose cells, cardiac cells, neural cells, epithelial cells, endothelial cells, and the like. Nucleic acids encoding factors associated with stem cells are known in the art. Nonlimiting examples of such factors associated with stem cells and pluripotency include Oct-3/4, the SOX family (*e.g.*, SOX1, SOX2, SOX3 and/or SOX15), the Klf family (*e.g.*, Klf1, Klf2, Klf4 and/or Klf5), the Myc family (*e.g.*, C-myc, L-myc and/or N-myc), NANOG and/or LIN28.

The invention can also be practiced to treat and/or prevent a metabolic disorder such as diabetes (*e.g.*, insulin), hemophilia (*e.g.*, Factor IX or Factor VIII), a lysosomal storage disorder such as a mucopolysaccharidosis disorder (*e.g.*, Sly syndrome [β-glucuronidase], Hurler Syndrome [α-L-iduronidase], Scheie Syndrome [α-L-iduronidase], Hurler-Scheie Syndrome [α-L-iduronidase], Hunter's Syndrome [iduronate sulfatase], Sanfilippo Syndrome A [heparan sulfamidase], B [N-acetylglucosaminidase], C [acetyl-CoA:α-glucosaminide acetyltransferase], D [N-acetylglucosamine 6-sulfatase], Morquio Syndrome A [galactose-6-sulfate sulfatase],

B [β-galactosidase], Maroteaux-Lamy Syndrome [N-acetylgalactosamine-4-sulfatase], *etc.*), Fabry disease (α-galactosidase), Gaucher's disease (glucocerebrosidase), or a glycogen storage disorder (*e.g.*, Pompe disease; lysosomal acid α-glucosidase).

Gene transfer has substantial potential use for understanding and providing therapy for disease states. There are a number of inherited diseases in which defective genes are known and have been cloned. In general, the above disease states fall into two classes: deficiency states, usually of enzymes, which are generally inherited in a recessive manner, and unbalanced states, which may involve regulatory or structural proteins, and which are typically inherited in a dominant manner. For deficiency state diseases, gene transfer can be used to bring a normal gene into affected tissues for replacement therapy, as well as to create animal models for the disease using antisense mutations. For unbalanced disease states, gene transfer can be used to create a disease state in a model system, which can then be used in efforts to counteract the disease state. Thus, virus vectors according to the present invention permit the treatment and/or prevention of genetic diseases.

The virus vectors according to the present invention may also be employed to provide a functional RNA to a cell *in vitro* or *in vivo*. Expression of the functional RNA in the cell, for example, can diminish expression of a particular target protein by the cell. Accordingly, functional RNA can be administered to decrease expression of a particular protein in a subject in need thereof. Functional RNA can also be administered to cells *in vitro* to regulate gene expression and/or cell physiology, *e.g.*, to optimize cell or tissue culture systems or in screening methods.

In addition, virus vectors according to the instant invention find use in diagnostic and screening methods, whereby a nucleic acid of interest is transiently or stably expressed in a cell culture system, or alternatively, a transgenic animal model.

The virus vectors of the present invention can also be used for various non-therapeutic purposes, including but not limited to use in protocols to assess gene targeting, clearance, transcription, translation, *etc.*, as would be apparent to one skilled in the art. The virus vectors can also be used for the purpose of evaluating safety (spread, toxicity, immunogenicity, *etc.*). Such data, for example, are considered by the United States Food and Drug Administration as part of the regulatory approval process prior to evaluation of clinical efficacy.

As a further aspect, the virus vectors of the present invention may be used to produce an immune response in a subject. According to this embodiment, a virus vector comprising a heterologous nucleic acid sequence encoding an immunogenic polypeptide can be administered to a subject, and an active immune response is

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mounted by the subject against the immunogenic polypeptide. Immunogenic polypeptides are as described hereinabove. In some embodiments, a protective immune response is elicited.

Alternatively, the virus vector may be administered to a cell *ex vivo* and the altered cell is administered to the subject. The virus vector comprising the heterologous nucleic acid is introduced into the cell, and the cell is administered to the subject, where the heterologous nucleic acid encoding the immunogen can be expressed and induce an immune response in the subject against the immunogen. In particular embodiments, the cell is an antigen-presenting cell (*e.g.*, a dendritic cell).

An "active immune response" or "active immunity" is characterized by "participation of host tissues and cells after an encounter with the immunogen. It involves differentiation and proliferation of immunocompetent cells in lymphoreticular tissues, which lead to synthesis of antibody or the development of cell-mediated reactivity, or both." Herbert B. Herscowitz, *Immunophysiology: Cell Function and Cellular Interactions in Antibody Formation, in* IMMUNOLOGY: BASIC PROCESSES 117 (Joseph A. Bellanti ed., 1985). Alternatively stated, an active immune response is mounted by the host after exposure to an immunogen by infection or by vaccination. Active immunity can be contrasted with passive immunity, which is acquired through the "transfer of preformed substances (antibody, transfer factor, thymic graft, interleukin-2) from an actively immunized host to a non-immune host." *Id.*

A "protective" immune response or "protective" immunity as used herein indicates that the immune response confers some benefit to the subject in that it prevents or reduces the incidence of disease. Alternatively, a protective immune response or protective immunity may be useful in the treatment and/or prevention of disease, in particular cancer or tumors (*e.g.*, by preventing cancer or tumor formation, by causing regression of a cancer or tumor and/or by preventing metastasis and/or by preventing growth of metastatic nodules). The protective effects may be complete or partial, as long as the benefits of the treatment outweigh any disadvantages thereof.

In particular embodiments, the virus vector or cell comprising the heterologous nucleic acid can be administered in an immunogenically effective amount, as described below.

The virus vectors of the present invention can also be administered for cancer immunotherapy by administration of a virus vector expressing one or more cancer cell antigens (or an immunologically similar molecule) or any other immunogen that

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produces an immune response against a cancer cell. To illustrate, an immune response can be produced against a cancer cell antigen in a subject by administering a virus vector comprising a heterologous nucleic acid encoding the cancer cell antigen, for example to treat a patient with cancer and/or to prevent cancer from developing in the subject. The virus vector may be administered to a subject *in vivo* or by using *ex vivo* methods, as described herein. Alternatively, the cancer antigen can be expressed as part of the virus capsid or be otherwise associated with the virus capsid (*e.g.*, as described above).

As another alternative, any other therapeutic nucleic acid (*e.g.*, RNAi) or polypeptide (*e.g.*, cytokine) known in the art can be administered to treat and/or prevent cancer.

As used herein, the term "cancer" encompasses tumor-forming cancers. Likewise, the term "cancerous tissue" encompasses tumors. A "cancer cell antigen" encompasses tumor antigens.

The term "cancer" has its understood meaning in the art, for example, an uncontrolled growth of tissue that has the potential to spread to distant sites of the body (*i.e.*, metastasize). Exemplary cancers include, but are not limited to melanoma, adenocarcinoma, thymoma, lymphoma (*e.g.*, non-Hodgkin's lymphoma, Hodgkin's lymphoma), sarcoma, lung cancer, liver cancer, colon cancer, leukemia, uterine cancer, breast cancer, prostate cancer, ovarian cancer, cervical cancer, bladder cancer, kidney cancer, pancreatic cancer, brain cancer and any other cancer or malignant condition now known or later identified. In representative embodiments, the invention provides a method of treating and/or preventing tumor-forming cancers.

The term "tumor" is also understood in the art, for example, as an abnormal mass of undifferentiated cells within a multicellular organism. Tumors can be malignant or benign. In representative embodiments, the methods disclosed herein are used to prevent and treat malignant tumors.

By the terms "treating cancer," "treatment of cancer" and equivalent terms it is intended that the severity of the cancer is reduced or at least partially eliminated and/or the progression of the disease is slowed and/or controlled and/or the disease is stabilized. In particular embodiments, these terms indicate that metastasis of the cancer is prevented or reduced or at least partially eliminated and/or that growth of metastatic nodules is prevented or reduced or at least partially eliminated.

By the terms "prevention of cancer" or "preventing cancer" and equivalent terms it is intended that the methods at least partially eliminate or reduce and/or delay the incidence and/or severity of the onset of cancer. Alternatively stated, the

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onset of cancer in the subject may be reduced in likelihood or probability and/or delayed.

In particular embodiments, cells may be removed from a subject with cancer and contacted with a virus vector expressing a cancer cell antigen according to the instant invention. The modified cell is then administered to the subject, whereby an immune response against the cancer cell antigen is elicited. This method can be advantageously employed with immunocompromised subjects that cannot mount a sufficient immune response *in vivo* (*i.e.*, cannot produce enhancing antibodies in sufficient quantities).

It is known in the art that immune responses may be enhanced by immunomodulatory cytokines (*e.g.*, α-interferon, β-interferon, γ-interferon, ω-interferon, τ-interferon, interleukin-1α, interleukin-1β, interleukin-2, interleukin-3, interleukin-4, interleukin 5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, interleukin 12, interleukin-13, interleukin-14, interleukin-18, B cell Growth factor, CD40 Ligand, tumor necrosis factor-α, tumor necrosis factor-β, monocyte chemoattractant protein-1, granulocyte-macrophage colony stimulating factor, and lymphotoxin). Accordingly, immunomodulatory cytokines (preferably, CTL inductive cytokines) may be administered to a subject in conjunction with the virus vector.

Cytokines may be administered by any method known in the art. Exogenous cytokines may be administered to the subject, or alternatively, a nucleic acid encoding a cytokine may be delivered to the subject using a suitable vector, and the cytokine produced *in vivo*.

Subjects, Pharmaceutical Formulations, and Modes of Administration.

Virus vectors and capsids according to the present invention find use in both veterinary and medical applications. Suitable subjects include both avians and mammals. The term "avian" as used herein includes, but is not limited to, chickens, ducks, geese, quail, turkeys, pheasant, parrots, parakeets, and the like. The term "mammal" as used herein includes, but is not limited to, humans, non-human primates, bovines, ovines, caprines, equines, felines, canines, lagomorphs, *etc.* Human subjects include neonates, infants, juveniles, adults and geriatric subjects.

In representative embodiments, the subject is "in need of" the methods of the invention.

In particular embodiments, the present invention provides a pharmaceutical composition comprising a virus vector and/or capsid of the invention in a

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pharmaceutically acceptable carrier and, optionally, other medicinal agents, pharmaceutical agents, stabilizing agents, buffers, carriers, adjuvants, diluents, *etc.* For injection, the carrier will typically be a liquid. For other methods of administration, the carrier may be either solid or liquid. For inhalation administration, the carrier will be respirable, and optionally can be in solid or liquid particulate form.

By "pharmaceutically acceptable" it is meant a material that is not toxic or otherwise undesirable, *i.e.*, the material may be administered to a subject without causing any undesirable biological effects.

One aspect of the present invention is a method of transferring a nucleic acid to a cell *in vitro*. The virus vector may be introduced into the cells at the appropriate multiplicity of infection according to standard transduction methods suitable for the particular target cells. Titers of virus vector to administer can vary, depending upon the target cell type and number, and the particular virus vector, and can be determined by those of skill in the art without undue experimentation. In representative embodiments, at least about 10^3 infectious units, optionally at least about 10^5 infectious units are introduced to the cell.

The cell(s) into which the virus vector is introduced can be of any type, including but not limited to neural cells (including cells of the peripheral and central nervous systems, in particular, brain cells such as neurons and oligodendricytes), lung cells, cells of the eye (including retinal cells, retinal pigment epithelium, and corneal cells), epithelial cells (*e.g.*, gut and respiratory epithelial cells), muscle cells (*e.g.*, skeletal muscle cells, cardiac muscle cells, smooth muscle cells and/or diaphragm muscle cells), dendritic cells, pancreatic cells (including islet cells), hepatic cells, myocardial cells, bone cells (*e.g.*, bone marrow stem cells), hematopoietic stem cells, spleen cells, keratinocytes, fibroblasts, endothelial cells, prostate cells, germ cells, and the like. In representative embodiments, the cell can be any progenitor cell. As a further possibility, the cell can be a stem cell (*e.g.*, neural stem cell, liver stem cell). As still a further alternative, the cell can be a cancer or tumor cell. Moreover, the cell can be from any species of origin, as indicated above.

The virus vector can be introduced into cells *in vitro* for the purpose of administering the modified cell to a subject. In particular embodiments, the cells have been removed from a subject, the virus vector is introduced therein, and the cells are then administered back into the subject. Methods of removing cells from subject for manipulation *ex vivo*, followed by introduction back into the subject are known in the art (*see, e.g.*, U.S. patent No. 5,399,346). Alternatively, the recombinant virus vector can be introduced into cells from a donor subject, into cultured cells, or into cells from

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any other suitable source, and the cells are administered to a subject in need thereof (*i.e.*, a "recipient" subject).

Suitable cells for *ex vivo* nucleic acid delivery are as described above. Dosages of the cells to administer to a subject will vary upon the age, condition and species of the subject, the type of cell, the nucleic acid being expressed by the cell, the mode of administration, and the like. Typically, at least about 10^2 to about 10^8 cells or at least about 10^3 to about 10^6 cells will be administered per dose in a pharmaceutically acceptable carrier. In particular embodiments, the cells transduced with the virus vector are administered to the subject in a treatment effective or prevention effective amount in combination with a pharmaceutical carrier.

In some embodiments, the virus vector is introduced into a cell and the cell can be administered to a subject to elicit an immunogenic response against the delivered polypeptide (*e.g.*, expressed as a transgene or in the capsid). Typically, a quantity of cells expressing an immunogenically effective amount of the polypeptide in combination with a pharmaceutically acceptable carrier is administered. An "immunogenically effective amount" is an amount of the expressed polypeptide that is sufficient to evoke an active immune response against the polypeptide in the subject to which the pharmaceutical formulation is administered. In particular embodiments, the dosage is sufficient to produce a protective immune response (as defined above). The degree of protection conferred need not be complete or permanent, as long as the benefits of administering the immunogenic polypeptide outweigh any disadvantages thereof.

A further aspect of the invention is a method of administering the virus vector and/or virus capsid to subjects. Administration of the virus vectors and/or capsids according to the present invention to a human subject or an animal in need thereof can be by any means known in the art. Optionally, the virus vector and/or capsid is delivered in a treatment effective or prevention effective dose in a pharmaceutically acceptable carrier.

The virus vectors and/or capsids of the invention can further be administered to elicit an immunogenic response (*e.g.*, as a vaccine). Typically, immunogenic compositions of the present invention comprise an immunogenically effective amount of virus vector and/or capsid in combination with a pharmaceutically acceptable carrier. Optionally, the dosage is sufficient to produce a protective immune response (as defined above). The degree of protection conferred need not be complete or permanent, as long as the benefits of administering the immunogenic polypeptide outweigh any disadvantages thereof. Subjects and immunogens are as described above.

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Dosages of the virus vector and/or capsid to be administered to a subject depend upon the mode of administration, the disease or condition to be treated and/or prevented, the individual subject's condition, the particular virus vector or capsid, and the nucleic acid to be delivered, and the like, and can be determined in a routine manner. Exemplary doses for achieving therapeutic effects are titers of at least about 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^3, 10^{14}, 10^{15} transducing units, optionally about $10^8 - 10^{13}$ transducing units.

In particular embodiments, more than one administration (*e.g.*, two, three, four or more administrations) may be employed to achieve the desired level of gene expression over a period of various intervals, *e.g.*, daily, weekly, monthly, yearly, *etc.*

Exemplary modes of administration include oral, rectal, transmucosal, intranasal, inhalation (*e.g.*, via an aerosol), buccal (*e.g.*, sublingual), vaginal, intrathecal, intraocular, transdermal, *in utero* (or *in ovo*), parenteral (*e.g.*, intravenous, subcutaneous, intradermal, intramuscular [including administration to skeletal, diaphragm and/or cardiac muscle], intradermal, intrapleural, intracerebral, and intraarticular), topical (*e.g.*, to both skin and mucosal surfaces, including airway surfaces, and transdermal administration), intralymphatic, and the like, as well as direct tissue or organ injection (*e.g.*, to liver, skeletal muscle, cardiac muscle, diaphragm muscle or brain). Administration can also be to a tumor (*e.g.*, in or near a tumor or a lymph node). The most suitable route in any given case will depend on the nature and severity of the condition being treated and/or prevented and on the nature of the particular vector that is being used.

Administration to skeletal muscle according to the present invention includes but is not limited to administration to skeletal muscle in the limbs (*e.g.*, upper arm, lower arm, upper leg, and/or lower leg), back, neck, head (*e.g.*, tongue), thorax, abdomen, pelvis/perineum, and/or digits. Suitable skeletal muscles include but are not limited to abductor digiti minimi (in the hand), abductor digiti minimi (in the foot), abductor hallucis, abductor ossis metatarsi quinti, abductor pollicis brevis, abductor pollicis longus, adductor brevis, adductor hallucis, adductor longus, adductor magnus, adductor pollicis, anconeus, anterior scalene, articularis genus, biceps brachii, biceps femoris, brachialis, brachioradialis, buccinator, coracobrachialis, corrugator supercilii, deltoid, depressor anguli oris, depressor labii inferioris, digastric, dorsal interossei (in the hand), dorsal interossei (in the foot), extensor carpi radialis brevis, extensor carpi radialis longus, extensor carpi ulnaris, extensor digiti minimi, extensor digitorum, extensor digitorum brevis, extensor digitorum longus, extensor hallucis brevis, extensor hallucis longus, extensor indicis, extensor pollicis brevis, extensor pollicis longus, flexor carpi radialis, flexor carpi ulnaris, flexor digiti minimi

brevis (in the hand), flexor digiti minimi brevis (in the foot), flexor digitorum brevis, flexor digitorum longus, flexor digitorum profundus, flexor digitorum superficialis, flexor hallucis brevis, flexor hallucis longus, flexor pollicis brevis, flexor pollicis longus, frontalis, gastrocnemius, geniohyoid, gluteus maximus, gluteus medius, gluteus minimus, gracilis, iliocostalis cervicis, iliocostalis lumborum, iliocostalis thoracis, illiacus, inferior gemellus, inferior oblique, inferior rectus, infraspinatus, interspinalis, intertransversi, lateral pterygoid, lateral rectus, latissimus dorsi, levator anguli oris, levator labii superioris, levator labii superioris alaeque nasi, levator palpebrae superioris, levator scapulae, long rotators, longissimus capitis, longissimus cervicis, longissimus thoracis, longus capitis, longus colli, lumbricals (in the hand), lumbricals (in the foot), masseter, medial pterygoid, medial rectus, middle scalene, multifidus, mylohyoid, obliquus capitis inferior, obliquus capitis superior, obturator externus, obturator internus, occipitalis, omohyoid, opponens digiti minimi, opponens pollicis, orbicularis oculi, orbicularis oris, palmar interossei, palmaris brevis, palmaris longus, pectineus, pectoralis major, pectoralis minor, peroneus brevis, peroneus longus, peroneus tertius, piriformis, plantar interossei, plantaris, platysma, popliteus, posterior scalene, pronator quadratus, pronator teres, psoas major, quadratus femoris, quadratus plantae, rectus capitis anterior, rectus capitis lateralis, rectus capitis posterior major, rectus capitis posterior minor, rectus femoris, rhomboid major, rhomboid minor, risorius, sartorius, scalenus minimus, semimembranosus, semispinalis capitis, semispinalis cervicis, semispinalis thoracis, semitendinosus, serratus anterior, short rotators, soleus, spinalis capitis, spinalis cervicis, spinalis thoracis, splenius capitis, splenius cervicis, sternocleidomastoid, sternohyoid, sternothyroid, stylohyoid, subclavius, subscapularis, superior gemellus, superior oblique, superior rectus, supinator, supraspinatus, temporalis, tensor fascia lata, teres major, teres minor, thoracis, thyrohyoid, tibialis anterior, tibialis posterior, trapezius, triceps brachii, vastus intermedius, vastus lateralis, vastus medialis, zygomaticus major, and zygomaticus minor, and any other suitable skeletal muscle as known in the art.

The virus vector and/or capsid can be delivered to skeletal muscle by intravenous administration, intra-arterial administration, intraperitoneal administration, limb perfusion, (optionally, isolated limb perfusion of a leg and/or arm; *see, e.g.* Arruda et al., (2005) *Blood* 105: 3458-3464), and/or direct intramuscular injection. In particular embodiments, the virus vector and/or capsid is administered to a limb (arm and/or leg) of a subject (*e.g.*, a subject with muscular dystrophy such as DMD) by limb perfusion, optionally isolated limb perfusion (*e.g.*, by intravenous or intra-articular administration). In embodiments of the invention, the virus vectors and/or

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capsids of the invention can advantageously be administered without employing "hydrodynamic" techniques. Tissue delivery (*e.g.*, to muscle) of prior art vectors is often enhanced by hydrodynamic techniques (*e.g.*, intravenous/intravenous administration in a large volume), which increase pressure in the vasculature and facilitate the ability of the vector to cross the endothelial cell barrier. In particular embodiments, the viral vectors and/or capsids of the invention can be administered in the absence of hydrodynamic techniques such as high volume infusions and/or elevated intravascular pressure (*e.g.*, greater than normal systolic pressure, for example, less than or equal to a 5%, 10%, 15%, 20%, 25% increase in intravascular pressure over normal systolic pressure). Such methods may reduce or avoid the side effects associated with hydrodynamic techniques such as edema, nerve damage and/or compartment syndrome.

Administration to cardiac muscle includes administration to the left atrium, right atrium, left ventricle, right ventricle and/or septum. The virus vector and/or capsid can be delivered to cardiac muscle by intravenous administration, intra-arterial administration such as intra-aortic administration, direct cardiac injection (*e.g.*, into left atrium, right atrium, left ventricle, right ventricle), and/or coronary artery perfusion.

Administration to diaphragm muscle can be by any suitable method including intravenous administration, intra-arterial administration, and/or intra-peritoneal administration.

Delivery to a target tissue can also be achieved by delivering a depot comprising the virus vector and/or capsid. In representative embodiments, a depot comprising the virus vector and/or capsid is implanted into skeletal, cardiac and/or diaphragm muscle tissue or the tissue can be contacted with a film or other matrix comprising the virus vector and/or capsid. Such implantable matrices or substrates are described in U.S. Patent No. 7,201,898.

In particular embodiments, a virus vector and/or virus capsid according to the present invention is administered to skeletal muscle, diaphragm muscle and/or cardiac muscle (*e.g.*, to treat and/or prevent muscular dystrophy, heart disease [for example, PAD or congestive heart failure]).

In representative embodiments, the invention is used to treat and/or prevent disorders of skeletal, cardiac and/or diaphragm muscle.

In a representative embodiment, the invention provides a method of treating and/or preventing muscular dystrophy in a subject in need thereof, the method comprising: administering a treatment or prevention effective amount of a virus vector of the invention to a mammalian subject, wherein the virus vector comprises a heterologous nucleic acid encoding dystrophin, a mini-dystrophin, a micro-dystrophin,

myostatin propeptide, follistatin, activin type II soluble receptor, IGF-1, anti-inflammatory polypeptides such as the Ikappa B dominant mutant, sarcospan, utrophin, a micro-dystrophin, laminin-α2, α-sarcoglycan, β-sarcoglycan, γ-sarcoglycan, δ-sarcoglycan, IGF-1, an antibody or antibody fragment against myostatin or myostatin propeptide, and/or RNAi against myostatin. In particular embodiments, the virus vector can be administered to skeletal, diaphragm and/or cardiac muscle as described elsewhere herein.

Alternatively, the invention can be practiced to deliver a nucleic acid to skeletal, cardiac or diaphragm muscle, which is used as a platform for production of a polypeptide (*e.g.*, an enzyme) or functional RNA (*e.g.*, RNAi, microRNA, antisense RNA) that normally circulates in the blood or for systemic delivery to other tissues to treat and/or prevent a disorder (*e.g.*, a metabolic disorder, such as diabetes [*e.g.*, insulin], hemophilia [*e.g.*, Factor IX or Factor VIII], a mucopolysaccharide disorder [*e.g.*, Sly syndrome, Hurler Syndrome, Scheie Syndrome, Hurler-Scheie Syndrome, Hunter's Syndrome, Sanfilippo Syndrome A, B, C, D, Morquio Syndrome, Maroteaux-Lamy Syndrome, *etc.*] or a lysosomal storage disorder such as Gaucher's disease [glucocerebrosidase] or Fabry disease [α-galactosidase A] or a glycogen storage disorder such as Pompe disease [lysosomal acid α glucosidase]). Other suitable proteins for treating and/or preventing metabolic disorders are described herein. The use of muscle as a platform to express a nucleic acid of interest is described in U.S. Patent publication US 2002/0192189.

Thus, as one aspect, the invention further encompasses a method of treating and/or preventing a metabolic disorder in a subject in need thereof, the method comprising: administering a treatment or prevention effective amount of a virus vector of the invention to skeletal muscle of a subject, wherein the virus vector comprises a heterologous nucleic acid encoding a polypeptide, wherein the metabolic disorder is a result of a deficiency and/or defect in the polypeptide. Illustrative metabolic disorders and heterologous nucleic acids encoding polypeptides are described herein. Optionally, the polypeptide is secreted (*e.g.*, a polypeptide that is a secreted polypeptide in its native state or that has been engineered to be secreted, for example, by operable association with a secretory signal sequence as is known in the art). Without being limited by any particular theory of the invention, according to this embodiment, administration to the skeletal muscle can result in secretion of the polypeptide into the systemic circulation and delivery to target tissue(s). Methods of delivering virus vectors to skeletal muscle is described in more detail herein.

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The invention can also be practiced to produce antisense RNA, RNAi or other functional RNA (*e.g.*, a ribozyme) for systemic delivery.

The invention also provides a method of treating and/or preventing congenital heart failure or PAD in a subject in need thereof, the method comprising administering a treatment or prevention effective amount of a virus vector of the invention to a mammalian subject, wherein the virus vector comprises a heterologous nucleic acid encoding, for example, a sarcoplasmic endoreticulum Ca^{2+}-ATPase (SERCA2a), an angiogenic factor, phosphatase inhibitor I (I-1) and fragments thereof (*e.g.*, I1C), RNAi against phospholamban; a phospholamban inhibitory or dominant-negative molecule such as phospholamban S16E, a zinc finger protein that regulates the phospholamban gene, β2-adrenergic receptor, β2-adrenergic receptor kinase (BARK), PI3 kinase, calsarcan, a β-adrenergic receptor kinase inhibitor (βARKct), inhibitor 1 of protein phosphatase 1 and fragments thereof (*e.g.*, I1C), S100A1, parvalbumin, adenylyl cyclase type 6, a molecule that effects G-protein coupled receptor kinase type 2 knockdown such as a truncated constitutively active bARKct, Pim-1, PGC-1α, SOD-1, SOD-2, EC-SOD, kallikrein, HIF, thymosin-β4, mir-1, mir-133, mir-206, mir-208 and/or mir-26a.

Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Alternatively, one may administer the virus vector and/or virus capsids of the invention in a local rather than systemic manner, for example, in a depot or sustained-release formulation. Further, the virus vector and/or virus capsid can be delivered adhered to a surgically implantable matrix (*e.g.*, as described in U.S. Patent Publication No. US-2004-0013645-A1).

The virus vectors and/or virus capsids disclosed herein can be administered to the lungs of a subject by any suitable means, optionally by administering an aerosol suspension of respirable particles comprised of the virus vectors and/or virus capsids, which the subject inhales. The respirable particles can be liquid or solid. Aerosols of liquid particles comprising the virus vectors and/or virus capsids may be produced by any suitable means, such as with a pressure-driven aerosol nebulizer or an ultrasonic nebulizer, as is known to those of skill in the art. *See, e.g.*, U.S. Patent No. 4,501,729. Aerosols of solid particles comprising the virus vectors and/or capsids may likewise be produced with any solid particulate medicament aerosol generator, by techniques known in the pharmaceutical art.

The virus vectors and virus capsids can be administered to tissues of the CNS (*e.g.*, brain, eye) and may advantageously result in broader distribution of the

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virus vector or capsid than would be observed in the absence of the present invention.

In particular embodiments, the delivery vectors of the invention may be administered to treat diseases of the CNS, including genetic disorders, neurodegenerative disorders, psychiatric disorders and tumors. Illustrative diseases of the CNS include, but are not limited to Alzheimer's disease, Parkinson's disease, Huntington's disease, Canavan disease, Leigh's disease, Refsum disease, Tourette syndrome, primary lateral sclerosis, amyotrophic lateral sclerosis, progressive muscular atrophy, Pick's disease, muscular dystrophy, multiple sclerosis, myasthenia gravis, Binswanger's disease, trauma due to spinal cord or head injury, Tay Sachs disease, Lesch-Nyan disease, epilepsy, cerebral infarcts, psychiatric disorders including mood disorders (*e.g.*, depression, bipolar affective disorder, persistent affective disorder, secondary mood disorder), schizophrenia, drug dependency (*e.g.*, alcoholism and other substance dependencies), neuroses (*e.g.*, anxiety, obsessional disorder, somatoform disorder, dissociative disorder, grief, post-partum depression), psychosis (*e.g.*, hallucinations and delusions), dementia, paranoia, attention deficit disorder, psychosexual disorders, sleeping disorders, pain disorders, eating or weight disorders (*e.g.*, obesity, cachexia, anorexia nervosa, and bulemia) and cancers and tumors (*e.g.*, pituitary tumors) of the CNS.

Disorders of the CNS include ophthalmic disorders involving the retina, posterior tract, and optic nerve (*e.g.*, retinitis pigmentosa, diabetic retinopathy and other retinal degenerative diseases, uveitis, age-related macular degeneration, glaucoma).

Most, if not all, ophthalmic diseases and disorders are associated with one or more of three types of indications: (1) angiogenesis, (2) inflammation, and (3) degeneration. The delivery vectors of the present invention can be employed to deliver anti-angiogenic factors; anti-inflammatory factors; factors that retard cell degeneration, promote cell sparing, or promote cell growth and combinations of the foregoing.

Diabetic retinopathy, for example, is characterized by angiogenesis. Diabetic retinopathy can be treated by delivering one or more anti-angiogenic factors either intraocularly (*e.g.*, in the vitreous) or periocularly(*e.g.*, in the sub-Tenon's region). One or more neurotrophic factors may also be co-delivered, either intraocularly (*e.g.*, intravitreally) or periocularly.

Uveitis involves inflammation. One or more anti-inflammatory factors can be administered by intraocular (*e.g.*, vitreous or anterior chamber) administration of a delivery vector of the invention.

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Retinitis pigmentosa, by comparison, is characterized by retinal degeneration. In representative embodiments, retinitis pigmentosa can be treated by intraocular (*e.g.*, vitreal administration) of a delivery vector encoding one or more neurotrophic factors.

Age-related macular degeneration involves both angiogenesis and retinal degeneration. This disorder can be treated by administering the inventive deliver vectors encoding one or more neurotrophic factors intraocularly (*e.g.*, vitreous) and/or one or more anti-angiogenic factors intraocularly or periocularly (*e.g.*, in the sub-Tenon's region).

Glaucoma is characterized by increased ocular pressure and loss of retinal ganglion cells. Treatments for glaucoma include administration of one or more neuroprotective agents that protect cells from excitotoxic damage using the inventive delivery vectors. Such agents include N-methyl-D-aspartate (NMDA) antagonists, cytokines, and neurotrophic factors, delivered intraocularly, optionally intravitreally.

In other embodiments, the present invention may be used to treat seizures, *e.g.*, to reduce the onset, incidence or severity of seizures. The efficacy of a therapeutic treatment for seizures can be assessed by behavioral (*e.g.*, shaking, ticks of the eye or mouth) and/or electrographic means (most seizures have signature electrographic abnormalities). Thus, the invention can also be used to treat epilepsy, which is marked by multiple seizures over time.

In one representative embodiment, somatostatin (or an active fragment thereof) is administered to the brain using a delivery vector of the invention to treat a pituitary tumor. According to this embodiment, the delivery vector encoding somatostatin (or an active fragment thereof) is administered by microinfusion into the pituitary. Likewise, such treatment can be used to treat acromegaly (abnormal growth hormone secretion from the pituitary). The nucleic acid (*e.g.*, GenBank Accession No. J00306) and amino acid (*e.g.*, GenBank Accession No. P01166; contains processed active peptides somatostatin-28 and somatostatin-14) sequences of somatostatins are known in the art.

In particular embodiments, the vector can comprise a secretory signal as described in U.S. Patent No. 7,071,172.

In representative embodiments of the invention, the virus vector and/or virus capsid is administered to the CNS (*e.g.*, to the brain or to the eye). The virus vector and/or capsid may be introduced into the spinal cord, brainstem (medulla oblongata, pons), midbrain (hypothalamus, thalamus, epithalamus, pituitary gland, substantia nigra, pineal gland), cerebellum, telencephalon (corpus striatum, cerebrum including the occipital, temporal, parietal and frontal lobes. cortex, basal ganglia, hippocampus

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and portaamygdala), limbic system, neocortex, corpus striatum, cerebrum, and inferior colliculus. The virus vector and/or capsid may also be administered to different regions of the eye such as the retina, cornea and/or optic nerve.

The virus vector and/or capsid may be delivered into the cerebrospinal fluid (*e.g.*, by lumbar puncture) for more disperse administration of the delivery vector. The virus vector and/or capsid may further be administered intravascularly to the CNS in situations in which the blood-brain barrier has been perturbed (*e.g.*, brain tumor or cerebral infarct).

The virus vector and/or capsid can be administered to the desired region(s) of the CNS by any route known in the art, including but not limited to, intrathecal, intra-ocular, intracerebral, intraventricular, intravenous (*e.g.*, in the presence of a sugar such as mannitol), intranasal, intra-aural, intra-ocular (*e.g.*, intra-vitreous, sub-retinal, anterior chamber) and peri-ocular (*e.g.*, sub-Tenon's region) delivery as well as intramuscular delivery with retrograde delivery to motor neurons.

In particular embodiments, the virus vector and/or capsid is administered in a liquid formulation by direct injection (*e.g.*, stereotactic injection) to the desired region or compartment in the CNS. In other embodiments, the virus vector and/or capsid may be provided by topical application to the desired region or by intra-nasal administration of an aerosol formulation. Administration to the eye, may be by topical application of liquid droplets. As a further alternative, the virus vector and/or capsid may be administered as a solid, slow-release formulation (*see, e.g.*, U.S. Patent No. 7,201,898).

In yet additional embodiments, the virus vector can used for retrograde transport to treat and/or prevent diseases and disorders involving motor neurons (e.g., amyotrophic lateral sclerosis (ALS); spinal muscular atrophy (SMA), etc.). For example, the virus vector can be delivered to muscle tissue from which it can migrate into neurons.

Having described the present invention, the same will be explained in greater detail in the following examples, which are included herein for illustration purposes only, and which are not intended to be limiting to the invention.

EXAMPLES

The inventors have exploited the plasticity of the heparin binding domain on AAV2 to generate synthetic AAV strains with unique systemic tissue tropisms. Using a domain swapping approach, the inventors substituted a linear heparin sulfate-binding motif on the AAV serotype 2 capsid with corresponding amino acid residues

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from various other AAV serotypes and variants as well as synthetic strains (see **Table 5**). Attenuation of heparin sulfate binding through this comprehensive mutagenesis approach yielded three groups of chimeric AAV capsids with overlapping phenotypes: (a) AAV mutants capable of systemic spread; (b) AAV mutants detargeted from the liver and (c) AAV mutants with low level transduction.

The inventors generated a panel of chimeric AAV2-derived capsids, deficient in heparin binding, by swapping the 585-RGNRQA-590 heparin binding domain with amino acid residues derived from the corresponding capsid domain on various AAV isolates. *In vivo* screening of the AAV vector panel following intravenous administration in mice was carried out through live animal bioluminescent imaging. This approach resulted in the discovery of several chimeric AAV capsids displaying distinct systemic transduction profiles as well as mutants with only low-level transduction for any tissue type.

One chimeric vector, AAV2i8, selectively transduces cardiac as well as skeletal muscle tissue with high efficiency. The AAV2i8 capsid, which is detargeted from the liver, appears to traverse the blood vasculature to efficiently transduce a wide variety of muscle groups. Comparative analysis of capsid surface topologies reveals a chimeric footprint at the three-fold axis of symmetry on the AAV2i8 capsid (data not shown) that may contribute to the observed atypical transduction profile. The chimeric nature of AAV2i8 is corroborated by the observation that AAV2i8 capsids are only modestly neutralized when exposed to anti-AAV2 serum or human serum (see **Tables 6** and **7**). In addition to being de-targeted from the liver, AAV2i8 demonstrates an ability to traverse the blood vasculature and transduce cardiac and skeletal muscle with high efficiency comparable to AAV8 vectors.

TABLE 5

Hexapeptide motif (585-590)	AAV strain(s)	Tropism**	Transduction Efficiency (Muscle)	Transduction Efficiency (Liver)
RGNRQA	AAV2	Liver	-	++
SSSTDP	2i1; 2i6	N/A	-	-
SSNTAP	2i3a/3b	N/A	-	-
SNSNLP	2i4	N/A	-	-
SSTTAP	2i5	N/A	-	-
SAQAQA	2i9	N/A	-	-
QGNTAP	2i8; 2irh43; 2irh49-53; 2irh57; 2irh58; 2irh64	Systemic Muscle	++++	+
QANTGP	2i10; 2irh40	Systemic Muscle	++	-
QTNTGP	2irh38	Systemic Muscle	++	-
QTNGAP	2irh2	Systemic Muscle	+	-
NATTAP	2i11; 2i12; 2irh32-34	Systemic Muscle	+	-
QGNTAP	AAV8	Systemic (Multi-organ)	++++	++++

* (-) No transduction observed, (+) one relative log unit; ** Based on image analysis, genome copy number and/or luciferase expression levels

TABLE 6

Vector	Sera AAV2
AAV2	1:2500
AAV8	<1:10
AAV2i8	1:40

TABLE 7

Subject ID	NA titer to AAV		
	AAV2	AAV8	AAV2i8
CHI 2-17	256	<20 (8)	<20 (16)
DEN 4-8	256	<20 (8)	<20 (8)
DEN 20-7	64	<20 (<2)	<20 (<2)
IND 1-5	512	<20 (16)	<20 (16)

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Example 1

Generation of AAV2 Inner Loop Mutants (AAV2i series)

The heparin binding motif, 585-RGNRQA-590, is located within loop IV at the three-fold axis of symmetry on the AAV2 capsid surface. Through site-directed mutagenesis, the hexapeptide motif was substituted with corresponding amino acids (located adjacent to a conserved glutamine residue) from different AAV serotypes and non-human primate isolates to generate a series of AAV2i mutants (see **Table 5**). Titers of all AAV2i mutants were comparable to that of parental AAV2 vectors.

Example 2

AAV2i Mutants are Deficient in Heparin Binding and Transduction *in vitro*

In the current study, AAV2i mutants containing amino acid residues Q, A, S or N in position 585 and T, N, A or G in position 588 were unable to bind heparin as demonstrated by affinity column binding assays. Representative elution profiles of parental AAV2 capsids and one such mutant, AAV2i8, are shown in **Figure 1a**. While the AAV2 peak fraction elutes at ~300mM NaCl, the AAV2i8 capsid is unable to bind heparin under physiological conditions (pH 7.4, 150mM NaCl).

In general, AAV2i mutants were deficient by several orders of magnitude at transducing HEK293 cells in comparison with the parental AAV2 vector. Several representative examples are shown in **Figure 1b**. This observation can be attributed to the inability of AAV2i mutants to bind cell surface heparin sulfate proteoglycans. Based on the rationale that certain AAV serotypes (1, 4, 5 and 6) utilize N- or O-linked sialic acid as a primary receptor, we also determined whether modest transduction efficiencies displayed by AAV2i mutants can be explained by sialic acid binding. However, as shown in **Figure 1b**, treatment of HEK293 cells with sialidase to remove surface-exposed sialic acid groups did not affect transduction efficiency of AAV2i1, 2i4 or 2i5. Transduction efficiencies of parental AAV2, AAV2i7 and 2i8 vectors also remain unaffected. In contrast, transduction by AAV4, which utilizes O-linked sialic acid as a primary receptor decreased by an order of magnitude. Lastly, no significant advantage was noted in the ability of AAV2i mutants to infect heparin sulfate-negative CHOpgsD cells, which are relatively non-permissive to parental AAV2 vectors (**Figure 1c**). Modest transduction levels by AAV2 can possibly be attributed to non-specific interaction with chondroitin sulfate, over-expressed on the surface of CHOpgsD cells.

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Example 3

AAV2i8 Displays a Distinct Phenotype *in vivo*

Despite the low levels of transduction observed *in vitro*, we determined the tissue tropism profiles of AAV2i mutants in normal Balb/C mice using live animal bioluminescence imaging. AAV2i mutants 1, 3, 4, 5, 7, 8 and parental AAV2 vectors packaging the firefly luciferase transgene driven by the cytomegalovirus (CMV) promoter were injected at a dose of $1x10^{10}$ vector genomes per mouse, through intramuscular route into the right hind limb or through intravenous route through the tail vein.

In general, most AAV2i mutants appeared to exhibit low-level transduction based on bioluminescent images obtained 1 week post-administration (**Figures 2a** and **2b**). One notable exception was AAV2i8.

Unlike AAV2, which displays preferential tropism for the murine liver, AAV2i8 demonstrates a systemic transduction profile. AAV2i8 transduces murine hind limb skeletal muscle with moderate efficiency following intramuscular administration (**Figure 2a**). Following intravenous administration, AAV2i8 displayed a systemic transduction profile (**Figure 2b**) regardless of the duration of gene expression or whether the vector was administered via the tail vein or the portal vein (**Figure 3**).

Example 4

AAV2i Mutants with a 585-Q/NXXTXP-590 Motif Display a Systemic Transduction Profile

Following preliminary observations with AAV2i8 vectors *in vivo*, we administered several AAV2i mutants with 585-QXXTXP-590 or 585-NXXTXP-590 motifs as well as parental AAV2 and AAV8 vectors as controls in mice. All vectors packaging the luciferase transgene driven by the chicken beta-actin (CBA) promoter were administered at a dose of $5x10^{10}$ vector genomes per mouse and live animal images obtained 10 days post-administration. As shown in **Figure 4**, AAV2i mutants with residues Q/N585, T588 and P590 appear to display systemic transduction profiles similar to AAV8 vectors. The significantly higher transduction efficiency exhibited by AAV2i8 in comparison to AAV2i10, AAV2i11, AAV2irh.2 and AAV2irh.38 vectors highlights the subtle synergy between residues located within the hexapeptide motif in conferring systemic tissue tropism. In contrast, AAV2 vectors display a preferential tropism for liver as established earlier.

It is noteworthy to mention that the 585-QQNTAP-590 motif was unable to confer systemic tropism when incorporated into the corresponding domain on AAV1 or AAV3 capsids (**Figure 5**).

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Example 5

AAV2i8 is Detargeted from the Liver and Displays Selective Muscle Tropism

Based on relatively similar systemic transduction patterns displayed by the aforementioned mutants following intravenous administration in mice, the lab-derived AAV2i8 strain was chosen as a lead candidate for further characterization. In order to determine the transduction efficiency of AAV2i8 in comparison with parental AAV2 and AAV8 vectors, we quantified luciferase transgene expression and genome copy numbers in cardiac, skeletal muscle and liver tissue lysates at 2 weeks post-administration. As shown in **Figure 6a**, AAV8 vectors ubiquitously transduced muscle and liver tissue with high efficiency corroborating the systemic transduction profile observed earlier in **Figure 4**. Although less efficient than AAV8, AAV2 vectors preferentially transduced liver and only displayed modest transduction levels in muscle tissue. In contrast, AAV2i8 appears to preferentially transduce muscle tissue with high efficiency similar to AAV8 and is simultaneously detargeted from the liver.

The aforementioned luciferase transgene expression profiles are corroborated by biodistribution of vector genome copies in muscle and liver tissues as determined by Q-PCR (**Figure 6b**). In the case of AAV2 and AAV8 vectors, a disproportionately high amount of vector genome copies were recovered from liver tissue in comparison to cardiac or skeletal muscle tissue. The latter observation attests to the preferential liver tropism of AAV2 and AAV8 vectors, although AAV8 also appears to transduce muscle tissue with similar efficiency. In the case of AAV2i8, the lack of sequestration of vector genomes in liver tissue and re-direction to muscle tissue is particularly striking. Low levels of AAV2i8 vector genome copies were recovered from other major organs such as brain, lung and spleen (data not shown).

Notable exceptions to AAV2i mutants containing the 585-Q/NXXTXP-590 motif, include AAV2i7 (585-AANTAA-590) and AAV2irh.36 (585-SSTAGP-590) that also displayed a systemic transduction profile. Despite efficient liver detargeting, AAV2i7 transduces muscle tissue with significantly lower efficiency in comparison to AAV2i8 and AAV8 vectors (data not shown). Interestingly, mutation of 585-AANTAA-590 to 585-QQNTAA-590 (lacking the P590 residue in AAV2i8) restores liver tropism and decreases overall transduction efficiency. On the other hand, while AAV2irh.36 displays moderate systemic transduction efficiency, this vector appears to have retained significant liver tropism (data not shown). Mutation of 585-SSTAGP-590 to 585-PSTAGP-590 in AAV2irh.36 resulted in very poor transduction *in vivo*. The latter

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observations suggest that in general, attenuation of heparin binding can result in liver detargeting and systemic dissemination of AAV2-derived vectors. However, specific domains such as the 585-Q/NXXTXP-590 motif might confer highly efficient systemic transduction.

5

Example 6

AAV2i8 Transduces a Wide Range of Muscle Groups

In order to determine the extent of the global spread of AAV2i8 following intravenous administration, we harvested different muscle groups from Balb/C mice 10 at 4 weeks post-administration. As shown in **Figure 7**, AAV2i8 transduces a wide range of muscle groups in the murine forearms and hind legs as well as intercostal, facial and abdominal muscles. Notably, cardiac and diaphragm muscle are transduced by AAV2i8 with high efficiency, whereas other major organs, such as the brain, lung and spleen, are transduced with low efficiency. These results distinguish 15 the tissue tropism of the AAV2i8 capsid from that of any naturally occurring AAV serotype or isolate that has thus far been characterized (**Figure 8**).

Example 7

AAV2i8 Traverses Blood Vessels with High Efficiency

20 An isolated hind limb perfusion technique was used to examine the efficiency with which AAV2i8 traverses the blood vessel barrier. AAV2i8 transduced hind limb skeletal muscle as efficiently as AAV8 at low volume of injection, at moderate and high vector dosage (**Figures 9a**). At low vector dose, AAV8 displayed three- to tenfold increases in transduction efficiency at higher volumes of injection. However, 25 AAV2i8 traversed blood vessels and transduced underlying skeletal muscle with high efficiency regardless of the volume of injection.

The atypical tropism of AAV2i8 distinguishes it from natural AAV serotypes 8 and 9 and suggests that engineered AAV vectors can be tailored for specific clinical applications. AAV2i8 showed markedly reduced blood clearance and appears to 30 persist well over 48 hours in blood (**Figure 9b**). Moreover, muscle-specific luciferase transgene expression levels increased gradually over the course of several weeks (**Figure 10**). In contrast, AAV8 vector genome copy number rapidly decreased, approaching background levels within the same time period. These results and previous observations that other AAV serotypes with systemic tissue tropism have 35 long circulation half-lives suggest that strategies to manipulate blood circulation time of AAV capsids might afford control over vector tropism.

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Example 8

Insertion/Substitution at Position 265 of the Capsid Protein Restores Liver Tropism to AAV2i8

New vectors were generated in which an aspartic acid or glutamic acid was inserted following amino acid position 264 (numbering with respect to the AAV2 VP1 capsid subunit) of the AAV2i8 vector (2i8D and 2i8E, respectively). Female (**Figure 11**; top) and male (**Figure 11**; bottom) BALB/c mice were injected intravenously via the tail vein with AAV2, AAV8, AAV9, 2i8D or 2i8E vectors (dose 1×10^{11} vg in 200 µl PBS) packaging the CBA-Luc cassette. Live animal bioluminescent imaging was used to evaluate vector tropism and luciferase expression 4 days after injection. The levels of systemic transduction for the 2i8D and 2i8E vectors were similar to those observed for the recombinant AAV8 and AAV9 vectors (**Figure 11**). Further, although there were some gender-dependent expression patterns, liver transduction efficiency was, in general, similar between the 2i8D and 2i8E vectors as compared with the AAV8 and AAV9 vectors (**Figure 11**). Thus, although substitution of the QQNTAP motif at positions 585 through 590 (inclusive) of the AAV2 capsid protein results in detargeting from the liver as compared with AAV2 or AAV8 vectors, the additional insertion of a aspartic acid (D) or glutamic acid (E) following amino acid 264 was able to restore liver tropism to levels similar to those seen with AAV8 or AAV9. Moreover, the 2i8D and 2i8E vectors were also able to maintain wide-spread muscle tropism as seen with vector AAV2i8.

In further experiments, other amino acids are inserted following position 264 in the AAV2i8 vector (*e.g.*, valine, leucine, lysine, arginine, threonine, serine, tyrosine, glycine, alanine, proline, asparagine, phenylalanine, tyrosine or glutamine), and transduction patterns and gene expression are evaluated as described above.

In other studies, the QQNTAP motif is substituted at the positions corresponding to amino acids 585 through 590 (inclusive) of the AAV capsid subunit from other AAV with and without the addition of an amino acid insertion/substitution at position 265 (*e.g.*, aspartic acid, glutamic acid, alanine, leucine, lysine, arginine, threonine, serine, tyrosine, glycine, alanine, proline, asparagine, phenylalanine, tyrosine or glutamine), and transduction patterns and gene expression are evaluated as described above.

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Example 9

Studies in Brain

In a further study, an AAV2 vector having an aspartic acid inserted following position 264 of the capsid protein was generated (AAV2-265D). Rats received AAV2, 2i8, AAV2-265D or 2i8D vector (described in Example 8) expressing a GFP transgene by stereotactic injection into the brain. AAV2-265D demonstrated higher levels of GFP expression as compared with AAV2; however, neither vector exhibited much spread beyond the site of injection (data not shown). In contrast, injection of the 2i8 vector into brain resulted in only low levels of transduction as assessed by GFP expression. Incorporation of the aspartic acid at position 265 in vector 2i8D restored transduction in the brain and also resulted in much more extensive spread from the injection site throughout the brain (*e.g.*, hippocampus and striatum) as compared with equivalent amounts of the AAV2 and AAV2-265D vectors (data not shown). For all vectors, neurons were the primary cell type transduced (data not shown).

In further experiments, other amino acids are inserted following position 264 in the AAV2i8 vector (*e.g.*, glutamic acid, valine, leucine, lysine, arginine, threonine, serine, tyrosine, glycine, alanine, proline, asparagine, phenylalanine, tyrosine or glutamine), and transduction patterns, vector spread, and gene expression in the brain are evaluated as described above.

In other studies, the QQNTAP motif is substituted at the positions corresponding to amino acids 585 through 590 (inclusive) of the AAV capsid subunit from other AAV with and without the addition of an amino acid insertion/substitution at position 265 (*e.g.*, aspartic acid, glutamic acid, alanine, leucine, lysine, arginine, threonine, serine, tyrosine, glycine, alanine, proline, asparagine, phenylalanine, tyrosine or glutamine), and transduction patterns, vector spread, and gene expression in the brain are evaluated as described above.

Example 10

Summary of Characteristics of AAV2i Mutants

AAV2i mutants from this study were unable to bind heparin (**Figure 1a**). The inability to bind heparin results in significant decrease in transduction efficiency *in vitro* as well as *in vivo* following intramuscular as well as intravenous administration in mice **(Figure 2a and 2b)**.

A striking exception in this regard is the AAV2i8 mutant, wherein a systemic transduction profile following intravenous administration is observed (**Figure 2b**).

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Further investigation led to the discovery that AAV2i mutants with a Q/NXXTXP motif demonstrate systemic tropism and superior transduction levels in contrast to parental AAV2 vectors and other AAV2i mutants in mice (Summarized in **Table 5**). Intriguingly, AAV2i8 and related mutants display an atypical transduction profile characterized by a switch in tropism from liver to muscle (**Figures 6a**, **6b**, **8**). Moreover, the latter transduction profile is also distinct from AAV8 and AAV9, which transduce multiple organs following systemic administration.

Example 11

Comparative Analysis of the Surface Map of AAV2i8 with AAV2 and AAV8 Capsids

A comparative analysis of the surface map of AAV2i8 with AAV2 and AAV8 capsids indicates that a unique footprint is generated upon incorporation of 585-QQNTAP-590 domain in the context of the AAV2 capsid template (data not shown). The resulting chimeric capsid surface can facilitate specific interactions with endogenous and/or alternative secondary receptors distinct from those mediated through AAV2-heparin interactions. In addition, the ability to significantly alter capsid surface topology by swapping a linear motif supports this approach for generating capsids with antigenic profiles distinct from parental serotypes (*see* **Tables 6 and 7**).

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

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That which is claimed is:

1. An adeno-associated virus (AAV) capsid protein, wherein the capsid protein comprises a modification resulting in the amino acid sequence

X^1-X^2-X^3-X^4-X^5-X^6 (SEQ ID NO:1)

at the amino acids corresponding to amino acid positions 585 to 590 (VP1 numbering) of the native AAV2 capsid protein,

wherein X^1 is Q, N, S, P, A or G;
wherein X^2 is any amino acid;
wherein X^3 is any amino acid;
wherein X^4 is T, A, G or N;
wherein X^5 is any amino acid; and
wherein X^6 is P or A;
and further wherein X^1-X^2-X^3-X^4-X^5-X^6 (SEQ ID NO:1) is not AGNAQA.

2. The capsid protein of claim 1, wherein X^1 is Q, S, N or A.

3 The capsid protein of claim 1, wherein X^2 is Q, S, N, A or G.

4. The capsid protein of claim 1, wherein X^3 is S, N, T or Q.

5. The capsid protein of claim 1, wherein X^5 is S, N, T or Q.

6. The capsid protein of claim 1, wherein X^6 is P.

7. The capsid protein of claim 1, wherein
X^1 is Q or N;
X^4 is T; and
X^6 is P.

8. The capsid protein of claim 1, wherein
X^2 is Q;
X^3 is N; and/or
X^5 is A.

9. The capsid protein of claim 1, wherein X^1-X^2-X^3-X^4-X^5-X^6 (SEQ ID NO:1) is:

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(a) QQNTAP;
(b) AANTAA;
(c) SSTAGP;
(d) QQNTAA;
(e) PSTAGP;
(f) SSSTDP;
(g) SNSNLP;
(h) SSTTAP;
(i) SAQAQA;
(j) QANTGP;
(k) NATTAP;
(l) NQNTAP;
(m) QAANAP;
(n) SIVGLP;
(o) AASTAA;
(p) SSNTAP;
(q) SSTAGP;
(r) SQNTTA;
(s) QQDTAP;
(t) QTNTGP;
(u) QTNGAP;
(v) QQNAAP;
(w) AANTQA; or
(x) AASTAA.

10. The capsid subunit of claim 1, wherein the capsid protein is an AAV2 capsid protein.

11. An AAV capsid comprising the AAV capsid protein of any one of claims 1 to 10.

12. A virus vector comprising:
(a) the AAV capsid of claim 11; and
(b) a nucleic acid comprising at least one terminal repeat sequence, wherein the nucleic acid is encapsidated by the AAV capsid.

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13. The virus vector of claim 12, wherein the virus vector exhibits systemic tropism for skeletal, cardiac and diaphragm muscle.

14. The virus vector of claim 13, wherein the virus vector exhibits systemic tropism for skeletal muscle.

15. The virus vector of claim 12, wherein the virus vector has reduced tropism for liver.

16. The virus vector of claim 12, wherein the virus vector has selective tropism for skeletal muscle, cardiac muscle and/or diaphragm muscle.

17. A pharmaceutical formulation comprising the virus vector of any of claims 12 to 16 in a pharmaceutically acceptable carrier.

18. A method of administering a nucleic acid to a cell, the method comprising contacting the cell with the virus vector of any of claims 12 to 16 or the pharmaceutical formulation of claim 17.

19. A method of delivering a nucleic acid to a subject, the method comprising administering to the subject the virus vector of any of claims 12 to 16 or the pharmaceutical formulation of claim 17.

20. The method of claim 19, wherein the subject is a human subject.

21. The method of claim 19, wherein the subject has or is at risk for a disorder selected from the group consisting of a muscular dystrophy including Duchenne or Becker muscular dystrophy, hemophilia A, hemophilia B, multiple sclerosis, diabetes mellitus, Gaucher disease, Fabry disease, Pompe disease, cancer, arthritis, muscle wasting, heart disease including congenital heart failure or peripheral artery disease, intimal hyperplasia, a neurological disorder including epilepsy, Huntington's disease, Parkinson's disease or Alzheimer's disease, an autoimmune disease, cystic fibrosis, thalassemia, Hurler's Syndrome, Sly syndrome, Scheie Syndrome, Hurler-Scheie Syndrome, Hunter's Syndrome, Sanfilippo Syndrome A, B, C, D, Morquio Syndrome, Maroteaux-Lamy Syndrome, Krabbe's disease, phenylketonuria, Batten's disease, spinal cerebral ataxia, LDL receptor deficiency, hyperammonemia, anemia, arthritis,

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a retinal degenerative disorder including macular degeneration, adenosine deaminase deficiency, and cancer including tumor-forming cancers.

22. The method of any of claims 19-21, wherein the virus vector or pharmaceutical formulation is administered to skeletal muscle, cardiac muscle and/or diaphragm muscle.

23. The method of any of claims 19-22, wherein the virus vector or pharmaceutical formulation is administered intravenously.

24. The method of claim 22 or claim 23, wherein the subject has or is at risk for muscular dystrophy.

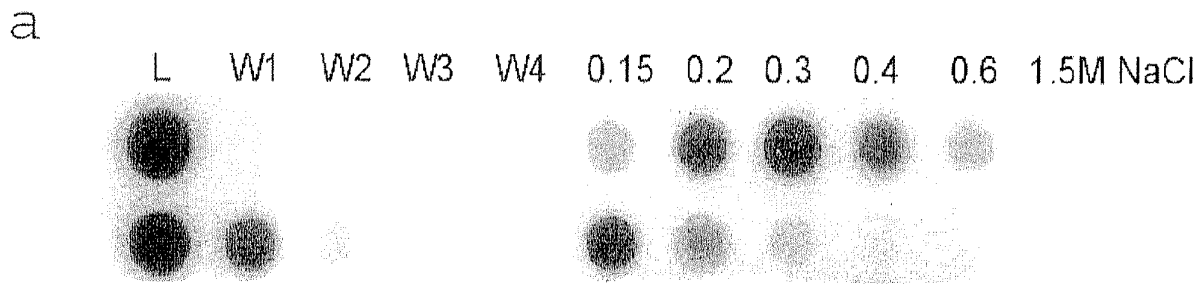
25. The method of claim 22 or claims 23, wherein the subject has or is at risk for heart disease.

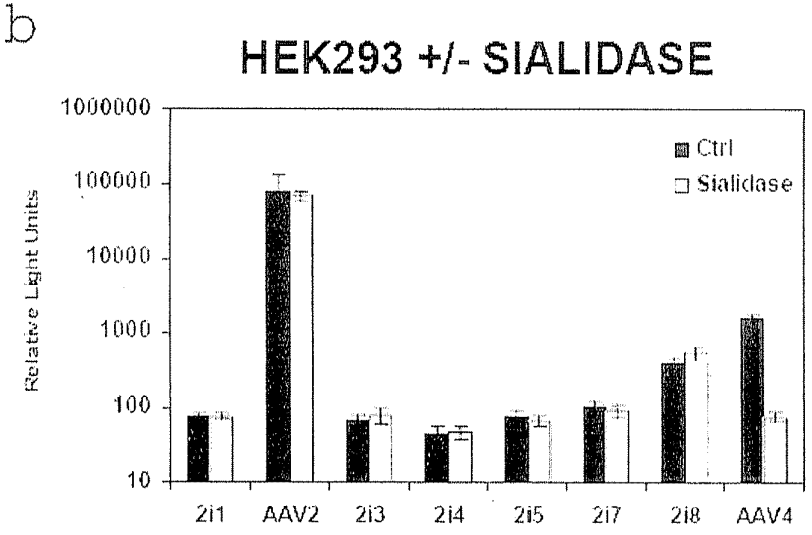
26. The method of claim 25, wherein the subject has or is at risk for congestive heart failure or peripheral artery disease.

27. The method of claim 22 or claim 23, wherein the subject has or is at risk for a metabolic disorder.

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Figure 1


a
L
W1
W2
W3
W4
0.15
0.2
0.3
0.4
0.6
1.5M NaCl


b
HEK293 +/- SIALIDASE
Relative Light Units
1000000
100000
10000
1000
100
10
Ctrl
Sialidase
2i1
AAV2
2i3
2i4
2i5
2i7
2i8
AAV4

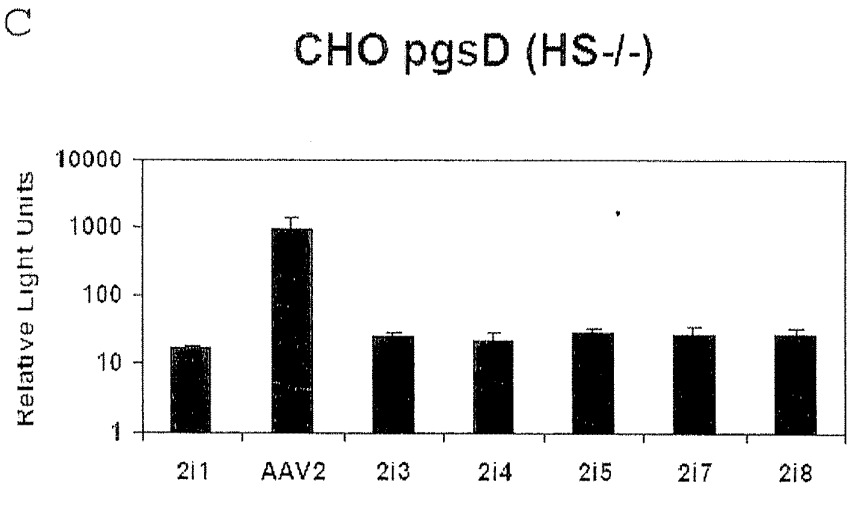
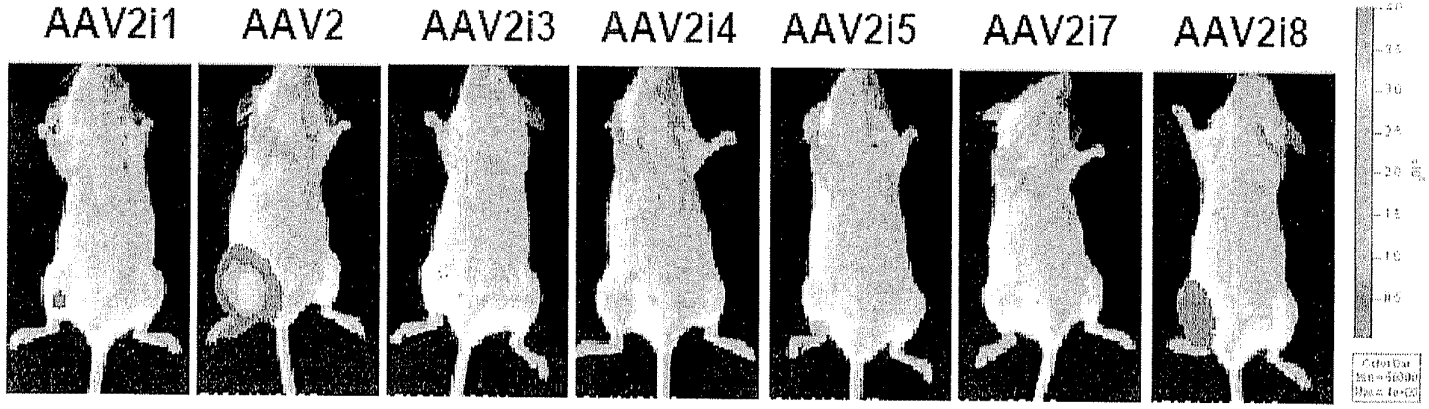

c
CHO pgsD (HS-/-)
Relative Light Units
10000
1000
100
10
1
2i1
AAV2
2i3
2i4
2i5
2i7
2i8

Figure 2

a


Route of Admn: Intramuscular
AAV2i1
AAV2
AAV2i3
AAV2i4
AAV2i5
AAV2i7
AAV2i8

b

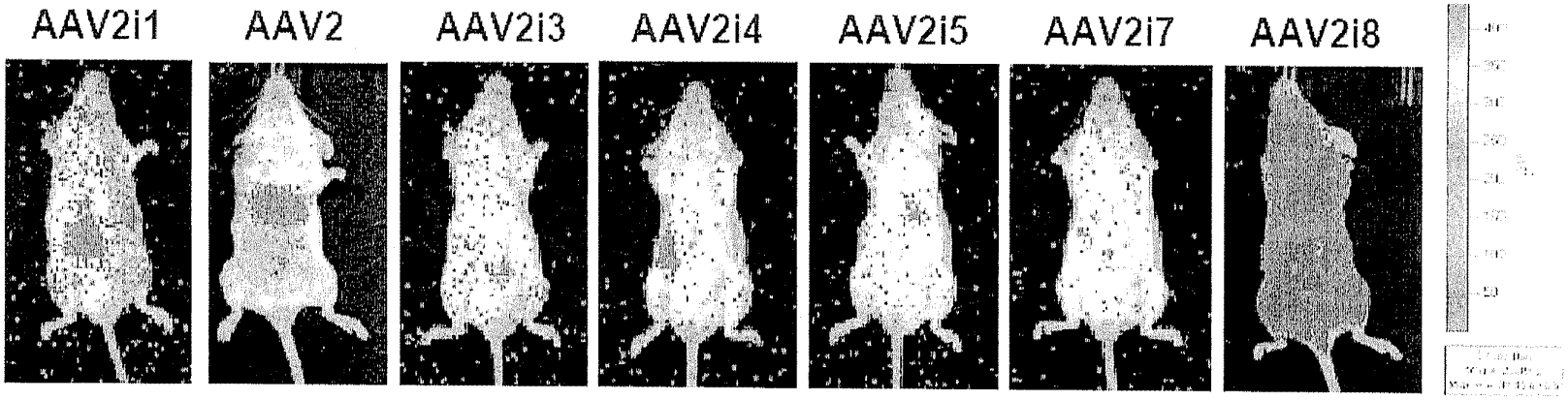

Route of Admn: Intravenous
AAV2i1
AAV2
AAV2i3
AAV2i4
AAV2i5
AAV2i7
AAV2i8

Figure 3

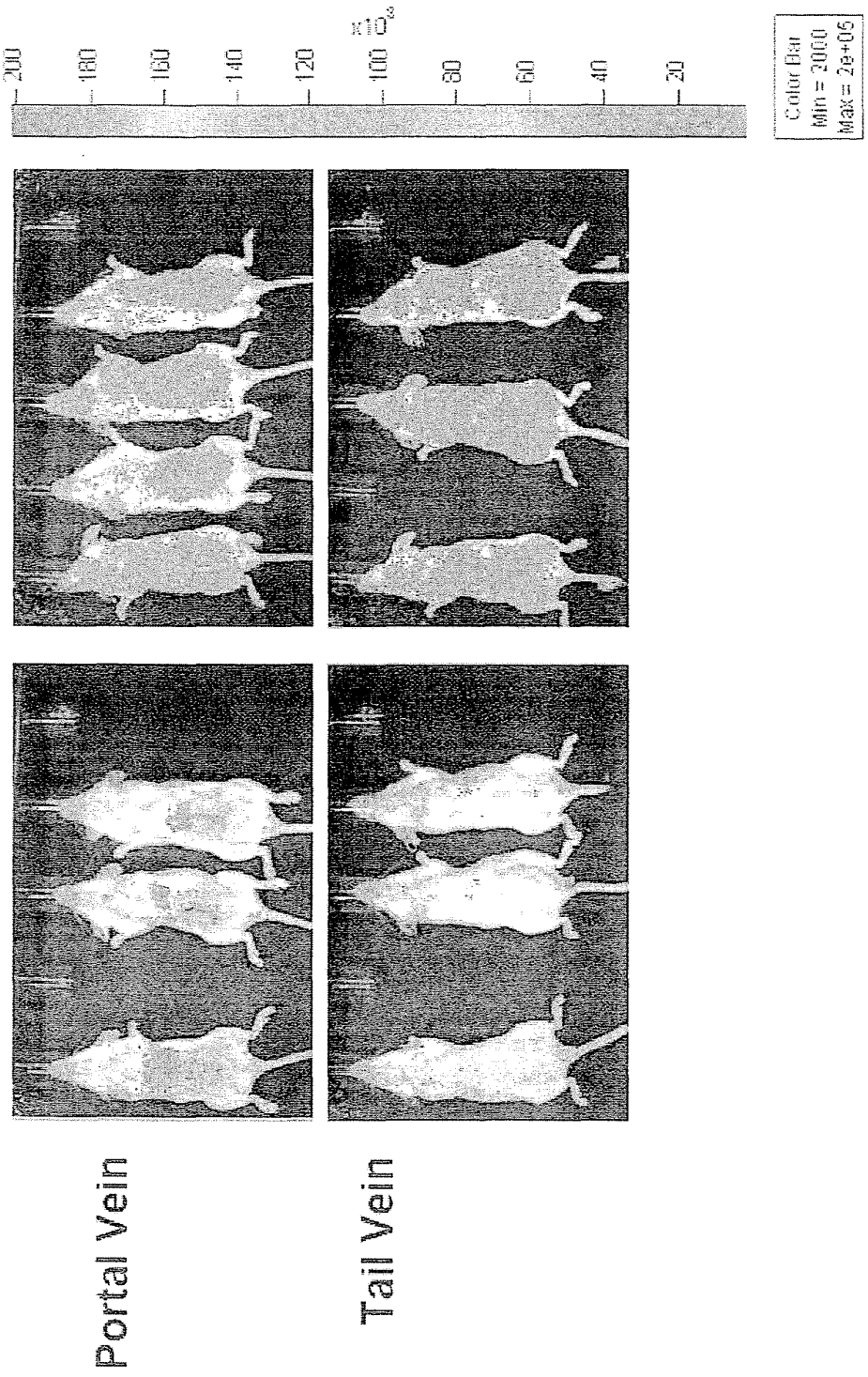

AAV2
AAV2i8
Portal Vein
Tail Vein
200
180
160
140
120
100
80
60
40
20
Color Bar
Min = 2000
Max = 2e+06

Figure 4

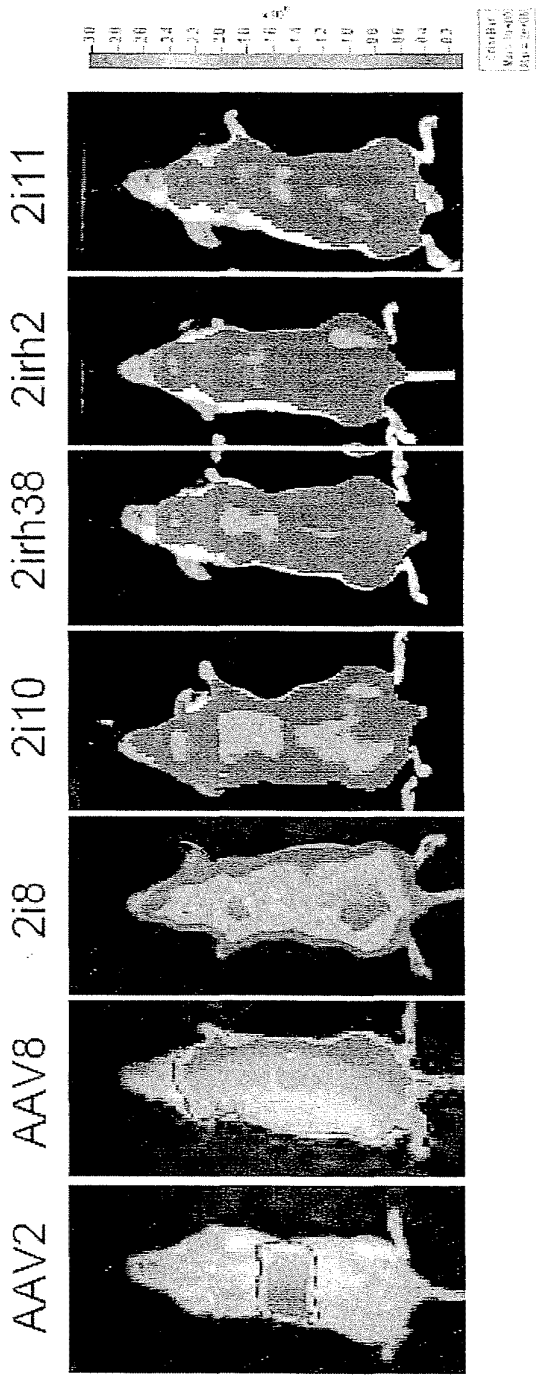


Figure 5

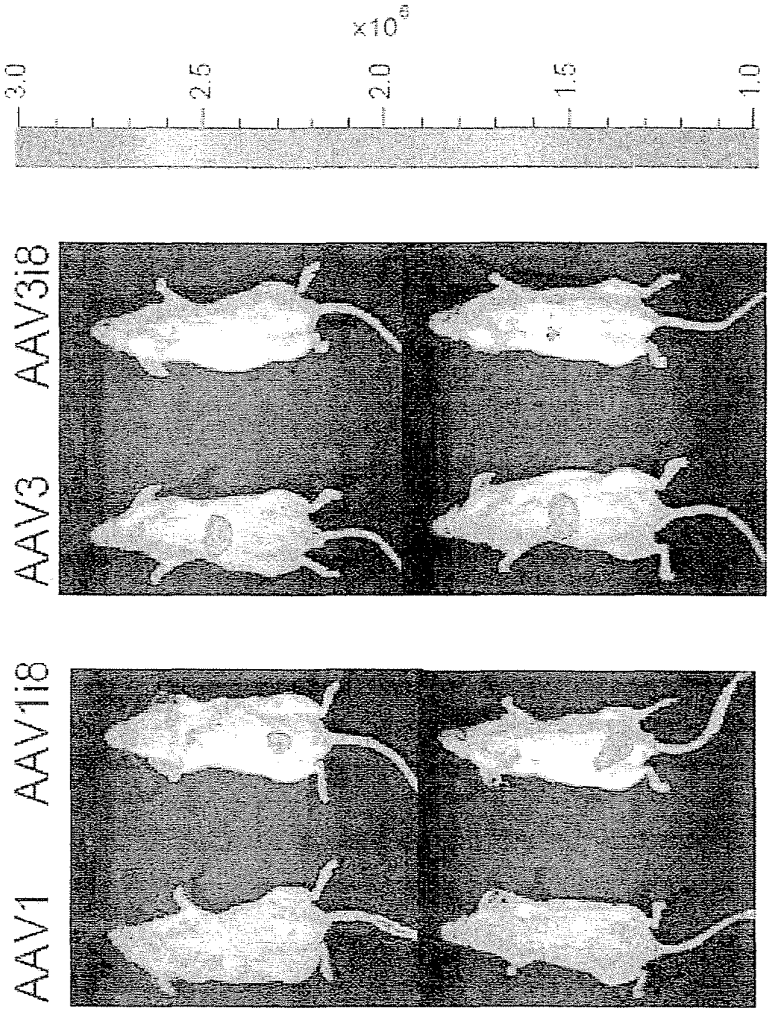


Figure 6a

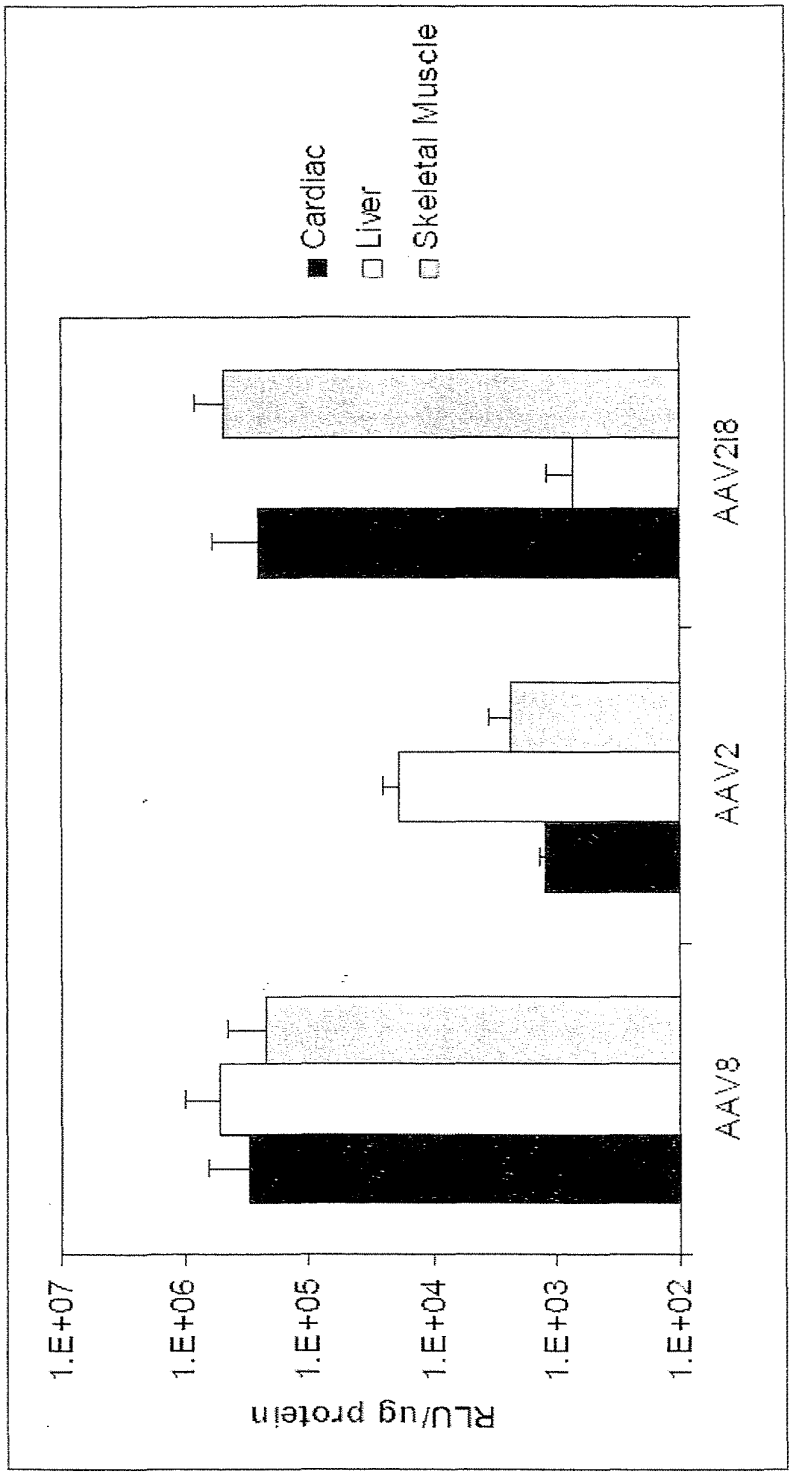

Cardiac
Liver
Skeletal Muscle
RLU/ug protein
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
AAV8
AAV2
AAV2i8

Figure 6b

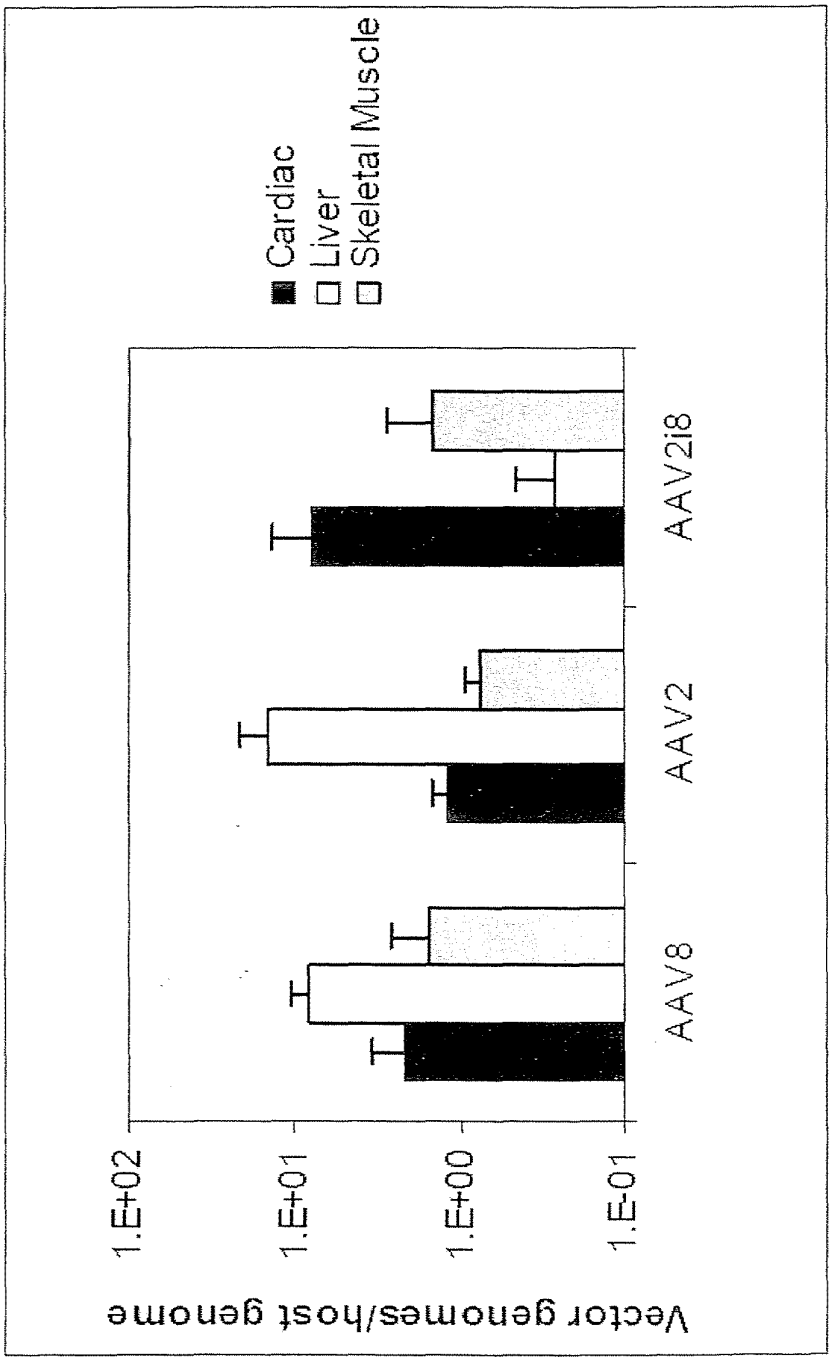

Vector genomes/host genome
1.E+02
1.E+01
1.E+00
1.E-01
AAV8
AAV2
AAV2i8
Cardiac
Liver
Skeletal Muscle

Figure 7

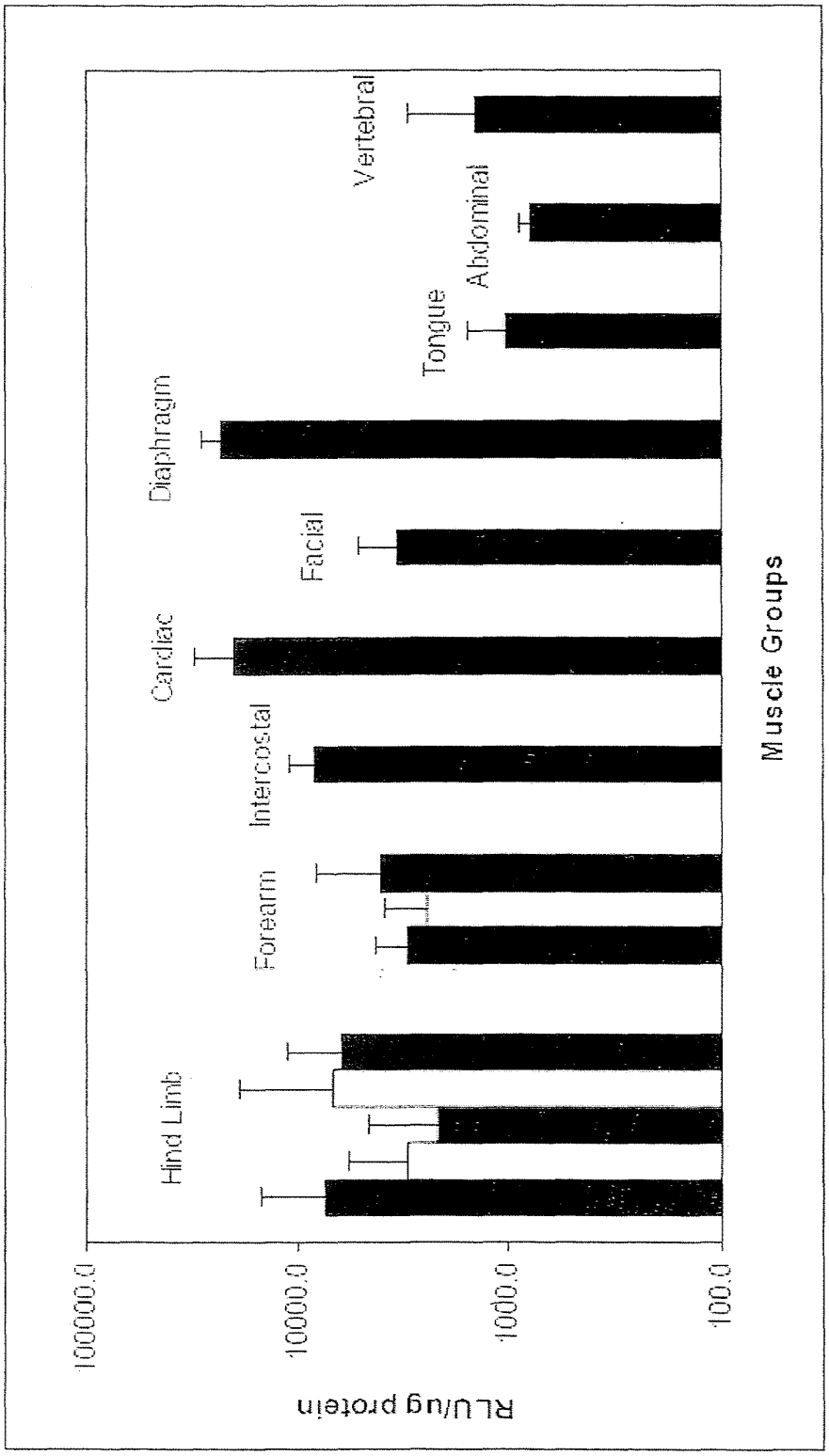

RLU/ug protein
100000.0
10000.0
1000.0
100.0
Hind Limb
Forearm
Intercostal
Cardiac
Diaphragm
Facial
Tongue
Abdominal
Vertebral
Muscle Groups

Figure 8

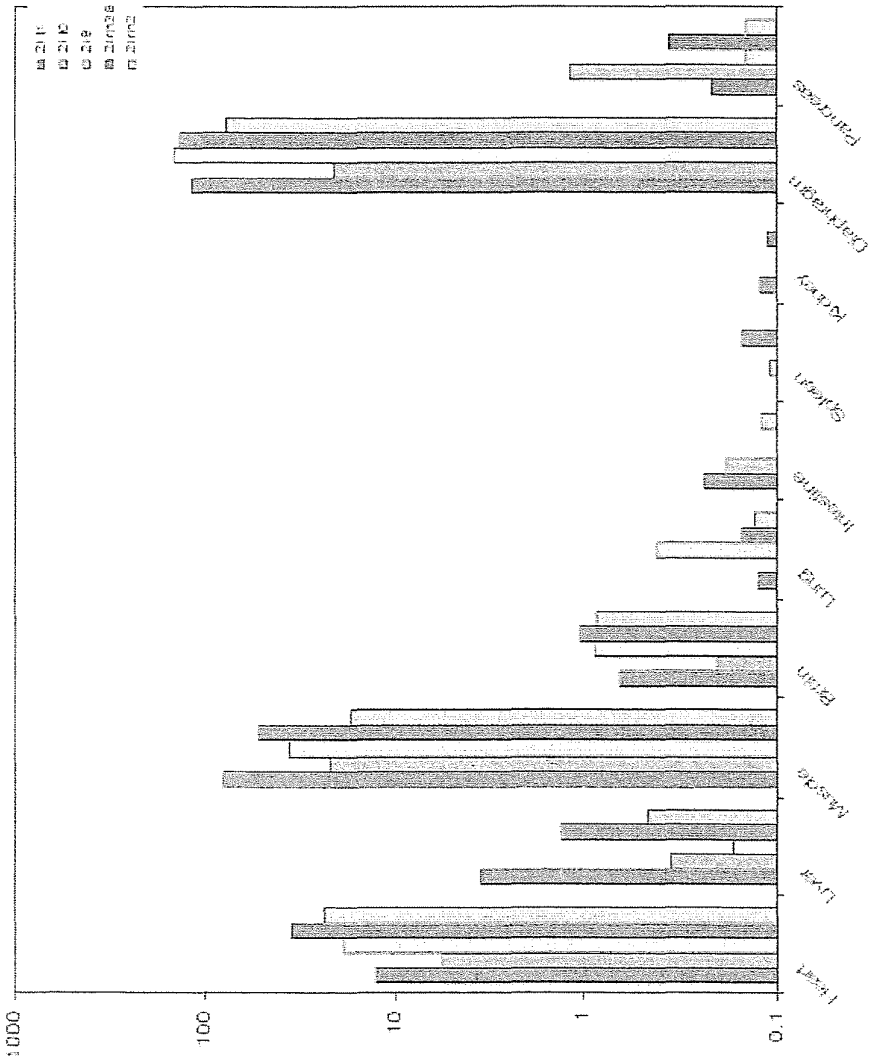

1000
100
10
1
0.1
Lung
Liver
Muscle
Brain
Lung
Muscle
Heart
Spleen
Kidney
Gastrocn
Pancreas

Figure 9

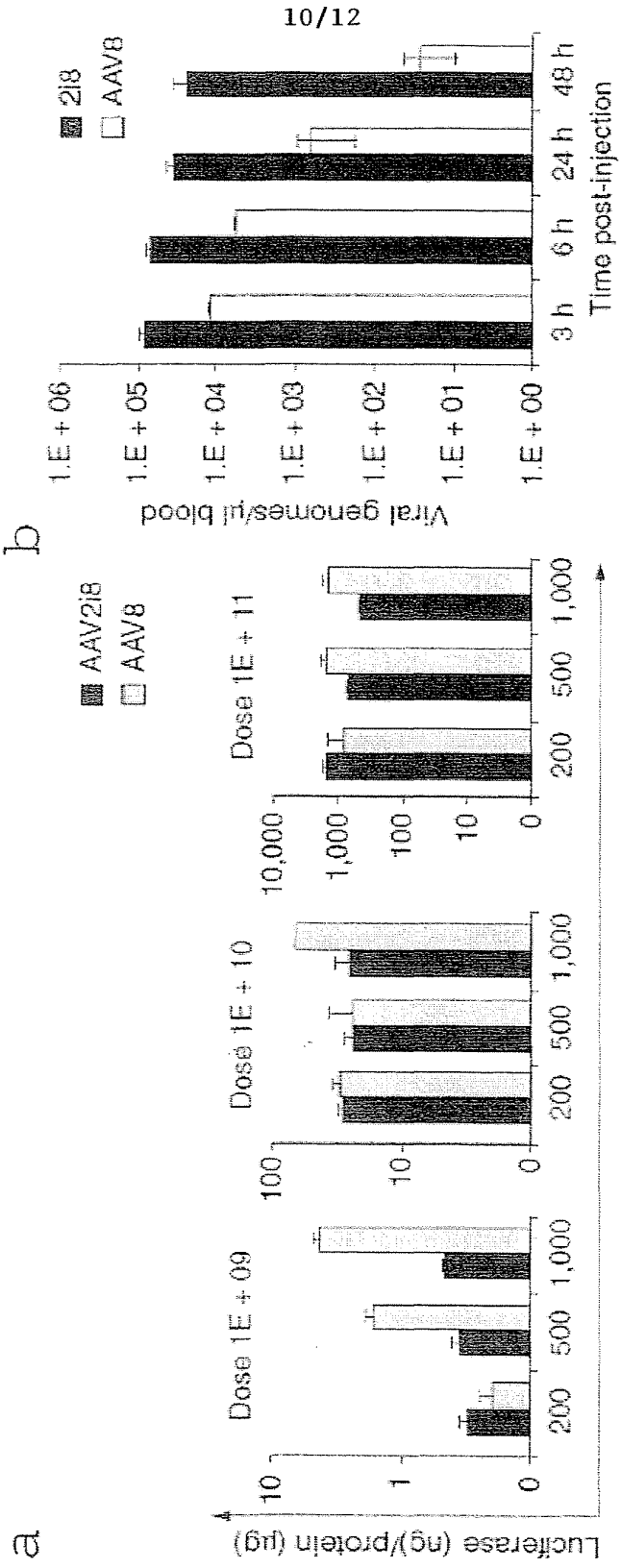

a
Luciferase (ng)/protein (µg)
AAV2i8
AAV8
Dose 1E + 09
Dose 1E + 10
Dose 1E + 11
0
200
500
1,000
10
1
100
10,000
1,000
b
Viral genomes/µl blood
1.E + 06
1.E + 05
1.E + 04
1.E + 03
1.E + 02
1.E + 01
1.E + 00
3 h
6 h
24 h
48 h
Time post-injection

Figure 10

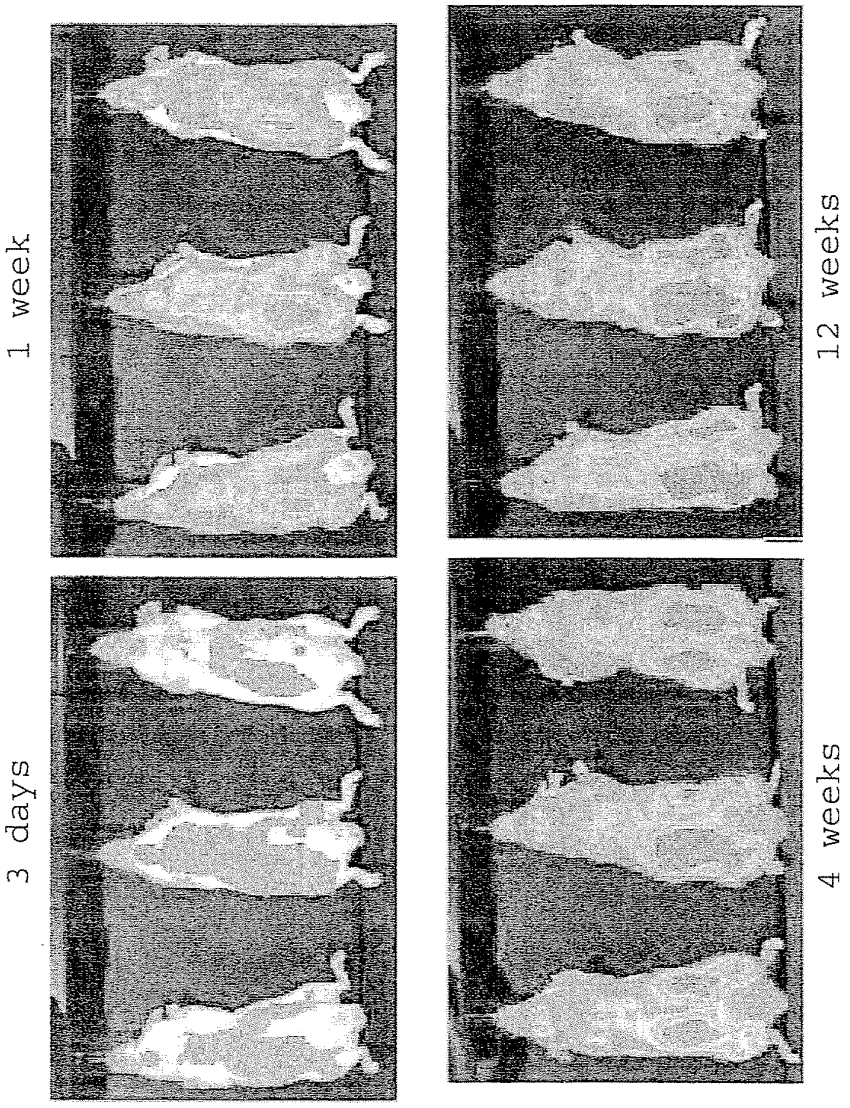
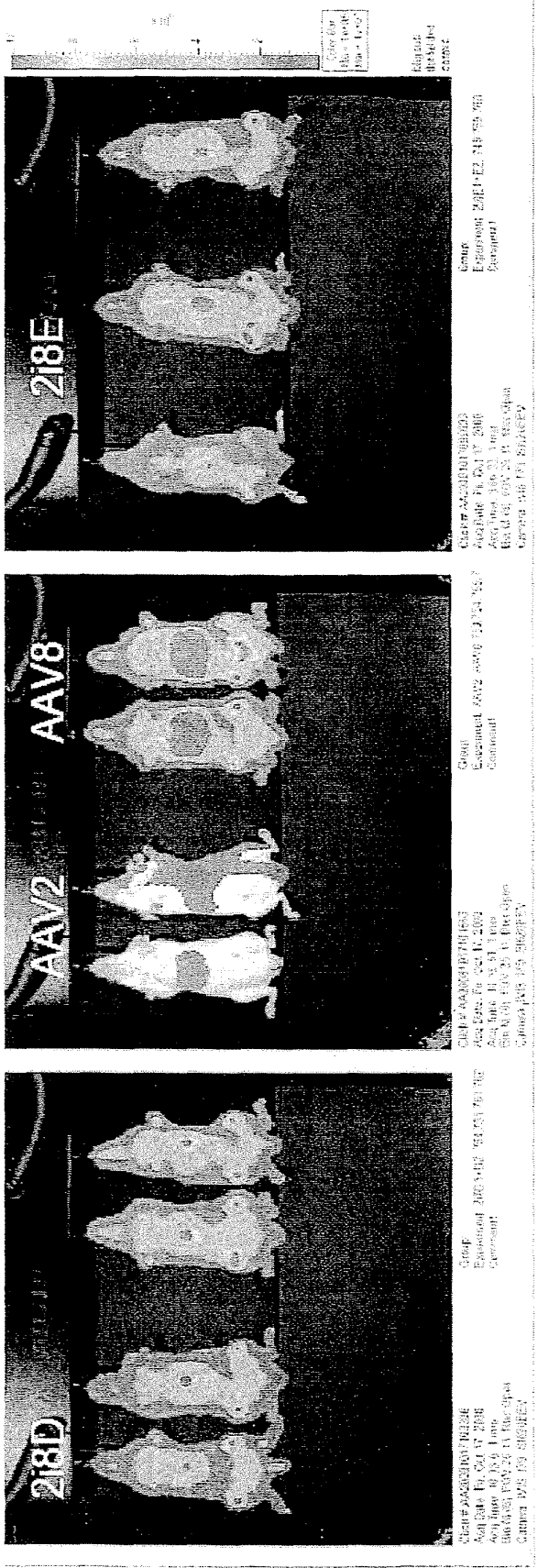
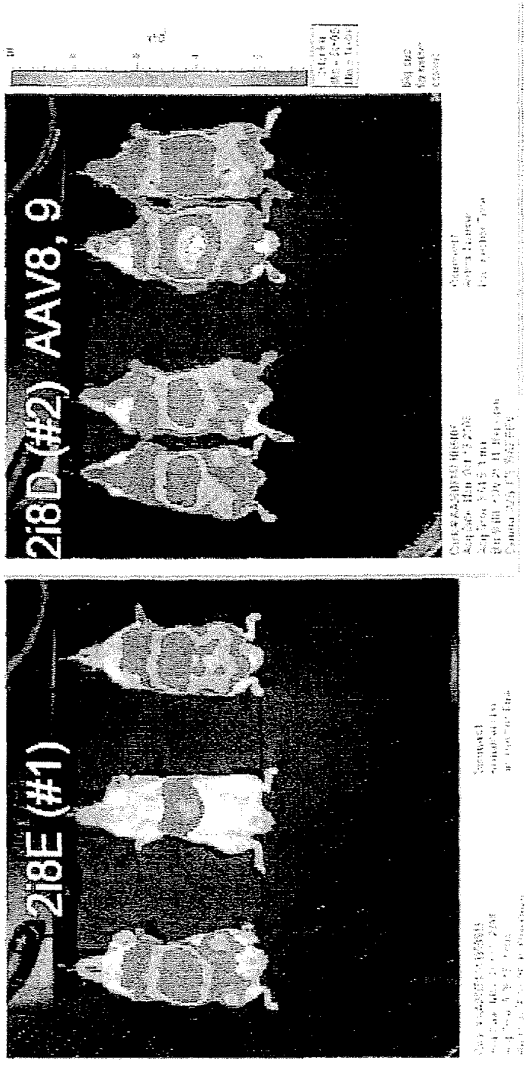


Figure 11





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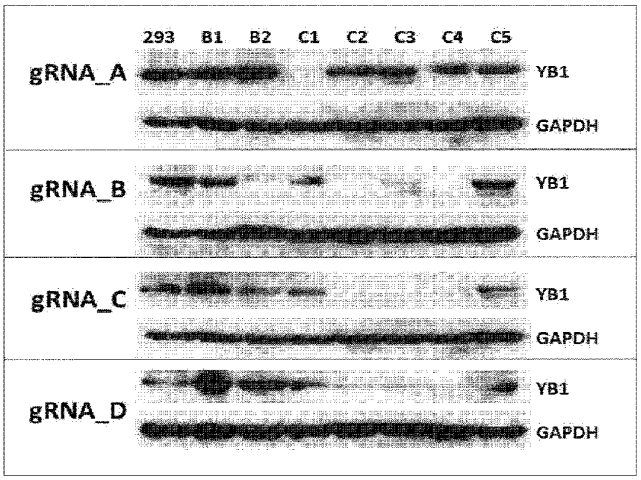
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(54) **Title**: HIGH TITER PRODUCTION OF ADENO-ASSOCIATED VIRAL VECTORS

Figure 12



(57) **Abstract**: This invention relates to adeno-associated viral (AVV) vectors, to producer cell lines for the production of AAV vectors and to methods of producing such vectors. More specifically, the invention relates to producer cell lines adapted to increase the titre of said vectors and methods of producing AAV vectors using said producer cell lines.

HIGH TITER PRODUCTION OF ADENO-ASSOCIATED VIRAL VECTORS

FIELD OF THE INVENTION

This invention relates to adeno-associated viral (AVV) vectors, to producer cell lines for the production of AAV vectors and to methods of producing such vectors. More specifically, the invention relates to producer cell lines adapted to increase the titre of said vectors and methods of producing AAV vectors using said producer cell lines.

BACKGROUND OF THE INVENTION

Adeno-associated viral (AAV) vectors have excellent safety profiles because wild type AAV has never been associated with any human disease. Thus, AAV are popular and successful vectors for gene therapies. AAV vectors have been extensively studied in clinical trials for many different conditions, including haemophilia B, heart disease and congenital blindness. In addition, the first EU licenced gene therapy drug, Glybera, is based on AAV vector for the treatment of familial lipoprotein lipase deficiency (LPLD), exemplifying the potential of AAV vectors in gene therapy.

Although many advances have been made in AAV vector design, barriers such as a pre-existing immune response have necessitated the administration of high titre AAV and, in many cases, a combined administration of an immune-suppressant to achieve clinical efficacy. This presents a significant challenge in AAV production and has considerable safety implications in the clinical use of AAV vectors.

AAV vectors are most commonly produced by a transient co-transfection of AAV plasmids and a helper plasmid derived from another virus, such as an adenovirus. Significant progress has recently been made in large scale production and robust purification of AAV to support clinical development. However, production of high titre AAV is still a significant challenge, requiring patients to receive repeated administrations of a vector to achieve the desired dosage. For example, for Glybera, it is required to administer the vector at a dose of $3x10^{12}$ vg /kg via 40 or 60 multiple injections.

Furthermore, as a result of current purification methods, AAV products typically contain high levels of protein aggregates or incompletely packaged empty capsids that lack vector DNA. The empty capsids in final products can often be as high as 40 fold over the level of complete particles. These impurities can trigger unwanted immune responses in patients. For example, recent studies have shown that cellular immune responses in mice and in human are directed to epitopes in the

AAV2 capsids, and the presence of empty capsids inhibits hepatocyte transduction in vivo following high dose vector administration. The potential adverse effects of the unwanted immunogenicity of empty capsids compromise product safety and efficacy.

Removal of empty capsids that have no therapeutic function by known methods is difficult due to the innate similarity of their particle size, affinity and protein composition to the complete particles containing vector DNA. There have been continuous efforts to separate empty AAV capsids from genome containing complete particles. Empty particle-free AAV2 has been achieved by differential $CHCl_3$. However, there may be problems using this method in scale up production and GMP manufacture. Ion exchange chromatography has also been reported for the separation of empty capsids in AAV2, 4, 5 and 8. However, from 20% up to 30 fold empty capsids remained in the final products.

Therefore, there is need to develop new methods of AAV vector production which decrease or eliminate the presence of empty capsids in the final product. This would improve the safety and efficacy of AAV products. Reduction of empty particles would also overcome the hurdle in high titre production.

SUMMARY OF THE INVENTION

Cellular and viral factors that contribute to the formation of two types of particles in AAV vector production are still largely unknown. In general, viruses package their genomes into protein capsids either via association of structural proteins with the viral genome or via insertion of viral genomes into preassembled capsids. AAVs are known to package their genome into preassembled capsids. It has been reported that specific amino acid interactions are required for efficient insertion of viral DNA and single amino acid mutations and gross conformation change of AAV capsid proteins have been shown to result in deficiency of packaging AAV genomes. There have also been very limited studies on the involvement of cellular proteins in AAV assembly, indicating that total producer cell proteins and DNA helicase are necessitated in AAV assembly. However, the actual mechanism by which AAV DNA is inserted into capsids is unknown.

In order to better understand the role of cellular proteins in AAV assembly and, ultimately improve the safety and quality of AAV products, the present inventors have analysed host cellular proteins co-produced and co-purified in AAV vectors. In particular, the present inventors have conducted the first systematic analysis and comparison of the protein composition between empty capsids and complete vectors in three AAV serotypes: AAV2; AAV5; and AAV8. The inventors have

demonstrated intrinsic similarity among the three AAV serotypes, although there are some notable differences between the different serotypes. Importantly, the inventors demonstrated for the first time that there are significant differences between empty capsids and complete vectors. Finally, the inventors have identified a number of host cell proteins that are inherently associated with AAV products. In particular, the inventors have demonstrated that host cell proteins YB1, NPM1 and NCL are found in AAV products, and that modulating the expression of these host cell proteins impacts the production of AAV vector particles.

Accordingly, the present invention provides a transgenic producer cell line in which the expression of at least one of YB1, NPM1 and NCL is modulated compared with a control producer cell line. Typically, the expression of: (i) NPM1 and NCL; (ii) YB1 and NPM1; (iii) YB1 and NCL; or (iv) YB1, NPM1 and NCL; is modulated in the producer cell line of the invention compared with a control producer cell line.

In a preferred embodiment, the expression of at least one of YB1, NPM1 and NCL is reduced compared with a control producer cell line, wherein the expression of YB1, NPM1 and/or NCL may be reduced using CRISPR genome editing, a double stranded RNA (dsRNA), a small interfering RNA (siRNA), a small hairpin RNA (shRNA), a micro RNA or an antisense RNA. Typically the expression of YB1, NPM1 and/or NCL is reduced using a shRNA, and preferably: YB1 expression is reduced using a shRNA comprising the nucleotide sequence SEQ ID NOs: 14 to 18 (Y1 to Y5); NPM1 expression is reduced using a shRNA comprising the nucleotide sequence SEQ ID NOs: 4 to 8 (NPM-N6 to NPM-N10); and/or NCL expression is reduced using a shRNA comprising the nucleotide sequence SEQ ID NOs: 9 to 13 (NCL-N1 to NCL-N5). The expression of YB1, NPM1 and/or NCL may be reduced using CRISPR genome editing. In one embodiment, the expression of YB1 is reduced using a gRNA pair selected from SEQ ID NOs: 33 and 34; SEQ ID NOs: 35 and 36; SEQ ID NOs: 37 and 38; and/or SEQ ID NOs: 39 and 40.

The expression of one or more additional genes and/or proteins listed in Table 2 herein may also be modulated in a producer cell line of the invention compared with a control producer cell line.

In a preferred embodiment, the transgenic producer cell line of the invention is a human embryonic kidney 293T cell line.

The invention also provides a method for producing an adeno-associated viral (AAV) vector comprising culturing an adeno-associated virus in a producer cell line in which the expression of at least one of YB1, NPM1 and NCL is modulated.

Typically the expression of: (i) NPM1 and NCL; (ii) YB1 and NPM1; (iii) YB1 and NCL; or (iv) YB1, NPM1 and NCL; in the producer cell line is modulated according to a method of the present invention. Preferably the modulation of YB1, NPM1 and/or NCL expression is a reduction in the expression of YB1, NPM1 and/or NCL, wherein the expression of YB1, NPM1 and/or NCL is reduced using CRISPR genome editing, a double stranded RNA (dsRNA), a small interfering RNA (siRNA), a small hairpin RNA (shRNA),a micro RNA or an antisense RNA. Typically the expression of YB1, NPM1 and/or NCL is reduced using a shRNA, and preferably: YB1 expression is reduced using a shRNA comprising the nucleotide sequence SEQ ID NOs: 14 to 18 (Y1 to Y5); NPM1 expression is reduced using a shRNA comprising the nucleotide sequence SEQ ID NOs: 4 to 8 (NPM-N6 to NPM-N10); and/or NCL expression is reduced using a shRNA comprising the nucleotide sequence SEQ ID NOs: 9 to 13 (NCL-N1 to NCL-N5). The expression of YB1, NPM1 and/or NCL may be reduced using CRISPR genome editing. In one embodiment, the expression of YB1 is reduced using a gRNA pair selected from SEQ ID NOs: 33 and 34; SEQ ID NOs: 35 and 36; SEQ ID NOs: 37 and 38; and/or SEQ ID NOs: 39 and 40.

The expression of one or more of the additional genes and/or proteins listed in Table 2 may also be modulated in the producer cell line used in a method of the present invention.

The method of the invention may increase the titre of AAV vector at least 2 fold compared with the titre of AAV vector produced by a control method, and/or the method of the invention may increase the ratio of complete: empty AAV vector by at least 20% compared with the ratio of complete: empty AAV vector produced by a control method.

In a preferred embodiment, the transgenic producer cell line used in a method of the invention is a human embryonic kidney 293T cell line.

Preferably, the method of the invention produces AAV2, AAV5 and/or AAV8 serotype AAV vectors.

The invention further provides a population of adeno-associated viral (AAV) vectors obtainable from a producer cell line of the invention and/or a method of the invention. Preferably, the ratio of complete: empty AAV vectors in the population of the invention is increased by at least 20% compared the ratio of complete: empty AAV vector in a population produced by a control method. Preferably the AAV vector population of the invention comprises AAV2, AAV5 and/or AAV8 serotype vectors.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Protein profile of three serotypes of AAV vectors before and after AVB Sepharose affinity chromatography. (a) A typical chromatography profile, showing the separation of AAV from unbounded cellular proteins; (b) protein profile of silver staining SDS-PAGE showing the purity of AAs vectors before (crude) and after (purified) chromatography. E: empty capsids, G: complete vectors carrying a reporter gene GFP, and AAV capsid proteins VP1, VP2 and VP3.

Figure 2. Immunoblotting analysis of cellular proteins showing the association of (a) Annexin A5, (b) CypA and RuvB2; (c) YB1 with AAV vectors before (crude) and after (purified) affinity chromatography. E: empty capsids, G: complete AAV carrying GFP and control 293T cell lysate; (d) association of YB1 with AAV vectors, using proteinase K to remove trace unincorporated cellular proteins that remained in purified vectors and were not protected by vector capsid.

Figure 3. Chronological analysis of protein expression in producer cells. (a) AAV proteins (VP1, VP2 and VP3) were detectable 24 hours after transfection with a slight increase from 24 to 72 hours after transfection; (b) Rep proteins were detectable 24 hours after transfection; (c) the expression of YB1 and CypA remains unchanged throughout a complete production cycle and; (d) overtime increase in Annexin A5 expression were observed in all three serotypes of AAV. Controls: 293T cell lysate without the transfection of any plasmids (293T), 293T cells with the transfection of pcDNA3 plasmids (Mock), and housekeeping gene GAPDH as a loading control.

Figure 4. Immunoblotting analysis of shRNA sequences targeting Annexin A5 and YB1. Significant gene knockdown was observed in producer cells carrying shRNA A2 or A5 and shRNA Y3, Y4 or Y5 sequences targeting (a) Annexin A5 and (b) YB1, respectively. 293T cells with a shRNA sequence targeting non-mammalian genes (Scramble) and without any shRNA sequences (293T) were used as controls. Housekeeping gene GAPDH was further used as a loading control.

Figure 5. Vector genome titres of AAV produced in YB1 gene knockdown cells relative to control shRNA scramble, showing (a) up to 50 fold increase in AAV2 and (b) 10 fold increase in AAV8 from 7 batches of gene knockdown producer cells in culture over 80 days.

Figure 6. Sustained down-regulation of YB1 in 6 batches of gene knockdown cells after cryo-preservation and in culture for up to 80 days. 293T cells with a shRNA sequence targeting non-

mammalian genes (Scramble) and without any shRNA sequences (293T) were used as controls. Housekeeping gene GAPDH was further used as a loading control.

Figure 7. Correlation of YB1 down-regulation and AAV production to the amount of YB1_shRNA viruses used, showing (a) a slight increase in YB1 deregulation when increasing shRNA viruses and (b) relative AAV2 genome titres to controls scramble and native 293T cells and, the maximum effect on vector genome titres in the producer cells treated with 50ml YB1-shRNA viruses. Control samples include 293T cells with a shRNA sequence targeting non-mammalian genes (Scramble), with LV empty particles but without a shRNA sequence (Mock) and native 293T cells without any LV and shRNA sequences (293T). Housekeeping gene GAPDH was further used as a loading control.

Figure 8. Immunoblotting analysis of shRNA sequences targeting NPM1. (A) Significant gene knockdown was observed in producer cells carrying shRNA N6 and N9, (B) down-regulation of NPM1 sustained for over 80 days and (C) after being recovered from cryopreservation. 293T cells with a shRNA sequence targeting non-mammalian genes (Scramble) and without any shRNA sequences (293T) were used as controls. Housekeeping gene GAPDH was further used as a loading control.

Figure 9. Immunoblotting analysis of shRNA sequences targeting NCL. (A) Significant gene knockdown was observed in producer cells carrying shRNA N4, (B) down-regulation of NCL sustained for over 80 days after in culture. 293T cells with a shRNA sequence targeting non-mammalian genes (Scramble) and without any shRNA sequences (293T) were used as controls. Housekeeping gene GAPDH was further used as a loading control.

Figure 10. Vector genome titres of AAV produced in NPM1 gene knockdown cells relative to control shRNA scramble, showing (a) up to 35 fold increase in AAV2 and (b) 30 fold increase in AAV8.

Figure 11. Vector genome titres of AAV produced in NCL gene knockdown cells relative to control shRNA scramble, showing a significant variation, ranging from 2-40 fold increase in AAV2 genome titres (A), and lesser up-regulating effects on vector titres (up to 10 fold increase in AAV5 (B) and AAV8 genome titres (C).

Figure 12. Immunoblotting analysis of YB1 gene knockout using CRISPR gRNA sequences. Two batches (B1 and B2) of CRISPR gRNA knockout cells were produced for each of the 4 CRISPR gRNA sequences A, B, C or D. Single cell clones were further derived from the parental B1 and B2

gRNA producer cells. YB1 gene knockout was observed in a total of 10 single cell clones. Parental 293T cells without any gRNA sequences (293T) were used as a control; housekeeping gene GAPDH was further used as a loading control.

Figure 13. Molecular analysis of YB1 knockdown cells for rep and cap expression and vector DNA production: (A) immunoblotting showing 12- and 4-fold increases in AAV2-Rep expression in YB1 knockdown cells 24 and 72 hours (H) after transient transfection, respectively; (B) ELISA showing a 7-fold decrease in cap expression in YB1 knockdown cell lysates compared to scramble cells and a comparable amount of Cap proteins detected in vectors harvested from YB1 and scramble cells and (C) real-time quantitative polymerase chain reaction (qPCR) showing 13 x increase in total vector DNA,8 x increase in unpackaged vector DNA, and 4 x increase in packaged vector DNA in YB1 knockdown cells compared to that in scramble cells. Y:S ratio between YB1 and scramble after being normalized by housekeeping gene GAPDH for immunoblotting or by cell numbers for qPCR analysis after being normalized by housekeeping gene actin. + PK, with proteinase K treatment; -PK, without proteinase K treatment; + DNaseI, with DNAse I treatment; S, control scramble; Y, YB1 knockdown cells.

Figure 14. Homo sapiens Y box binding protein 1 (YB1/YBX1) mRNA (SEQ ID NO: 3) and amino acid (SEQ ID NO: 29) sequences.

Figure 15. Homo sapiens nucleophosmin (nucleolar phosphoprotein B23, numatrin, NPM1) (cDNA clone MGC:22724 IMAGE:4081364), complete cds, mRNA (SEQ ID NO: 1) and amino acid (SEQ ID NO: 27) sequences.

Figure 16. Human nucleolin gene, complete cds, mRNA (SEQ ID NO: 2) and amino acid (SEQ ID NO: 28) sequences.

DETAILLED DESCRIPTION OF THE INVENTION

Adeno-Associated Virus Vectors

Adeno-associated viruses (AAV) are a family of small viruses which infect humans and some other primate species. AAV belong to the genus *Dependovirus*, of the family *Parvoviridae*. AAV are small (approximately 20nm), non-enveloped, replication-deficient viruses. AAV possess a single-

stranded linear DNA genomes approximately 4.7 kilobases (kb) in length that may be either positive or negative-sensed.

The AAV genome comprises inverted terminal repeats (ITRs) at both ends of the DNA strand, and two open reading frames (ORFs): *rep* and *cap*. The former is composed of four overlapping genes encoding Rep proteins required for the AAV life cycle, and the latter contains overlapping nucleotide sequences of capsid proteins: VP1, VP2 and VP3, which interact together to form a capsid of an icosahedral symmetry.

The genes for the VP1, VP2 and VP3 capsid proteins are generally controlled by a single promoter, designated p40, and all three are translated from a single mRNA. The molecular weights of VP1, VP2 and VP3 are typically about 87, 72 and 62 kiloDaltons (kDa) respectively. The AAV capsid proteins are typically encoded on the AAV DNA genome.

Despite the high seroprevalence of AAV in the human population (approximately 80% of humans are seropositive for AAV2) the virus has not been linked to any human illness. AAV vectors can infect both dividing and quiescent cells. The virus may persist in an extrachromosomal state without integrating into the genome of the host cell. Alternatively, the virus may stably integrate into the host cell genome at a specific site in human chromosome 19 (AAVS1). In contrast to adenoviruses, AAV usually does not trigger an immune response to cells infected with it, and thus can deliver genes to sites of interest, including the brain in the context of gene therapy for diseases of muscle and eye. These features make AAV a very attractive candidate for creating viral vectors for gene therapy, and for developing disease models.

During the process of AAV assembly and production, AAV vectors acquire, inherently and submissively, various cellular proteins, but the identity of these proteins has previously been poorly characterised. The present inventors have, for the first time, identified and characterised host proteins inherently associated with AAV vectors, with the aim of improving the production of AAV vectors for gene therapy. In particular, the present inventors have investigated three serotypes of recombinant AAV, namely AAV2, AAV5 and AAV8 and have demonstrated an important role for NPM1, NCL and YB1 in AAV vector production. Using liquid chromatography-mass spectrometry (LC/MS/MS), the inventors have identified 66 AAV-associated human cellular proteins including NPM1, NCL and YB1. Introducing shRNA sequences for NPM1, NCL and/or YB1 targets and down-regulates the respective genes and increases AAV titres.

As described herein, the present invention provides a new production system for the production of AAV vectors which increase the viral titre produced. The production systems of the

invention may also increase the ratio of complete: empty viral vector particles produced. The production systems of the invention comprise producer cell lines adapted for the production of AAV vectors, as well as methods of producing AAV vectors using said producer cell lines.

The present invention also provides AAV vectors produced by said production systems, producer cell lines and methods. Typically, the AAV vectors of the present invention differ from AAV vectors produced by standard methods known in the art, because the AAV vectors of the present invention have a higher titre, improved complete: empty AAV vector ratio and/or a modulated level of cellular proteins, particularly proteins from the producer cell line, in the AAV vector.

According to the present invention, a complete viral vector particle is a viral vector particle which comprises a payload for delivery to an individual. Typically, the payload is a nucleotide sequence as described herein. According to the present invention, an empty viral vector particle is a viral vector particle which lacks said payload. Typically, an empty viral vector particle of the invention is an empty AAV capsid or protein coat.

An AAV vector of the invention may be useful in methods of gene therapy and gene manipulation and modification in many disciplines. The vector may comprise one or more therapeutic nucleotide sequence, which may be in any appropriate form, including single stranded DNA (ssDNA) or self-complementary DNA. For example, an AAV vector of the invention may comprise a therapeutic DNA sequence useful in therapy or prevention of a disease or disorder. An AAV vector of the invention may also be useful in other way, for example in drug development and research. Such non-therapeutic applications include for gene manipulation and delivery, for example to deliver shRNA, oncogenes, etc. to generate induced pluripotent stem cells (IPS).

The AAV vector may be of any AAV serotype. For instance, the AVV vector of the invention may be any one of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10 or AAV11. In a preferred embodiment, the AAV vector is selected from AAV2, AAV5 or AAV8. A combination of any two or more AAV serotypes is also provided by the present invention. The combination of AAV serotypes may include AAV2 and AAV5; AAV5 and AAV8; AAV2 and AAV8; or AAV2, AAV5 and AAV8.

The invention also provides a population of adeno-associated viral (AAV) vectors of the invention. In particular, the present invention provides a population of AAV vectors obtainable by a method of the invention as disclosed herein. According to the present invention, a population of AAV vectors may be defined as multiple copies of an AAV of the invention. For example, a population of AAV vectors of the invention may comprise at least 10^9 vg/ml, at least 10^9 vg/ml, at least 20^9 vg/ml, at least 30^9 vg/ml, at least 40^9 vg/ml, at least 50^9 vg/ml, at least 10^{10} vg/ml or more

AAV vector particles. An AAV population of the invention may comprise of a single type of AAV vector of the invention. For example, the AAV population of the invention may comprise a single serotype of AAV vector, and may comprise a single payload (e.g. one medicine). The AAV population may be produced in one or more batch or one or more production cycle.

The capsid of the AAV vectors of the invention may comprise any combination of the proteins listed in Table 2 herein. Typically, the capsid of the AAV vectors of the invention comprise any combination of YB1, NPM1 and NCL as disclosed herein. Said capsid may also comprise one or more of the genes and/or proteins listed in Table 2 which are shared by four or more of the AAV serotypes investigated. The one or more gene and/or protein may already be known to have a function in AAV, or may have been associated with AAV for the first time herein. Typically the one or more gene and/or protein that is modulated in addition to at least one of YB1, NPM1 and NCL is selected from heterogeneous nuclear ribonucleoprotein K (hnRNPK), single-stranded DNA binding protein 1, nascent polypeptide-associated complex alpha subunit (CypA), peptidyl-prolyl cis-trans isomerase, alpha-enolase, Annexin A5, RuVB like 1 and like 2.

Any of the disclosure herein in relation to AAV vectors of the present invention may also be applied to an AAV vector population of the invention. For example, in one embodiment of the invention, modulation of one or more gene and/or protein of a producer cell line may increase the ratio of complete: empty AAV vector in an AAV vector population by 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 150%, at least 200% or more. Preferably, modulation of one or more gene and/or protein of a producer cell line increases the ratio of complete: empty AAV vector particles in an AAV vector population of the invention by at least 50%. Any increase in viral titre resulting from the modulation of one or more gene and/or protein may be compared with the viral titre obtained from a control method as described herein. A control method may be any standard method known in the art for producing AAV vectors. For example, a control method may use producer cell lines which have not been adapted according to the present invention. AAV vectors and AAV vector populations produced by such control/standard methods may be used as control vectors and populations as described herein.

Standard methods known in the art can produce AAV vector populations in which up to 100 % of the AAV vectors produced are empty, or with 100 fold more empty AAV vectors than complete AAV vectors. Even with further processing, the standard methods produce AAV vector populations in which at least 20% of the AAV vectors are empty. The methods of the present invention may produce AAV vector populations in which at least 50%, at least 60%, at least 70%, at least 80%, at

least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, up to 100% of the AAV vectors are complete (i.e. less than 50%, less than 40%, less than 30%, less than 20%, less than 15%, less than 10%, less than 5%, less than 4%, less than 3%, less than 2%, less than 1%, up to none of the AAV vectors produced are empty). The methods of the invention may produce AAV vector populations in which there are less than 50-fold, less than 40-fold, less than 30-fold, less than 20-fold, less than 10-fold, or less empty AAV vectors compared with the number of complete AAV vectors.

At least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, up to and including all of the AAV vector particles in an AAV vector population of the invention may have a capsid which comprises at least one of the proteins listed in Table 2. Typically, in an AAV vector population of the invention at least 50% of the AAV vector particles have a capsid which comprises at least one of the proteins listed in Table 2. Preferably the at least one protein is a protein one or more of YB1, NPM1 and/or NCL as described herein, more preferably at least 50% of the AAV vector particles have a capsid which comprises YB1, even more preferably at least 50% of the AAV vector particles have a capsid which comprises YB1, NPM1 and NCL.

The amount of at least one of the proteins listed in Table 2 in the capsid of the AAV vectors in an AAV vector or AAV vector population of the invention may be modified compared with the amount of said at least one protein in the capsid of control AAV vectors or in the capsid of AAV vectors in a control AAV vector population. For example, the amount of the at least one proteins may differ by 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 150%, at least 200% or more compared to the amount of the at least one protein in the capsid of control AAV vectors or in the capsid of AAV vectors in a control AAV vector population. This modification may be an increase or a decrease in the at least one protein. When multiple proteins are modified relative to a control, all may be decreased, some may be decreased and others increased, or all may be increased relative to the control.

The presence and/or amount of any of the proteins listed in Table 2 in the capsid of the AAV vectors in an AAV vector population of the invention may be determined and/or quantified, using any standard technique. Any increase in the presence and/or amount of any of the proteins listed in Table 2 resulting from the modulation of one or more gene and/or protein in the producer cell line according to the present invention may be compared with presence and/or amount of any of the

proteins listed in Table 2 in the capsids of AAV vectors in an AAV vector population obtained from a control method as described herein.

The AAV vector or AAV vector population of the invention may have a titre that is increased by at least 2 fold, at least 5 fold, at least 10 fold, at least 20 fold, at least 30 fold, at least 40 fold, at least 50 fold, at least 60 fold, at least 70 fold or more compared to a control AAV vector or AAV vector population. Preferably, the titre of the AAV vector or AAV vector population is increased by at least 2 fold, at least 5 fold or at least 10 fold. The AAV vector or AAV vector population titre may be compared with the titre of a control AAV vector or AAV vector population, or with the viral titre obtained from a control method as described herein.

Methods for Producing Adeno-Associated Viral Vectors

The present inventors have shown for the first time that modulating the expression of certain genes and/or proteins in a producer cell line used to produce an AAV vector can have an effect on the titre of viral vector produced from said producer cell line. Modulation of the expression of certain genes and/or proteins in a producer cell line used to produce an AAV vector may also increase the ratio of complete: empty viral vector particles produced from said producer cell line. Modulation of the expression of certain genes and/or proteins in a producer cell line used to produce an AAV vector may also modify the presence and/or amount of the proteins in the AAV vector capsid, particularly proteins derived from the producer cell line.

Modulation of One or More Gene and/or Protein

As described herein, the present invention provides a new production system for the production of AAV vectors which increase the viral titre produced. The production systems of the invention may also increase the ratio of complete: empty viral vector particles produced. The production systems of the invention may also modify the presence and/or amount of the proteins in the AAV vector capsid, particularly proteins derived from the producer cell line. The production systems of the invention comprise producer cell lines adapted for the production of AAV vectors, as well as methods of producing AAV vectors using said producer cell lines. The present invention also provides AAV vectors produced by said production systems, producer cell lines and methods.

Accordingly, the present invention provides a method of producing an AAV vector comprising modulating the expression of one or more genes and/or proteins in a producer cell line.

Modulation may be increasing or reducing (decreasing) the expression of the one or more gene and/or protein. In cases where multiple genes and/or proteins are modulated, all the gene/proteins may be increased, or all the genes/proteins may be decrease, or one or more genes/proteins may be increased and others of the genes/proteins may be decreased. In a preferred embodiment, the modulation is decreasing the expression of the one or more gene and/or protein.

Modulation, whether an increase or a reduction in the expression of one or more gene and/or protein in a producer cell line, may be measured relative to a control. Thus, the expression of one or more gene and/or protein in a producer cell line of the invention may be compared with the expression of said one or more gene and/or protein in a control. The actual amount of the one or more gene and/or protein, such as the mass, molar amount, concentration or molarity of the one or more gene and/or protein in the producer cell line of the invention and the control may be assessed and compared with the corresponding value from the control. Alternatively, the expression of one or more gene and/or protein in a producer cell line of the invention may be compared with that of the control without quantifying the mass, molar amount, concentration or molarity of the one or more gene and/or protein.

Typically the control is an equivalent producer cell line in which no modulation of the one or more gene and/or protein has been effected. For example, in the case where the producer cell line of the invention is a transgenic cell line in which YB1 expression has been reduced, a suitable control would be the same cell line in which YB1 expression has not been altered. Such control cell lines may be wild-type cell lines. A control method according to the present invention typically uses a control producer cell line as described herein. Conventional methods for the production of AVV, including known methods may be considered control methods according to the present invention.

The expression of the one or more gene and/or protein in a producer cell line of the invention may differ by 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 150%, at least 200% or more compared with a control.

For example, if the expression of one or more gene and/or protein in a producer cell line of the invention is reduced compared with a control, the expression may be reduced partially or totally compared with the control. Typically the expression is reduced by 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 95%, at least 99%, up to total elimination (knockout) of expression of the one or more gene and/or protein.

If the expression of one or more gene and/or protein in a producer cell line of the invention is increased compared with a control, the expression may be increased by 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 150%, at least 200% compared with the control.

The expression of one or more gene and/or protein in a producer cell line of the invention may be determined by quantitative and/or qualitative analysis. Typically, gene expression may be expressed in terms of mRNA levels.

The expression level of the one/or more gene and/or protein in a producer cell line of the invention encompasses the mass of the one/or more gene and/or protein, the molar amount of the one/or more gene and/or protein, the concentration of the one/or more gene and/or protein and the molarity of the one/or more gene and/or protein. This expression level may be given in any appropriate units. For example, the concentration of the one or more gene and/or protein may be given in pg/ml, ng/ml or µg/ml.

The expression level of the one/or more gene and/or protein in a producer cell line of the invention may be measured directly or indirectly.

The relative expression of the one or more modulated gene and/or protein in a producer cell line of the invention relative to a control may be determined using any appropriate technique. Suitable standard techniques are known in the art, for example Western blotting and enzyme-linked immunosorbent assays (ELISAs).

The expression level of the one or more gene and/or protein to be modulated may be altered compared with a control for at least 12 hours, at least 24 hours, at least 30 hours, at least 48 hours, at least 72 hours, at least 96 hours, at least 120 hours, at least 144 hours, at least 1 week, at least 2 weeks, at least 3 weeks, at least 4 weeks, at least 5 weeks, at least 6 weeks, at least 7 weeks, at least 8 weeks, at least 9 weeks, at least 10 weeks, at least 11 weeks, at least 12 weeks, at least 13 weeks, at least 14 weeks, at least 15 weeks or more.

The expression level of the one or more gene and/or protein to be modulated may be altered compared with a control for at least one, at least two, at least three, at least four, at least five, at least ten, at least 20, at least 30, at least 40 or more passages of the producer cell line in culture. The expression level of the one or more gene and/or protein to be modulated may be altered indefinitely.

Modulated Genes and/or Proteins

The inventors have carried out the first systematic analysis of the non-viral proteins contained in the capsid of AAV, and have identified 66 proteins that are found in the capsid or one or more of AAV serotypes 2, 5 and 8. These proteins are identified herein in Table 2. Typically these non-viral proteins are derived from the producer cell line in which the AAV vector particles are made.

Modulating the expression of one or more of these 66 proteins in a producer cell line may be useful in increasing the titre of AAV vector produced from said producer cell line. Accordingly, the present invention provides a method for producing an AAV vector comprising modulating the expression of at least one of the proteins listed in Table 2.

The methods of the invention may comprise modulating the expression at least one of the proteins listed in Table 2 that is a DNA binding protein. Alternatively or in addition, the methods of the invention may comprise modulating the expression at least one of the proteins listed in Table 2 that is not a DNA binding protein.

The method of the invention may comprise modulating the expression of at least 2, at least 3, at least 4, at least 5, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, up to all of the genes and/or proteins listed in Table 2.

Of the proteins listed in Table 2, several are known to bind to DNA. For example, NPM1 (also known as nucleophosmin, nucleolar phosphoprotein B23 and numatrin), NCL and YB1 all bind to DNA and/or RNA.

NPM1 is associated with nucleolar ribonucleoprotein structures and binds to both single- and double-stranded DNA. The genomic DNA sequence of the human NPM1 gene is given in SEQ ID NO: 1 (Genbank Accession No. BC016768, version BC016768.1 GI:16876991). NPM1 is generally located in the nucleolus, but can be translocated to the nucleoplasm in some circumstances.

The NCL gene encodes the nucleolar phosphoprotein nucleolin. Nucleolin is a DNA binding protein involved in the synthesis and maturation of ribosomes. Any reference herein to the NCL protein may be understood as a reference to nucleolin. The genomic DNA sequence of the human NCL gene is given in SEQ ID NO: 2 (Genbank Accession No. M60858, version M60858.1 GI:189305).

The YB1 gene encodes the Y box binding protein 1 (also known as Y box transcription factor and nuclease sensitive element binding protein 1). The genomic DNA sequence of the human YB1 gene is given in SEQ ID NO: 3 (Genbank Accession No. NM_004559.3, version NM_004559.3

GI:109134359). Any reference herein to the YB1 protein may be understood as a reference to Y box binding protein 1. YB1 is a DNA and RNA binding protein involved in many DNA and mRNA dependent processes. YB1 packs and stabilises mRNA and is involved in gene regulation at different levels.

Adenoprotein E1B is known to interact with YB1, and has been shown to result in the accumulation of YB1 in cell nuclei and the activation of adenoviral gene E2A. Overexpression of YB1 regulated the adenoviral E2 promoter in an E1-independent manner, and has been shown to increase adenoviral DNA replication and an increase in the production of infectious viral particles from E1-deleted adenoviral vectors. As a result, overexpression of YB1 has been exploited in adenovirus-based vector development and virotherapy.

However, as demonstrated in the Examples herein, the present inventors have found that knockdown of YB1 results in increased AAV vector titres. Further, the present inventors demonstrated that down-regulation of YB1 expression in producer cells resulted in an increase in AAV rep expression, an increase in AAV vector DNA production and a decrease in AAV cap expression. Without wishing to be bound by theory, it is believed that DNA binding proteins, such as YB1, NPM1 and NCL, from a producer cell line compete with adenoviral components for binding to DNA, particularly single-stranded DNA (ssDNA). Such adenoviral components include adenoproteins E2a and E4, which are early function proteins required for viral replication, and VA, which is a region of the adenoviral genome that codes for small RNA molecules which are not translated by which regulate the translation of viral mRNAs for binding to DNA. Open-reading frame 6 of the E4 region is important for the conversion of the single-stranded AAV genome into a double-stranded form which is the substrate for subsequent steps in DNA replication. Protein E2A plays a key role in viral DNA replication via binding to AAV viral DNA, promoting DNA elongation and displacement of the elongating strand from its template.

Adenoviral components such as E2a, E4 and VA are needed for AAV vector particle production, and by competing with these adenoviral components for binding to DNA, producer cell line DNA binding proteins can decrease AAV vector particle production. Consequently, without being bound by theory, decreasing expression of producer cell line DNA binding proteins such as NPM1, NCL and YB1 reduces the competition of these producer cell line DNA binding proteins with E2A binding to AAV DNA, resulting in the enhancement of E2A-AAV DNA interaction, the efficiency of AAV DNA replication and ultimately increase in AAV vector genome titres.

YB1 binding to the ssDNA region of a promoter has been shown to result in the stabilisation of ssDNA that also inhibited gene transcription and translation. Therefore, it is possible that down regulation of YB1 (or another producer cell DNA binding protein such as NPM1 or NCL) promotes E2A binding to the AAV2p5 promoter that synergistically contribute to an increase in AAV2 and AAV8 titres (as observed by the present inventors).

Accordingly, typically the method of the invention comprises modulating the expression of at least one of NPM1, NCL and YB1. Typically the method of the invention comprises modulating the expression of YB1. Methods in which a combination of NPM1, NCL and YB1 are modulated are also encompassed. For example, a method of the invention may comprise modulating the expression of: (i) NPM1 and NCL; (ii) NPM1 and YB1; (iii) NCL and YB1; or (iv) NPM1, NCL and YB1. In a preferred embodiment, the method of the invention comprises modulating the expression of YB1, NPM1 and NCL.

In a preferred embodiment, the method of the invention comprises reducing the expression of at least one of NPM1, NCL and YB1. Typically the method of the invention comprises reducing the expression of YB1. Methods in which a combination of NPM1, NCL and YB1 are reduced are also encompassed. For example, a method of the invention may comprise reducing the expression of: (i) NPM1 and NCL; (ii) NPM1 and YB1; (iii) NCL and YB1; or (iv) NPM1, NCL and YB1. In a preferred embodiment, the method of the invention comprises reducing the expression of YB1, NPM1 and NCL.

The method of the invention may further comprise modulating the expression of one or more additional gene and/or protein in the producer cell line. For example, the method may further comprise modulating the expression of one or more additional protein listed in Table 2 in the producer cell line. The additional protein modulated according to the present invention may be a DNA binding protein. The additional protein modulated according to the present invention may have a function other than DNA binding. Thus, the method of the invention may involve the modulation of two or more genes and/or proteins that have different functions in the producer cell line, at least one of which may be a DNA binding protein. The method of the invention may involve modulating the expression of at least one of NPM1, NCL and YB1 and also the modulation of one or more additional gene and/or protein in the producer cell line, wherein the one or more additional gene and/or protein may be listed in Table 2.

The method of the invention may comprise modulating the expression of any combination of NPM1, NCL and YB1 as disclosed herein, and also modulating the expression of one or more of the

genes and/or proteins listed in Table 2. In a preferred embodiment, the one or more gene and/or protein in Table 2 are shared by four or more of the AAV serotypes investigated. The one or more gene and/or protein may already be known to have a function in AAV, or may have been associated with AAV for the first time herein. Typically the one or more gene and/or protein that is modulated in addition to at least one of NPM1, NCL and YB1 is selected from heterogeneous nuclear ribonucleoprotein K (hnRNPK), single-stranded DNA binding protein 1, nascent polypeptide-associated complex alpha subunit (CypA), peptidyl-prolyl cis-trans isomerase, alpha-enolase, Annexin A5, RuVB like 1 and like 2.

The one or more gene and/or protein to be modulated according to the present invention may be modulated by any appropriate means. Suitable standard techniques are known in the art. Modulation may take place via any suitable mechanism, depending for example on the nature (see below) of the modulator used, e.g. steric interference in any direct or indirect interaction or modulation of the one or more gene and/or protein.

Modulators of the invention may be specific for a gene or protein to be modulated. By specific, it will be understood that the modulator binds to a gene or protein to be modulated, such as the YB1, NPM1 or NCL gene, with no significant cross-reactivity to any other molecule, particularly any other protein. For example, modulator that is specific for NPM1 will show no significant cross-reactivity with human neutrophil elastase. Cross-reactivity may be assessed by any suitable method. Cross-reactivity of modulator for a gene or protein to be modulated with a molecule other that gene or protein may be considered significant if the modulator binds to the other molecule at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 100% as strongly as it binds to the gene or protein to be modulated. A modulator that is specific for a gene or protein to be modulated may bind to another molecule such as human neutrophil elastase at less than 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25% or 20% the strength that it binds to the gene or protein to be modulated. Preferably, the modulator binds to the other molecule at less than 20%, less than 15%, less than 10% or less than 5%, less than 2% or less than 1% the strength that it binds to the gene or protein to be modulated.

Any suitable modulator may be used according to the invention, for example peptides and peptidomimetics, antibodies, small molecule inhibitors, double- stranded RNA, antisense (single stranded) RNA, aptamers and ribozymes. Transcriptional and post-transcriptional gene silencing technologies may be used to modulate one or more gene of the invention. Post-transcriptional gene silencing is also known as RNA interference (RNAi). In a preferred embodiment, modulation is carried out by Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) genome editing,

which is typically used to decrease expression of the one or more genes of the invention. Preferred antagonists include double- stranded RNA and chimeric guide RNA transcripts (gRNA). gRNA combines bacterial CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) which are recruited to a gene of interest during CRISPR genome editing, together with an endonuclease (typically a CRISPR associated nuclease (Cas), such as Cas9). The one or more genes of the invention may be modulated using the same category of modulator, or by different modulators. As a non-limiting example, YB1, NPM1 and/or NCL may all be modulated using CRISPR genome editing; or YB1 may be modulated by CRISPR genome editing and NPM1 and/or NCL may be modulated using shRNA.

Double-stranded RNA

Double-stranded RNA (dsRNA) molecules may be used to modulate expression of one or more gene in a producer cell line. Typically, dsRNAs are used to reduce expression of the one or more gene as described herein. dsRNA molecules may be used in RNAi to modulate one or more gene of the invention.

Using known techniques and based on a knowledge of the sequence of the one or more gene to be modulated, dsRNA molecules can be designed to antagonise the one or more gene by sequence homology-based targeting of the corresponding RNA sequence. Such dsRNAs will typically be small interfering RNAs (siRNAs), small hairpin RNAs (shRNAs), or micro-RNAs (miRNAs). The sequence of such dsRNAs will comprise a portion that corresponds with that of a portion of the mRNA encoding the one or more gene to be modulated. This portion will usually be 100% complementary to the target portion within the mRNA transcribed from the one or more gene, but lower levels of complementarity (e.g. 90% or more or 95% or more) may also be used. Typically the % complementarity is determined over a length of contiguous nucleic acid residues. A dsRNA molecule of the invention may, for example, have at least 80% complementarity to the target portion within the mRNA transcribed from the one or more gene measured over 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, or more nucleic acid residues, up to the dsRNA molecule having at least 80% complementarity the mRNA transcribed from the one or more gene of the invention over the entire length of the dsRNA molecule.

In a preferred embodiment, the dsRNA is a shRNA. ShRNA can be delivered to a producer cell line of the invention by any appropriate means. Suitable techniques are known in the art and include the use of plasmid, viral and bacterial vectors to deliver the shRNA to the producer cell line.

Typically, the shRNA is delivered using a viral vector delivery system. In a preferred embodiment, the viral vector is a lentiviral vector.

Generally, once the shRNA has been delivered to a producer cell, it is then transcribed in the nucleus and processed. The resulting pre-shRNA is exported from the nucleus and then processed by dicer and loaded into the RNA-induced silencing complex (RISC). The sense (passenger) strand is degraded. The antisense (guide) strand directs RISC to mRNA that has a complementary sequence. In the case of perfect complementarity, RISC cleaves the mRNA. In the case of imperfect complementarity, RISC represses translation of the mRNA. In both of these cases, the shRNA leads to target gene silencing.

shRNA is used to modulate the expression of one or more of the genes listed in Table 2. Multiple shRNAs may be used to modulate the expression of any one of the genes listed in Table 2. Typically, shRNA is used to modulate the expression of at least one of YB1, NPM1 and/or NCL and/or YB1. Multiple shRNAs may be used to modulate the expression ofYB1, NPM1 and/or NCL.

The shRNA used to modulate YB1 expression may comprise a nucleotide sequence selected from SEQ ID NOs: 14 to 18 (YB1-Y1 to YB1-Y5), or a variant thereof. Multiple shRNAs selected from SEQ ID NOs: 14 to 18, or a variant thereof, may be used to modulate YB1 expression. In a preferred embodiment, the shRNA used to modulate YB1 expression is SEQ ID NO: 17 and/or 18 (YB1-Y4 and Y5) or a variant thereof.

The shRNA used to modulate NPM1 expression may comprise a nucleotide sequence selected from SEQ ID NOs: 4 to 8 (NPM-N6 to NPM-N10), or a variant thereof. Multiple shRNAs selected from SEQ ID NOs: 4 to 8, or a variant thereof, may be used to modulate NPM1 expression. In a preferred embodiment, the shRNA used to modulate NPM1 expression is SEQ ID NO: 4 and/or 7 (NPM-N6 and N9) or a variant thereof.

The shRNA used to modulate NCL expression may comprise a nucleotide sequence selected from SEQ ID NOs: 9 to 13 (NCL-N1 to NCL-N5), or a variant thereof. Multiple shRNAs selected from SEQ ID NOs: 9 to 13, or a variant thereof, may be used to modulate NCL expression. In a preferred embodiment, the shRNA used to modulate NCL expression is SEQ ID NO: 9 and/or 12 (NCL-N1 and N4) or a variant thereof.

The sequences of NPM-N6 to N10, NCL-N1 to N5 and YB1-Y1 to Y5 (SEQ ID NOs: 4 to 18) are shown in Table 1 below.

Any combination of the shRNAs of SEQ ID NOs: 14 to 18 (YB1-Y1 to YB1-Y5), SEQ ID NOs: 4 to 8 (NPM-N6 to NPM-N10) and SEQ ID NOs: 9 to 13 (NCL-N1 to NCL-N5), or variants thereof, may be used.

A variant sequence may have at least 80% sequence identity to a sequence of the invention, measured over any appropriate length of sequence. Typically the % sequence identity is determined over a length of contiguous nucleic acid or amino acid residues. A variant sequence of the invention may, for example, have at least 80% sequence identity to a sequence of the invention measured over 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, or more nucleic acid or amino acid residues.

For example, a variant shRNA molecule of the invention may have at least 80% sequence identity with an shRNA molecule of the invention measured over at least 10, at least 20, at least 30, at least 40, at least 50, at least 60 or more nucleic acid residues, up to the variant shRNA molecule having at least 80% sequence identity with the shRNA molecule of the invention over the entire length of the variant shRNA molecule. Typically the variant shRNA molecule of the invention is a variant of one or more of the shRNA molecules of SEQ ID NOs: 4 to 18.

CRISPR Genome Editing

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system for genome editing typically comprises two distinct components: (1) a guide RNA and (2) an endonuclease, specifically a CRISPR associated (Cas) nuclease, such as Cas9. The guide RNA is a combination of the endogenous bacterial crRNA and tracrRNA into a single chimeric guide RNA (gRNA) transcript. When the gRNA and the Cas are expressed in the cell, the genomic target sequence can be modified or permanently disrupted.

The gRNA/Cas complex is recruited to the target sequence by the base-pairing between the gRNA sequence and the complement to the target sequence in one or more gene of the invention in the genomic DNA. For successful binding of Cas, the genomic target sequence must also contain the correct Protospacer Adjacent Motiff (PAM) sequence immediately following the target sequence. The binding of the gRNA/Cas complex localises the Cas to the genomic target sequence in the one or more gene of the invention so that the wild-type Cas can cut both strands of DNA causing a double strand break. This can be repaired through one of two general repair pathways: (1) the non-homologous end joining DNA repair pathway or (2) the homology directed repair pathway. The non-

Table 1: Sequences of exemplary shRNA molecules of the invention

Gene	shRNA designation	SEQ ID NO:	Sequence (5' – 3')
NPM1	NPM-N6	4	CCGGGCCAAGAATGTGTTGTCCAAACTCGAGTTTGGACAACACATTCTTGGCTTTTTG
	NPM-N7	5	CCGGGCGCCAGTGAAGAAATCTATACTCGAGTATAGATTTCTTCACTGGCGCTTTTTG
	NPM-N8	6	CCGGGCAAAGGATGAGTTGCACATTCTCGAGAATGTGCAACTCATCCTTTGCTTTTTG
	NPM-N9	7	CCGGCCTAGTTCTGTAGAAGACATTCTCGAGAATGTCTTCTACAGGAACTAGGTTTTTG
	NPM-N10	8	CCGGCCTAGTTCTGTAGAAGACATTCTCGAGAATGTCTTCTACAGAACTAGGTTTTTG
NCL	NCL-N1	9	CCGGCCTTGGAAATCCGTCTAGTTACTCGAGTAACTAGACGGATTTCCAAGGTTTTTG
	NCL-N2	10	CCGGCCGGTGAAATTGATGGAAATAACTCGAGTTATTTCCATCAATTTCACCGTTTTTG
	NCL-N3	11	CCGGCGGTGAAATTGATGGAAATAACTCGAGTTATTTCCATCAATTTCACCGTTTTTG
	NCL-N4	12	CCGGAGTAAAGGGATTGCTTATATTCTCGAGAATATAAGCAATCCCTTTACTTTTTTG
	NCL-N5	13	CCGGCGTTCGGGGCAAGGATAGTTACCTCGAGGTAACTATCCTTGCCCGAACGTTTTTG
YB1	YB-Y1	14	CCGGGCAGTTCAAGGCAGTAAATATCTCGAGATATTTACTGCCTTGAACTGGTTTTTG
	YB-Y2	15	CCGGAGCAGACCGTAACCATTATAGCTCGAGCTATAATGGTTACGGTCTGCTTTTTTG
	YB-Y3	16	CCGGGCTTACCATCTCTACCATCATCTCGAGATGATGGTAGAGATGGTAAGCTTTTT
	YB-Y4	17	CCGGGACGGCAATGAAGAAGATAAACTCGAGTTTATCTTCTTCATTGCCGTCTTTTT
	YB-Y5	18	CCGGCCAGTTCAAGGCAGTAAATATCTCGAGATATTTACTGCCTTGAACTGGTTTTT
Annexin A5	A-A1	19	CCGGGCCATCAAACAAGTTTATGAACTCGAGTTCATAAACTTGTTTGATGGCTTTTT
	A-A2	20	CCGGGCTGGAATTGATGAAGCTCAACTCGAGTTGAGCTTCATCAATTCCAGCTTTTT
	A-A3	21	CCGGCCATGATTAAGGGAGATACATCTCGAGATGTATCTCCCTTAATCATGGTTTTT
	A-A4	22	CCGGCGCGAGACTTCTGGCAATTTACTCGAGTAAATTGCCAGAAGTCTCGCGTTTTT
	A-A5	23	CCGGGCATCCTGACTCTGTTGACATCTCGAGATGTCAACAGAGTCAGGATGCTTTTT

homologous repair pathway often results in inserts/deletions at the double strand break that can lead to frameshifts and/or premature stop codons, effectively disrupting the open reading frame of the one or more gene of the invention. The homology directed repair pathway requires the presence of a repair template, which is used to fix the double strand break.

Any appropriate gRNA pair may be used for CRISPR genome editing according to the present invention, provided it modulates one or more gene of the invention as described herein. Typically gRNA pairs are used to reduce expression of one or more gene of the invention as described herein. Preferably any appropriate gRNA pair is used to modulate (typically reduce, preferably eliminate/knockout) expression of YB1, NPM1 and/or NCL, more preferably YB1.

gRNA pairs can be designed using known techniques and based on a knowledge of the sequence of the one or more gene to be modulated, typically using an appropriate computer programme, such as the CRISPR/Cas9 programme (https://chopchop.rc.fas.harvard.edu/). For example, gRNAs for modulatingYB1 may be designed using the CRISPR/Cas9 programme (https://chopchop.rc.fas.harvard.edu/) and targeting the entire Homo sapiens chromosome 1 sequence (Accession number NC_000001.11) that includes YB1 gene sequence. Knock out producer cells may be generated using any appropriate technique, with standard techniques being known in the art and suitable kits being commercially available.

gRNA pairs can be delivered to a producer cell line of the invention by any appropriate means. Suitable techniques are known in the art and include the use of plasmid, viral and bacterial vectors to deliver the gRNA pairs to the producer cell line. Typically, a gRNA pair is delivered using a plasmid DNA.

gRNA pairs may be used to modulate the expression of one or more of the genes listed in Table 2. Multiple gRNA pairs may be used to modulate the expression of any one of the genes listed in Table 2. Typically, gRNA pairs are used to modulate the expression of at least one of YB1, NPM1 and/or NCL and/or YB1. Multiple gRNA pairs may be used to modulate the expression of YB1, NPM1 and/or NCL.

The gRNA pairs used to modulate YB1 expression may comprise a nucleotide sequence pairs selected from SEQ ID NOs: 33 and 34; SEQ ID NOs: 35 and 36; SEQ ID NOs: 37 and 38; and/or SEQ ID NOs: 39 and 40, or variants thereof. Multiple gRNA pairs selected from SEQ ID NOs: 33 and 34; SEQ ID NOs: 35 and 36; SEQ ID NOs: 37 and 38; and SEQ ID NOs: 39 and 40, or variants thereof, may be used to modulate YB1 expression.

A variant sequence may have at least 80% sequence identity to a sequence of the invention, measured over any appropriate length of sequence. Typically the % sequence identity is determined over a length of contiguous nucleic acid or amino acid residues. A variant gRNA sequence of the invention may, for example, have at least 80% sequence identity to a sequence of the invention measured over at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, or more nucleic acid residues,, up to the variant gRNA molecule having at least 80% sequence identity with the shRNA molecule of the invention over the entire length of the variant gRNA molecule. Typically a variant gRNA molecule of the invention is a variant of one or more of the gRNA molecules of SEQ ID NOs: 33 to 40. gRNA pairs of the invention may comprise a variant of one or both of the gRNA sequences disclosed herein. For example, a variant of the gRNA pair of SEQ ID NOs: 33 and 34 may comprise a variant of SEQ ID NO: 33, a variant of SEQ ID NO: 34 or a variant of SEQ ID NOs: 33 and 34. This principle applies to all gRNA pairs disclosed herein.

Antisense RNA

Single-stranded DNA (ssDNA) molecules, also known as antisense RNA, may be used to modulate expression of one or more gene in a producer cell line. Typically, antisense RNAs are used to reduce expression of the one or more gene as described herein.

Using known techniques and based on a knowledge of the sequence of the one or more gene to be modulated, antisense RNA molecules can be designed to antagonise the one or more gene by sequence homology-based targeting of the corresponding RNA. The sequence of such antisense will comprise a portion that corresponds with that of a portion of the mRNA transcribed from the one or more gene. This portion will usually be 100% complementary to the target portion within the transcribed mRNA but lower levels of complementarity (e.g. 90% or more or 95% or more) may also be used.

Aptamers

Aptamers are generally nucleic acid molecules that bind a specific target molecule. Aptamers can be engineered completely in vitro, are readily produced by chemical synthesis, possess desirable storage properties, and elicit little or no immunogenicity in therapeutic applications. These characteristics make them particularly useful in pharmaceutical and therapeutic utilities.

As used herein, "*aptamer*" refers in general to a single or double stranded oligonucleotide or a mixture of such oligonucleotides, wherein the oligonucleotide or mixture is capable of binding specifically to a target. Oligonucleotide aptamers will be discussed here, but the skilled reader will appreciate that other aptamers having equivalent binding characteristics can also be used, such as peptide aptamers.

In general, aptamers may comprise oligonucleotides that are at least 5, at least 10 or at least 15 nucleotides in length. Aptamers may comprise sequences that are up to 40, up to 60 or up to 100 or more nucleotides in length. For example, aptamers may be from 5 to 100 nucleotides, from 10 to 40 nucleotides, or from 15 to 40 nucleotides in length. Where possible, aptamers of shorter length are preferred as these will often lead to less interference by other molecules or materials.

Aptamers may be generated using routine methods such as the Systematic Evolution of Ligands by Exponential enrichment (SELEX) procedure. SELEX is a method for the *in vitro* evolution of nucleic acid molecules with highly specific binding to target molecules. It is described in, for example, US 5,654, 151, US 5,503,978, US 5,567,588 and WO 96/38579.

The SELEX method involves the selection of nucleic acid aptamers and in particular single stranded nucleic acids capable of binding to a desired target, from a collection of oligonucleotides. A collection of single- stranded nucleic acids (e.g., DNA, RNA, or variants thereof) is contacted with a target, under conditions favourable for binding, those nucleic acids which are bound to targets in the mixture are separated from those which do not bind, the nucleic acid-target complexes are dissociated, those nucleic acids which had bound to the target are amplified to yield a collection or library which is enriched in nucleic acids having the desired binding activity, and then this series of steps is repeated as necessary to produce a library of nucleic acids (aptamers) having specific binding affinity for the relevant target.

Variant Nucleic Acid Sequences

A sequence identity of at least 80% includes at least 82%, at least 84%, at least 86%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and 100% sequence identity (to each and every nucleic acid sequence presented herein and/ or to each and every SEQ ID NO presented herein).

Any of a variety of sequence alignment methods can be used to determine percent identity, including, without limitation, global methods, local methods and hybrid methods, such as, e.g.,

segment approach methods. Protocols to determine percent identity are routine procedures within the scope of one skilled in the. Global methods align sequences from the beginning to the end of the molecule and determine the best alignment by adding up scores of individual residue pairs and by imposing gap penalties. Non-limiting methods include, e.g., CLUSTAL W, see, e.g., Julie D. Thompson et al., CLUSTAL W: Improving the Sensitivity of Progressive Multiple Sequence Alignment Through Sequence Weighting, Position- Specific Gap Penalties and Weight Matrix Choice, 22 (22) Nucleic Acids Research 4673-4680 (1994); and iterative refinement, see, e.g., Osamu Gotoh, Significant Improvement in Accuracy of Multiple Protein. Sequence Alignments by Iterative Refinement as Assessed by Reference to Structural Alignments, 264(4) J. Mol. Biol. 823-838 (1996). Local methods align sequences by identifying one or more conserved motifs shared by all of the input sequences. Non-limiting methods include, e.g., Match-box, see, e.g., Eric Depiereux and Ernest Feytmans, Match-Box: A Fundamentally New Algorithm for the Simultaneous Alignment of Several Protein Sequences, 8(5) CABIOS 501 -509 (1992); Gibbs sampling, see, e.g., C. E. Lawrence *et al.*, Detecting Subtle Sequence Signals: A Gibbs Sampling Strategy for Multiple Alignment, 262 (5131) Science 208-214 (1993); Align-M, see, e.g., Ivo Van Walle *et al.*, Align-M - A New Algorithm for Multiple Alignment of Highly Divergent Sequences, 20 (9) Bioinformatics:1428-1435 (2004). Thus, percent sequence identity is determined by conventional methods. See, for example, Altschul *et al.*, Bull. Math. Bio. 48: 603-16, 1986 and Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915-19, 1992.

Variants of the specific sequences provided above may alternatively be defined by reciting the number of nucleotides that differ between the variant sequences and the specific reference sequences provided above. Thus, in one embodiment, the sequence may comprise (or consist of) a nucleotide sequence that differs from the specific sequences provided above at no more than 5, no more than 4, no more than 3, no more than 2 nucleotide positions, for example at no more than 1 nucleotide position. Conservative substitutions are preferred.

The variant nucleic acid molecules of the invention, such as the variant shRNA molecules and/or variant gRNA molecules and/or variant gRNA pairs of the invention typically still retain the activity of the corresponding molecules of the invention. Thus, for example, the variant shRNA molecules of the invention retain the ability of the corresponding shRNA molecules to modulate the expression of the one or more gene of the invention. The variant shRNA molecules may retain at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, up to and including 100% of the modulatory activity of the shRNA molecules of the invention. This applies equally to the gRNA molecules and/or gRNA pairs of the invention.

The nucleic acid molecules of the invention, such as the shRNA molecules, gRNA molecules and/or gRNA pairs of the invention may be labelled (or tagged) in order to facilitate the removal of producer cells lacking the shRNA molecules, gRNA molecules and/or gRNA pairs. Puromycin is an example of a suitable tag. The two gRNA molecules making up a gRNA pair of the invention may be labelled with the same tag. Alternatively, two gRNA molecules making up a gRNA pair of the invention may be labelled with different tags to enable the two gRNA molecules to be distinguished.

Effect of Modulation

Modulators of the invention can increase or reduce the expression of one or more gene and/or protein of a producer cell line. Typically the one or more gene and/or protein is listed in Table 2 as disclosed herein. Typically the modulation of the one or more gene and/or protein of a producer cell line will result in an increase titre of AAV vector production by the producer cell line according to the present invention.

Modulation of one or more gene and/or protein of a producer cell line may increase the titre of AAV vector production by at least 2 fold, at least 3 fold, at least 4 fold, at least 5 fold, at least 6 fold, at least 7 fold, at least 8 fold, at least 9 fold, at least 10 fold, at least 15 fold, at least 20 fold, at least 25 fold, at least 30 fold, at least 35 fold, at least 40 fold, at least 45 fold, at least 50 fold, at least 55 fold, at least 60 fold, at least 65 fold, at least 70 fold or more. Preferably, modulation of one or more gene and/or protein of a producer cell line increases the titre of AAV vector production by at least 2 fold, at least 5 fold or at least 10 fold. Any increase in viral titre resulting from the modulation of one or more gene and/or protein may be compared with the viral titre obtained from a control method as described herein.

Modulation of one or more gene and/or protein of a producer cell line may increase the titre of AAV vector production for at least 5 days, at least 10 days, at least 20 days, at least 30 days, at least 40 days, at least 50 days, at least 60 days, at least 70 days, at least 80 days, at least 90 days, at least 100 days or more. Preferably, modulation of one or more gene and/or protein of a producer cell line increases the titre of AAV vector production for at least 40 days. Again, the duration of the increased viral titre may be compared with a control, as described herein.

Modulation of one or more gene and/or protein of a producer cell line may increase the titre of AAV vector production for at least one, at least two, at least three, at least four, at least five, at least ten, at least 20, at least 30, at least 40 or more passages of the producer cell line in culture.

Modulation of one or more gene and/or protein of a producer cell line may increase the titre of AAV vector production indefinitely.

There may be a time and dose dependence of the modulation of the one or more gene and/or protein on viral titre. Typically, even at a low dose and early time point, it may be possible to achieve a significant fold increase in AAV vector titre of at least 2 fold, at least 5 fold or at least 10 fold (see, for example, Figure 6B).

The increase in AAV vector titre may be measured using any appropriate technique. Standard techniques for measuring viral titres are known in the art, for example, using cell based assay, e.g. a plaque assay and/or quantitative PCR (qPCR).

The modulation of the one or more gene and/or protein of the producer cell line may increase the ratio of complete: empty AAV vector produced by the producer cell line according to the present invention. The increase in AAV vector titre and/or the increase in the ratio of complete: empty AAV vector may be compared with a control as described herein.

Modulation of one or more gene and/or protein of a producer cell line may increase the ratio of complete: empty AAV vector produced by the producer cell line by 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 150%, at least 200% or more. Preferably, modulation of one or more gene and/or protein of a producer cell line increases the ratio of complete: empty AAV vector produced by the producer cell line by at least 50%.

Modulation of one or more gene and/or protein of a producer cell line may increase the ratio of complete: empty AAV vector produced by the producer cell line such that the AAV vector population comprises less than 50-fold, less than 40-fold, less than 30-fold, less than 20-fold, less than 10-fold, or less empty AAV vectors compared with the number of complete AAV vectors.

Any increase in viral titre resulting from the modulation of one or more gene and/or protein may be compared with the viral titre obtained from a control method as described herein.

Modulation of one or more gene and/or protein of a producer cell line may increase the ratio of complete: empty AAV vector produced by the producer cell line for at least 5 days, at least 10 days, at least 20 days, at least 30 days, at least 40 days, at least 50 days, at least 60 days, at least 70 days, at least 80 days, at least 90 days, at least 100 days or more. Preferably, modulation of one or more gene and/or protein of a producer cell line increases the ratio of complete: empty AAV

vector produced by the producer cell line for at least 40 days. Again, the duration of the increased viral titre may be compared with a control, as described herein.

Modulation of one or more gene and/or protein of a producer cell line may increase the ratio of complete: empty AAV vector produced by the producer cell line for at least one, at least two, at least three, at least four, at least five, at least ten, at least 20, at least 30, at least 40 or more passages of the producer cell line in culture.

Modulation of one or more gene and/or protein of a producer cell line may increase the ratio of complete: empty AAV vector produced by the producer cell line indefinitely.

The ratio of complete: empty AAV vector produced by the producer cell line may be measured using any appropriate technique. Standard techniques for measuring viral titres are known in the art. For example, a combination of qPCR and ELISA may be used to quantify the ratio of complete: empty AAV vector.

Modulation of one or more gene and/or protein of a producer cell line may independently increase AAV rep expression, increase AAV vector DNA production and/or decrease AAV cap expression. Typically the change in AAV rep expression, increase AAV vector DNA production and/or decrease AAV cap expression is at least 2 fold, at least 3 fold, at least 4 fold, at least 5 fold, at least 6 fold, at least 7 fold, at least 8 fold, at least 9 fold, at least 10 fold, at least 11 fold, at least 12 fold, at least 13 fold, at least 14 fold, at least 15 fold, at least 20 fold, at least 25 fold, at least 30 fold, at least 35 fold, at least 40 fold, at least 45 fold, at least 50 fold, at least 55 fold, at least 60 fold, at least 65 fold, at least 70 fold or more compared with a control cell line or method as defined herein.

Modulation of one or more gene and/or protein of a producer cell line may independently increase AAV rep expression, increase AAV vector DNA production and/or decrease AAV cap expression for at least 5 days, at least 10 days, at least 20 days, at least 30 days, at least 40 days, at least 50 days, at least 60 days, at least 70 days, at least 80 days, at least 90 days, at least 100 days or more. Preferably, modulation of one or more gene and/or protein of a producer cell line independently increases AAV rep expression, increases AAV vector DNA production and/or decreases AAV cap expression for at least 40 days. Again, the duration of the effect may be compared with a control, as described herein.

Modulation of one or more gene and/or protein of a producer cell line may independently increase AAV rep expression, increase AAV vector DNA production and/or decrease AAV cap expression for at least one, at least two, at least three, at least four, at least five, at least ten, at least 20, at least 30, at least 40 or more passages of the producer cell line in culture.

Modulation of one or more gene and/or protein of a producer cell line may independently increase AAV rep expression, increase AAV vector DNA production and/or decrease AAV cap expression indefinitely.

There may be a time and dose dependence of the modulation of the one or more gene and/or protein on AAV rep expression, AAV vector DNA production and/or AAV cap expression. Typically, even at a low dose and early time point, it may be possible to achieve a significant fold effect on AAV rep expression, AAV vector DNA production and/or AAV cap expression.

The increase AAV rep expression, increase AAV vector DNA production and/or decrease AAV cap expression may be measured using any appropriate technique. Standard techniques for measuring viral titres are known in the art.

Modulation of one or more gene and/or protein of a producer cell line may independently increase AAV rep expression, increase AAV vector DNA production and/or decrease AAV cap expression by 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 150%, at least 200% or more. Preferably, modulation of one or more gene and/or protein of a producer cell line independently increases AAV rep expression, increases AAV vector DNA production and/or decreases AAV cap expression by at least 50%.

Production of the AAV Vector

As described herein, the present invention provides a producer cell line in which the expression of one or more gene and/or protein listed in Table 2 is modulated compared with a control.

In a preferred embodiment, modulation of the one or more gene and/or protein is achieved by shRNA or CRISPR genome editing. If shRNA is used, typically the shRNA is tagged to facilitate removal of producer cells lacking the shRNA. If CRISPR genome editing is used, PCR primers can be designed which can generate two products (bands) due to the cleavage of gDNA in the positive knockout cells compared to the single PCR product/band in the non-cleaved/non-gRNA editing control cells. Again, this facilitates removal of producer cells in which the one or more gene of the invention is not modulated.

Once such a producer cell line of the invention has been established, it may be used to produce AAV vectors according to the present invention. Any appropriate technique may be used to generate AAV vectors according to the present invention. Standard techniques are known in the art.

The AAV vectors of the present invention may be engineered to carry any therapeutic polynucleotide for delivery and expression to a target cell or target cells. The therapeutic polynucleotide may be engineered into various sites within the AAV vectors, including but not limited to, the E1 region, the E2 region, the E3 region and the E4 region, using techniques that are well known to those skilled in the art (Current Protocols in Molecular Biology, Ausubel, F. et al., eds., Wiley and Sons, New York, 1995). The therapeutic polynucleotide cloned into the AAV vector may be engineered as a complete transcriptional unit, including a suitable promoter and polyadylation signal.

Thus, the AAV vectors of the present invention may comprise a therapeutic polynucleotide gene and a promoter between a pair of AAV-derived terminal repeats. The combination of a promoter and therapeutic polynucleotide is also referred to herein as a cassette.

The promoter sequence is operably linked to the therapeutic polynucleotide in a manner to effect expression of the gene. Hence, the promoter sequence can be at either or both ends of the therapeutic polynucleotide. Furthermore, more than one promoter and therapeutic polynucleotide can be present in one AAV vector, i.e. there can be two or more cassettes between the terminal repeats. Accordingly, more than one heterologous gene can be expressed by one vector.

Any promoter may be used in the AAV vector of the invention, provided the promoter is capable of driving expression of the therapeutic polynucleotide when they are operably linked. Such promoters are known in the art, including the AAV E1 promoter or E4 promoter, for example, as well as others including, but not limited to, the CMV promoter and the PGK promoter. The promoter may be tissue or cell preferred or specific, meaning that it drives expression of the therapeutic polynucleotide in either a particular tissue or cell type of interest. Again, such promoters are known in the art.

Suitable polyadenylation signals at the 3' end of the therapeutic polypeptide include, but are not limited to, the AAV polyadenylation signals. The E3 region of the AAV genome may be deleted in order to increase the cloning capacity of a vector, or it may be left in the vector construct.

The AAV vector of the invention typically comprises: 1) the terminal repeats mediate stable, site-specific integration into the cellular genome of an individual to be treated; and 2) the promoter

mediates expression of a therapeutic polynucleotide, or the promoter mediates transcription of an antisense RNA or a sense RNA encoding a polypeptide of interest.

The AAV vectors of the present invention can be constructed by a variety of standard methods known in the art, and the order of the ligation of the elements can be varied. A promoter and therapeutic polynucleotide may be ligated together to provide a cassette which can be inserted between two AAV inverted terminal repeats (ITRs). Standard techniques for the construction of AAV vectors of the invention are known in the art and can be found in references such as Sambrook et al. (1989: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y.), or any of the myriad of laboratory manuals on recombinant DNA technology that are widely available. A variety of strategies are available for ligating fragments of DNA, the choice of which depends on the nature of the termini of the DNA fragments and can be readily determined.

For example, standard techniques for producing AAV vectors according to the present invention include the transfection of DNA plasmid(s) into the producer cells to express AAV Capsid and Rep proteins; the co-transfection of a DNA plasmid to express a AAV vector genome that is in the forms of single stranded DNA or self-complementary DNA and comprises two AAV inverted terminal repeats (ITR) derived from any AAV serotypes flanking a therapeutic and/or reporter gene(s) to exert biological or therapeutic functions in recipient cells or patients; and with or without (i.e. in a helper-free system) the introduction, via plasmid DNA co-transfection or virus infection, of the AAV helper element(s) from a AAV helper virus, e.g. Adenovirus and Herpes simplex virus.

Other nucleotide sequence elements which facilitate integration of DNA into chromosomes, expression of the DNA, and cloning of the vector may be included in the AAV vector of the invention. For example, the presence of enhancers upstream of the promoter or terminators downstream of the therapeutic polynucleotide can facilitate expression.

As disclosed herein, the AAV vectors of the invention also comprise AAV capsid proteins. Typically, these AAV capsid proteins are designated VP1, VP2 and VP3. These AAV capsid proteins are typically encoded on the AAV DNA genome, enabling the production of assembled AAV viral particles.

Producer Cell Lines

According to the present invention, a producer cell line may be defined as a cell line capable of replicating and packaging an AAV vector. Any appropriate producer cell line may be modified and

used according to the present invention. A producer cell line of the invention is a eukaryotic cell line, and typically a mammalian cell line. The AAV vectors produced by the present invention are usually intended for therapy in humans. Therefore, preferably a producer cell line of the invention is a human cell line. A producer cell line of the invention may be selected from NIH3T3, HT1080, A549, HeLa cells, and HEK 293T cell lines. A producer cell line of the invention may be in an adherent or suspension form.

In a preferred embodiment, the producer cell line is the human embryonic kidney (HEK) 293T cell line. The HEK293T cell line expresses the SV40 early region under the transcriptional control of the Rous sarcoma virus long terminal repeat promoter.

As referred to herein, a control producer cell line is one which has not been modified or adapted according to the present invention.

Therapeutic Applications

The AAV vectors of the invention may be used as gene therapy vectors. Gene therapy involves the transfer and insertion of new genetic information into cells. The genetic information may be transiently or stably inserted into cells. The AAV vectors of the present invention are safe for gene therapy. Thus, the AAV vectors of the present invention may be capable of site-specific integration into a mammalian chromosome without substantial cytotoxicity, and which direct host cell-specific expression of a therapeutic polynucleotide. Preferably the AAV vectors of the invention are used in gene therapy in mammals, more preferably humans.

The AAV vectors of the invention may comprise any polynucleotide which can be used to treat or prevent a disease or condition. The therapeutic polynucleotide may be DNA or RNA. Typically the therapeutic polynucleotide is DNA. The therapeutic polynucleotide is preferably a biologically functional gene which targets (replaces) a non-functional and/or mutated gene in the individual to be treated. In preferred embodiments, when the individual to be treated is human, the therapeutic polynucleotide is also human.

The therapeutic polynucleotide may encode a biologically functional protein, i.e. a polypeptide or protein which affects the cellular mechanism of a cell in which the biologically functional protein is expressed. For example, the biologically functional protein can be a protein which is essential for normal growth of the cell or for maintaining the health of an individual. The biologically functional protein can also be a protein which improves the health of an individual by

either supplying a missing protein, by providing increased quantities of a protein which is under-produced in the individual or by providing a protein which inhibits or counteracts an undesired molecule which may be present in the individual. The biologically functional protein can also be a protein which is a useful protein for investigative studies for developing new gene therapies or for studying cellular mechanisms.

The biologically functional protein can be a protein which is essential for normal growth or repair of the body. The biologically functional protein may also be one which is useful in fighting diseases such as cancer. The biologically functional protein may also be a selectable marker for antibiotic resistance such as a selectable marker for neomycin resistance in eukaryotes. Other types of selectable markers may also be used. The therapeutic polynucleotide encoding these proteins can be provided by any of a variety of methods, such as routine cloning procedures (Sambrook et al.), excision from a vector containing the gene of interest, or chemical or enzymatic synthesis based on published sequence information. In many instances the DNA encoding the protein of interest is commercially available.

The biologically functional protein can affect cellular mechanism by providing a new or altered function to a cell. For example, the therapeutic polynucleotide can be a multidrug resistance gene (mdr) which encodes P-glycoprotein. P-glycoprotein is a cell membrane glycoprotein which affects intracellular drug accumulation and is responsible for the phenomenon of multidrug resistance.

The therapeutic polynucleotide can encode a non-biologically functional protein. For example, a hybrid gene comprising various domains and functions from a variety of sources can be designed and produced by recombinant technology or enzymatic or chemical synthesis.

The therapeutic polynucleotide may be capable of being transcribed into an RNA molecule which is sufficiently complementary to hybridize to an mRNA or DNA of interest, i.e. a sense or antisense RNA. Such RNA molecules may be useful in preventing or limiting the expression of overproduced, defective, or otherwise undesirable molecules. The AAV vector of the present invention can comprise, as the therapeutic polynucleotide, a sequence encoding an antisense RNA which is sufficiently complementary to a target sequence such that it binds to the target sequence. For example, the target sequence can be part of the mRNA encoding a polypeptide such that it binds to and prevents translation of mRNA encoding the polypeptide. The target sequence may be a segment of a gene that is essential for transcription such that the antisense RNA binds the segment (e.g. a promoter or coding region) and prevents or limits transcription. Hence, the antisense RNA

must be of sufficient length and complementarity to prevent translation of its target mRNA or transcription of its target DNA. Antisense RNAs having sufficient complementarity to a target sequence such that the antisense RNA is capable of binding to the target and thereby inhibiting translation or transcription can be determined using standard techniques. The therapeutic polynucleotide can be provided, for example, by chemical or enzymatic synthesis, or from commercial sources.

The function of the AAV vectors of the present invention, i.e. the ability to mediate transfer and expression of therapeutic polynucleotide, can be evaluated by monitoring the expression of the therapeutic polynucleotide in transduced cells. For example, cells may be transfected with an AAV vector of the present invention or infected with varying concentrations of virions containing said AAV vector and then assessed for the expression of the therapeutic polynucleotide.

The assay for expression depends upon the nature of the therapeutic polynucleotide. Expression can be monitored by a variety of methods including immunological, histo-chemical or activity assays. For example, Northern analysis can be used to assess transcription using appropriate DNA or RNA probes. If antibodies to the polypeptide encoded by the therapeutic polynucleotide are available, Western blot analysis, immunohistochemistry or other immunological techniques can be used to assess the production of the polypeptide. Appropriate biochemical assays can also be used if the therapeutic polynucleotide is an enzyme. For example, if the therapeutic polynucleotide encodes antibiotic resistance, a determination of the resistance of infected cells to the antibiotic can be used to evaluate expression of the antibiotic resistance gene.

In addition to assessing that the heterologous gene is expressed in the appropriate cells, the correct promoter specificity of the AAV vectors can be evaluated by monitoring the expression of the therapeutic polynucleotide, or lack of expression, in cells in which the promoter is not expected to be active.

The therapeutic polynucleotide of the invention may be a gene selected from CFTR for CF, α1-antitrypsin for emphysema, soluble CD4 for AIDS, ADA for adenosine deaminase deficiency and any other gene recognised as being potentially useful for gene therapy.

The AAV vectors of the present invention can be adapted to *ex vivo* and *in vitro* gene therapy applications.

The AAV vectors of the present invention may also be used to deliver other therapeutics. For example, the AAV vectors of the present invention may be used to deliver small molecule, peptide and/or protein therapeutics.

The AAV vectors of the present invention may be used in combination with other therapeutic agents and/or therapeutic methods. In particular, the AAV vectors of the invention may be used in combination with standard therapeutic agents and/or methods in the treatment of diseases including, but not limited, to cancer. AAV induced immunity can enhance the antibody function. Therefore, one example of a therapeutic use according to the present invention is the use of AAV vectors of the invention to deliver therapeutic antibodies, or the use of AAV vectors of the invention in combination with therapeutic antibodies.

Accordingly, the present invention provides an AAV vector as described herein for use in a method of therapy. The invention also provides the use of an AAV vector as described herein in the manufacture of a medicament for gene therapy.

The following Examples illustrate the invention

EXAMPLES

Example 1 – Vector Production

Two forms of vector particles, that is, complete and empty (without any transgene sequence) particles from each of the three AAV serotypes, i.e. AAV2, AAV5 and AAV8, were produced and investigated. The complete vector particles contained a vector sequence encoding GFP and the empty vector particles were capsids without a vector sequence.

Complete AAV particles were expressed from 3 plasmids pHelper (Stratagene, USA), pAAV-RC encoding AAV Rep-Cap and pAAV-hrGFP (Stratagene, USA) and empty AAV capsids expressed from only pHelper and pAAV-RC. Plasmid pAAV2/2 Rep-Cap was purchased from Stratagene (USA) and, plasmids pAAV-2/5-RC and AAV-2/8-RC were kindly provided by Professor James Wilson (NIDCR /Pennsylvania, USA).

All vectors were produced by transient transfection of human embryonic kidney 293T cells (Stratagene, USA) using the Calcium phosphate-BBS method (Chen et al., (1988) *Biotechniques* 6: 632-638). Vector producer cells were harvested and were subjected to 5 cycles of freeze thaw to release vector particles and the cellular debris was removed by centrifugation (2000g).

The supernatant containing vector particles was then filtered through 0.45µm filters (Millipore, UK) and diluted with 20mM Bis-Tris Propane buffer prior to chromatography. A Gilson HPLC system (Anachem, UK) was used for chromatography, which was equipped with a UV-Detector (Gilson, 119), pump (Gilson 306), autosampler (Gilson 231XL) and fraction collector (Gilson FC203B) both fitted with temperature controlled racks connected to a refrigerated re-circulating water bath (Grant, SLS) which also cooled the water-jacketed column. The system was controlled using Gilson Unipoint software. An XK 16/26 column (Amersham, UK) was packed to contain a bed volume of 5ml AVB Sepharose High Performance medium (GE Healthcare, Sweden), and performed following the manufacturer's protocol.

The AVB Sepharose affinity chromatography used in this study was designed for the purification of Adeno-associated viruses. Figure 1A shows a typical protein separation using a 5ml column containing AVB Sepharose coupled with AAV antibodies and that majority proteins were not bound to AAV antibodies and thus flowed through the column before the elution buffer (50mM Glycine, pH2.7) being applied (Figure 1A). Both empty capsids and complete vectors were eluted at 25-35 minutes of migration time (Figure 1A). The protein profile of eluted samples was then visualised using SDS-PAGE and silver staining (Figure 1B), showing significant purity of AAV samples after chromatography. As expected, three dominant AAV capsids proteins VP1 (81kDa), VP2 (72kDa) and VP3 (62kDa) were evident. Crude un-purified AAV samples showed many protein bands.

Example 2 – Identification of Proteins from the AAV Vectors using LC-MS/MS

Purified vector particles from Example 1 were pooled and dialysis to equilibrate into PBS in a 10K MWCO Slide-A-Lyzer dialysis cassette (Thermo scientific, USA) and concentrated to 1/20 of initial volume using Ultra 5K MWCO centrifugal filter devices (Millipore, UK) before being subjected to vector quantitation.

Vector quantitation Genome titre of purified vectors was determined using SYBR green dye real-time qPCR with AAV2 ITR primers GGAACCCCTAGTGATGGAGTT (SEQ ID NO: 24) and CGGCCTCAGTGAGCGA (SEQ ID NO: 25) and probe CACTCCCTCTCTGCGCGCTCG (SEQ ID NO: 26), with the probe labelled at the 5' end with 6-carboxyfluoescein (6-FAM) and at the 3' end with carboxytetramethylrhodamine (TAMRA). A standard curve was generated with plasmid pAAV2-hr-GFP. Total protein was measured using Pierce BCA protein assay kit (Thermo scientific, USA) and following manufacturer's protocol. The identity and the predicted amount of capsids protein were visualised using SilverXpress staining kit (Invitrogen, USA) according to manufacturer's protocol.

Purified and concentrated vector samples were digested with trypsin in the presence of 1% Rapigest and 50 mM ammonium bicarbonate (ABC) pH 8.5 for 3 hrs at 37°C. The digestion was then terminated by adding HCl before the samples were subjected to MS analysis.

LC-MS/MS was carried out using a mass spectrometry system (Thermo Fisher, UK) equipped with a nano-electrospray ion source and two mass detectors i.e. linear trap (LTQ) and orbitrap, coupled with an Ultimate 3000 nano-LC system, comprising a solvent degasser, a loading pump, a nano-pump, and a thermostated autosampler. After an automated injection, the extracted peptides from each digestion were desalted in a trapping cartridge (PepMap reversed phase C18, 5 µm 100 Å, 300µ id x 5 mm length) (Thermo) and eluted on to a C18 reversed phase nano-column (3 µm, 100Å, 5 cm length) (Thermo), and followed by a 60 min separation under a column flow rate of 0.3 µl/min using linear gradient from 5–70% acetonitrile and 0.1% formic acid. After a first survey MS scan (from m/z 400 – 2000) in the LTQ, the 5 most intense ions were sequentially isolated and passed to the Orbi-trap for accurate mass measurement with the resolution of 30,000ppm. These were then fragmented in the linear ion trap at collision induced energy of 35%. The total cycle time was approximately 30 milliseconds. Data was collected in data dependent MS/MS mode with dynamic exclusion set to 2 counts.

Data analysis including mass spectra processing and database searching was carried out using Thermo Proteome Discoverer 1.2.with built-in Sequest. Initial mass tolerances for protein identification by MS were set to 10 parts per million (ppm). Up to two missed tryptic cleavages were considered and methionine oxidation was set as dynamic modification. Peptide sequences by MS/MS were only included when Xcorrelation scores were greater than 1.5, 2 or 2.2 for charge states 1, 2 and 3, respectively. An unambiguous identification was considered when at least two peptides matched to the protein. The protein FASTA databases were downloaded from www.uniprot.org, release 2012-03 including the complete entries from *Homo sapiens* (taxon identifier 9606) *Bos taurus* (9913), green fluorescent protein (P42212) and AAV2 (648242), AAV5 (82300) and AAV8 (202813).

Equal amount of total proteins from six different types of purified AAV vector samples, i.e. AAV2-GFP, AAV2-Empty, AAV5-GFP, AAV5-Empty, AAV8-GFP and AAV8-Empty, were subjected to LC-MS/MS analysis. To minimise data variation, three batches of samples were prepared for each type of vectors with each batch pooled from 40 tissue culture plates (150mm diameter). Three MS runs were performed for each batch of samples. The results showed that 66 proteins were detected in at least 2/3 runs of three batches of samples and were thus considered to be significant and to be further studied. These proteins are listed in Table 2.

Among the significant proteins:

- four proteins, i.e. GapDH, Heat shock 70 kDa protein 1A/1B, Histotone H2Atype 1H and Histone H2B were common to all 6 types of AAV vectors regardless of serotypes and particle forms;
- six proteins shared by 5 types of vectors;
- out of 10 proteins shared by four forms of samples, six proteins shared by both empty and complete forms of AAV2 and AAV5, indicating a relative similarity between AAV2 and AAV5 vectors;
- 20 proteins were common to two types of vectors, the majority of which were common to the empty and complete forms of the same serotype; and
- 21 proteins were unique to individual types of vectors.

Table 2 – List of Proteins Identified in MS/MS Studies

	Proteins ID	AAV2E	AAV2G	AAV5E	AAV5G	AAV8E	AAV8G
Shared by 6 (4 proteins)	Glyceraldehyde-3-phosphate dehydrogenase (Fragment) OS=Homo sapiens GN=GAPD PE=2 SV=1 - [Q5ZEY3_HUMAN]	+	+	+	+	+	+
	Heat shock 70 kDa protein 1A/1B OS=Homo sapiens GN=HSPA1A PE=1 SV=5 - [HSP71_HUMAN]	+	+	+	+	+	+
	Histone H2A type 1-H OS=Homo sapiens GN=HIST1H2AH PE=1 SV=3 - [H2A1H_HUMAN]	+	+	+	+	+	+
	Histone H2B OS=Homo sapiens PE=2 SV=1 - [A8K9J7_HUMAN]	+	+	+	+	+	+
Shared by 5 (6 proteins)	60S acidic ribosomal protein P2 OS=Homo sapiens GN=RPLP2 PE=1 SV=1 - [RLA2_HUMAN]	+	+	+	+		+
	Alpha-enolase OS=Homo sapiens GN=ENO1 PE=1 SV=2 - [ENOA_HUMAN]	+	+	+	+		+
	Nucleolin, isoform CRA_c OS=Homo sapiens GN=NCL PE=2 SV=1 - [B3KM80_HUMAN]	+	+	+	+		+
	Nucleophosmin OS=Homo sapiens GN=NPM1 PE=1 SV=2 - [NPM_HUMAN]	+	+	+	+		+
	YBX1 protein (Fragment) OS=Homo sapiens GN=YBX1 PE=2 SV=1 - [Q6PKI6_HUMAN]	+	+	+	+		+

	RuvB-like 2 OS=Homo sapiens GN=RUVBL2 PE=1 SV=3 - [RUVB2_HUMAN]	+	+	+	+		+
shared by 4 (10 proteins)	Annexin A5 OS=Homo sapiens GN=ANXA5 PE=1 SV=2 - [ANXA5_HUMAN]	+	+	+	+		
	Brain acid soluble protein 1 OS=Homo sapiens GN=BASP1 PE=1 SV=2 - [BASP1_HUMAN]	+	+	+	+		
	Histone H2A.x OS=Homo sapiens GN=H2AFX PE=1 SV=2 - [H2AX_HUMAN]	+	+			+	+
	MARCKS protein (Fragment) OS=Homo sapiens GN=MARCKS PE=2 SV=1 - [Q6NVI1_HUMAN]	+	+	+	+		
	MYL6 protein OS=Homo sapiens GN=MYL6 PE=2 SV=1 - [Q6IBG5_HUMAN]	+	+	+	+		
	Nascent polypeptide-associated complex alpha subunit (CypA) (Fragment) OS=Homo sapiens GN=NACA PE=4 SV=1 - [F8W1N5_HUMAN]	+	+	+	+		
	Peptidyl-prolyl cis-trans isomerase (Fragment) OS=Homo sapiens GN=PPIH PE=3 SV=1 - [C9JQD4_HUMAN]	+	+			+	+
	Putative uncharacterized protein (Fragment) OS=Homo sapiens PE=2 SV=1 - [Q8WVW5_HUMAN]	+		+		+	+
	RuvB-like 1 (Fragment) OS=Homo sapiens GN=RUVBL1 PE=2 SV=1 - [B5BUB1_HUMAN]	+	+	+		+	
	Triosephosphate isomerase (Fragment) OS=Homo sapiens PE=2 SV=1 - [Q53HE2_HUMAN]	+	+	+		+	
shared by 3 (5 proteins)	78 kDa glucose-regulated protein OS=Homo sapiens GN=HSPA5 PE=1 SV=2 - [GRP78_HUMAN]			+	+	+	
	ATP synthase subunit beta (Fragment) OS=Homo sapiens GN=ATP5B PE=2 SV=1 - [Q0QEN7_HUMAN]			+	+	+	
	Chaperonin 10-related protein (Fragment) OS=Homo sapiens GN=EPFP1 PE=2 SV=1 - [Q9UNM1_HUMAN]			+	+	+	

	L-lactate dehydrogenase B chain OS=Homo sapiens GN=LDHB PE=1 SV=2 - [LDHB_HUMAN]	+	+			+	
	Synaptic vesicle membrane protein VAT-1 homolog OS=Homo sapiens GN=VAT1 PE=1 SV=2 - [VAT1_HUMAN]	+	+	+			
shared by 2 (20 proteins)	Actin, gamma 1 OS=Homo sapiens GN=ACTG1 PE=3 SV=1 - [F5H0N0_HUMAN]			+	+		
	Annexin A2 (Fragment) OS=Homo sapiens GN=ANXA2 PE=4 SV=1 - [H0YKZ7_HUMAN]			+	+		
	ATP synthase subunit alpha OS=Homo sapiens PE=2 SV=1 - [B4DY56_HUMAN]			+	+		
	ATP synthase-coupling factor 6, mitochondrial OS=Homo sapiens GN=ATP5J PE=1 SV=1 - [ATP5J_HUMAN]			+	+		
	Capsid protein VP1 OS=Adeno-associated virus 2 (isolate Srivastava/1982) PE=1 SV=2 - [CAPSD_AAV2S]	+	+				
	Capsid protein OS=Adeno-associated virus - 5 GN=cap PE=1 SV=1 - [Q9YIJ1_9VIRU]			+	+		
	Capsid protein OS=Adeno-associated virus - 8 PE=1 SV=1 - [Q8JQF8_9VIRU]					+	+
	Cofilin 1 (non-muscle) (Fragment) OS=Homo sapiens GN=CFL1 PE=4 SV=1 - [E9PLJ3_HUMAN]			+	+		
	Complement component 1 Q subcomponent-binding protein, mitochondrial OS=Homo sapiens GN=C1QBP PE=1 SV=1 - [C1QBP_HUMAN]			+	+		
	Erythrocyte membrane protein band 4.1-like 3 OS=Homo sapiens GN=EPB41L3 PE=2 SV=1 - [A8K968_HUMAN]			+	+		
	Heterogeneous nuclear ribonucleoprotein A1 (Fragment) OS=Homo sapiens GN=HNRNPA1 PE=4 SV=1 - [F8VTQ5_HUMAN]					+	+

	Heterogeneous nuclear ribonucleoprotein K (hnRNPK) OS=Homo sapiens GN=HNRNPK PE=2 SV=1 - [Q5T6W5_HUMAN]				+		+
	Myosin-10 OS=Homo sapiens GN=MYH10 PE=1 SV=3 - [MYH10_HUMAN]			+	+		
	Myosin-9 OS=Homo sapiens GN=MYH9 PE=1 SV=4 - [MYH9_HUMAN]			+	+		
	Pyruvate kinase OS=Homo sapiens PE=2 SV=1 - [B4DNK4_HUMAN]			+		+	
	Ribosomal protein L5, isoform CRA_b OS=Homo sapiens GN=RPL5 PE=2 SV=1 - [B3KTM6_HUMAN]	+				+	
	Serine/arginine repetitive matrix protein 1 OS=Homo sapiens GN=SRRM1 PE=1 SV=2 - [SRRM1_HUMAN]	+	+				
	Single-stranded DNA binding protein 1 (Fragment) OS=Homo sapiens GN=SSBP1 PE=4 SV=1 - [C9K0U8_HUMAN]					+	+
	Tubulin, alpha 1b (Fragment) OS=Homo sapiens GN=TUBA1B PE=4 SV=1 - [F8VRK0_HUMAN]	+				+	
	Uncharacterized protein OS=Homo sapiens GN=SLC1A5 PE=2 SV=1 - [B4DWS4_HUMAN]			+	+		
Unique (21 proteins)	Adenylyl cyclase-associated protein OS=Homo sapiens PE=2 SV=1 - [B4DI38_HUMAN]					+	
	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (Fragment) OS=Homo sapiens GN=ATP5B PE=4 SV=1 - [H0YH81_HUMAN]			+			
	DNA-binding protein A OS=Homo sapiens GN=CSDA PE=1 SV=4 - [DBPA_HUMAN]					+	
	Elongation factor Tu, mitochondrial OS=Homo sapiens GN=TUFM PE=1 SV=2 - [EFTU_HUMAN]			+			
	Enolase (Fragment) OS=Homo sapiens GN=ENO1 PE=2 SV=1 - [Q9BT62_HUMAN]			+			
	EPB41 protein (Fragment) OS=Homo sapiens GN=EPB41 PE=2 SV=1 - [Q4VB87_HUMAN]						

			+			
HSP90AB1 protein (Fragment) OS=Homo sapiens GN=HSP90AB1 PE=2 SV=1 - [Q6PK50_HUMAN]			+			
IMP (inosine 5'-monophosphate) dehydrogenase 2 (Fragment) OS=Homo sapiens GN=IMPDH2 PE=4 SV=1 - [H0Y4R1_HUMAN]			+			
Insulin-like growth factor 2 mRNA binding protein 1, isoform CRA_a OS=Homo sapiens GN=IGF2BP1 PE=4 SV=1 - [D3DTW3_HUMAN]			+			
Liver histone H1e OS=Homo sapiens PE=2 SV=1 - [A3R0T7_HUMAN]					+	
Major coat protein Aa OS=Adeno-associated virus 2 (isolate Srivastava/1982) PE=4 SV=1 - [Q89269_AAV2S]					+	
Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase OS=Homo sapiens GN=MTHFD1 PE=3 SV=1 - [G3V2B8_HUMAN]				+		
Nucleolar RNA helicase 2 OS=Homo sapiens GN=DDX21 PE=1 SV=5 - [DDX21_HUMAN]				+		
Ornithine aminotransferase, mitochondrial OS=Homo sapiens GN=OAT PE=1 SV=1 - [OAT_HUMAN]			+			
Proteasome (prosome, macropain) 26S subunit, ATPase, 3 OS=Homo sapiens GN=PSMC3 PE=3 SV=1 - [E9PM69_HUMAN]			+			
Rep78 OS=Adeno-associated virus-Go.1 PE=4 SV=1 - [Q2LD61_9VIRU]			+			
SFPQ protein (Fragment) OS=Homo sapiens GN=SFPQ PE=2 SV=2 - [Q9BSV4_HUMAN]					+	
Signal recognition particle 72kDa, isoform CRA_c OS=Homo sapiens GN=SRP72 PE=4 SV=1 - [G5E9Z8_HUMAN]			+			

	Small nuclear ribonucleoprotein polypeptide B OS=Homo sapiens PE=2 SV=1 - [Q5XPV6_HUMAN]			+			
	THO complex subunit 4 OS=Homo sapiens GN=ALYREF PE=1 SV=3 - [THOC4_HUMAN]			+			
	TUBA1B protein OS=Homo sapiens GN=TUBA1B PE=2 SV=1 - [Q8WU19_HUMAN]			+			

Example 3 – Validation of MS data

In order to validate the mass spectrometry data obtained in Example 2, two categories of proteins identified using MS were selected for immunoblotting: (i) proteins NPM1, NCL1 and ATP5A that have an documented role in AAV life cycle; and (ii) proteins Annexin V, RuvB, CypA, hnRNPK and YB1 that have no reported association with AAV but have a relatively high score and confidence in MS analysis.

10% SDS polyacrylamide gel was used to resolve protein samples and the gels then subjected to immunoblotting. Samples for immunoblotting were electrophoresed and electroblotted to Hybond ECL membranes (Amersham,UK). The following primary antibodies were used: mouse anti AAV2, mouse anti Annexin V (Abcam, UK), anti nucleolin (Abcam, UK), anti nucleophosmin (Abcam, UK), anti EF1β2 (Abcam, UK), anti CypA (Abnova, UK), anti RuBV2 (Abcam, UK), anti hnRNP K (Abcam, UK), anti ATP5A (Abcam, UK and anti YB1 (Abcam, UK). The immunoblots were further incubated with goat anti-mouse, anti-rabbit or rabbit anti-goat horseradish peroxidase conjugates (Sigma, UK). The immuno-reactive proteins were detected using ECL chemiluminescence reagents (Amersham, UK).

For each sample, 10 µg of total protein from the same samples used for MS/MS were resolved using SDS/PAGE before being subjected for immunoblotting. The total protein was visualised using SDS/PAGE and silver staining (Figure 1B) , confirming sample purity and a comparable amount of AAV capsid proteins VP1, VP2 and VP3

The immunoblotting showed that RuvB2, CypA and Annexin A5 were detected in all samples tested (Figures 2A and B). YB1 was detected in all samples except AAV8 empty capsids (Figure 2C), regardless of them being crude or purified. To discriminate between incorporated and co-purified but unincorporated cellular proteins in AAV vectors, proteinase K (PK) was used to remove trace unincorporated cellular proteins that remained in purified vectors and were not protected by vector capsid. Figure 2D showed a comparable detection of YB1 proteins in purified AAV vectors before and

after PK treatment, indicating YB1 incorporation in AAV vectors. Furthermore, there was no detection of YB1 proteins in purified control 293T cell that had been treated and HPLC-purified in parallel with AAV vectors (lane unpurified, Figure 2D), highlighting the specificity of the adopted AVB column for AAV vector purification and further demonstrating the incorporation of YB1 proteins in AAV vectors. hnRNPK was not detected in any of the samples tested (data not shown).

The results from the immunoblotting and MS studies were further summarized and compared in Table 3, showing a 22.9% (11/48 sample s tested, highlighted with circles) difference and 77.1% agreement in protein detection between these two different methods.

Example 4 – Influence of AAV Production on Protein Expression in Producer Cells

In order to investigate potential influences of AAV production on the expression of both viral and cellular proteins in producer cells, changes in the expression of AAV capsid proteins, Annexin A5, CypA and YB1 were analysed throughout one complete cycle of AAV production, i.e. from 0 hour before the transfection of AAV and helper plasmids to 4, 6, 24 30, 48, and 72 hours after transfection when vector production process was terminated and AAV vectors harvested. AAV capsid proteins (VP1, VP2 and VP3) and Rep proteins could be detected 24 hours after transfection in AAV2 producer cells (Figures 3A and 3B). A slight increase in the capsid proteins in producer cells was observed from 24 to 72 hours after transfection. Throughout a complete cycle of AAV production, no significant changes were observed in YB1 and CypA expression (Figure 3C); over-time increase in Annexin A5 expression was observed in all three serotypes (Figure 3D).

Table 3 – Comparison of Immunoblotting and MS Results

Serotype / Forms / Protein	Immunoblotting						MS					
	AAV2		AAV5		AAV8		AAV2		AAV5		AAV8	
	E	G	E	G	E	G	E	G	E	G	E	G
RuVB	+	+	+	+	+	+	+	+	+	+	(-)	+
CypA	+	+	+	+	+	+	+	+	+	+	(-)	(-)
Annexin A5	+	+	+	+	+	+	+	+	+	+	(-)	(-)
ATPA5	-	-	+	+	+	+	+	+	+	+	(-)	+
NPM1	+	-	+	-	+	-	+	(+)	+	(+)	+	-
YB1	+	+	+	+	+	+	+	+	+	+	(-)	+
NCL	+	+	+	+	+	-	+	+	+	+	+	-
hnRNPK	-	-	-	-	-	-	-	-	-	(+)	-	(+)

Example 5 – shRNA Knockdown of NPM1, NCL and YB1

Functional shRNA specifically recognizing a target gene results in down-regulation of the expression of that gene. This strategy was selected as an alternative to study the role of cellular proteins in AAV assembly, as it was not possible to establish cells in culture to overexpress NCL and NPM1(data not shown) .

On the assumption that shRNA knockdown of NPM1, NCL, Annexin V and/or YB1 in producer cells would impair the (MS/MS and immunoblot identified) association of NPM1, NCL, Annexin V and/or YB1 with AAV virus and might subsequently influence AAV production in the gene-knockdown cells, a Lentiviral vector delivery system was used to screen shRNA sequences.

Functional shRNA specifically recognizing a target gene causes down-regulation of the targeted gene expression. To identify the role and importance of YB1 and Annexin A5 in AAV production, experiments were designed knockdown YB1 or Annexin A5 in producer cells using shRNA, the hypothesis being that this knockdown would impair the association of YB1 or Annexin A5 with AAV virus (as identified from the MS/MS and immunoblotting studies) and would subsequently influence AAV assembly in the gene-knockdown cells.

A Lentiviral vector delivery system was used to screen 10 shRNA sequences (A1 to A5, SEQ ID NOs: 19 to 23, and Y1 to Y5, SEQ ID NOs: 14 to 18) targeting Annexin A5 and YB1 for their ability to deregulate Annexin A5 and YB1 respectively.

In more detail, the shRNAs were expressed from plasmid pLKO.1-puro (Sigma, USA) using a Lentiviral expression system. shRNA and lentiviral vector packaging plasmids were transfected into 293T cells using the $CaCl_2$ transfection method. Control cells were transfected with empty capsids without a shRNA sequence (mock) or a scramble shRNA sequence targeting non-mammalian gene sequences (scramble). 293T cells were transduced with LV-shRNA vectors, and were subjected to puromycin selection 48h after transduction.

AAV vector genome titre was determined by q-PCR with primers and probe targeting the CMV promoter or AAV2 ITR sequences. CMV primers: TTC CTA CTT GGC AGT ACA TCT ACG (SEQ ID NO: 30) and GTC AAT GGG GTG GAG ACT TGG (SEQ ID NO: 31); and CMV probe: TGA GTC AAA CCG CTA TCC ACG CCC A (SEQ ID NO: 32). AAV2 ITR primers GGAACCCCTAGTGATGGAGTT (SEQ ID NO: 24) and CGGCCTCAGTGAGCGA (SEQ ID NO: 25) and probe CACTCCCTCTCTGCGCGCTCG (SEQ ID NO: 26). Again, a 5' 6-FAM and a 3' TAMRA label were used. The plasmid DNA pAAV-hrGFP (Stratagene, USA) was used as reference standard in 10-fold serial dilution ranging from 102 to 108 copies. qPCR was carried out using a LightCycler480 (Roche, USA) under the condition of one 10 min cycle at 950C, followed by 45 cycles of 15 sec at 950C, 30sec at 600C and 5sec at 720C. The level of gene

knockdown was evaluated on 10ug total proteins from shRNA virus transduced cells following the immunoblotting method as described earlier.

As there was no reporter gene in the LV shRNA system to perform the conventional method to titre shRNA viruses, the gene knockdown studies had to be controlled and validated using a direct comparison by simultaneously producing all sample and control shRNA viruses under an identical condition and subsequently using equal volume of shRNA viruses to treat the same number of cells. Figure 4 shows that the shRNA sequences A2 and A5 significantly down-regulated Annexin A5 expression (Figure 4A). Likewise, introducing Y3, Y4 or Y5 into cells resulted in significant down-regulation of YB1 (Figure 4B) compared to controls with sequences targeting non-mammalian genes (scramble) or without a shRNA sequence (293T). Therefore, these 4 shRNA sequences, i.e. A2, A5, Y4 and Y5, were used for subsequent studies.

Producer cells carrying a shRNA sequence, i.e. A2, A5, Y4, Y5 or scramble, were established for AAV production by culture in the presence of puromycin for at least 10 days to remove the cells without puromycin-tagged shRNA. Up to 7 independent knockdown cell lines were generated for each shRNA sequence and were used to produce AAV vectors. To minimise variation, AAV vectors were produced from 15cm-diameter plates containing over 50 million cells and simultaneously from knockdown and control cell lines produced under identical conditions and were then quantified for AAV genome titres using Real Time PCR.

As shown in Figure 5, introducing shRNA sequence YB1_Y4 to producer cells resulted in up to 50 fold increase in AAV2 (Figure 5A) and 10 fold increase in AAV8 (Figure 5B) vector genome titres relative to control shRNA scramble and that the same Y4 shRNA sequence had no significant influence on AAV5 production (data not shown). This indicates an intrinsic difference among the three serotypes of AAV. Although the reduction of YB1 expression in the knockdown cells continued for over 80 days, the maximum effect of Y4 was only obtained in producer cells that had been in culture for 20-40 days (Figure 5A). A similar time-dependent pattern was also observed in AAV8 producer cells (Figure 5B).

Figure 6 shows significant down-regulation of YB1 in 7 batches of knockdown cells that had been recovered from cryo-preservation and had been in culture for 20 to 80 day, demonstrating the comparability among batches and the sustainability of YB1 knockdown cells.

Figure 7 shows that there was a slight increase in YB1 deregulation when increasing shRNA viruses from 5 to 100ml per 10^5 cells (Figure 7A) and that the maximum effect on vector genome titres was observed when 50ml viruses containing shRNA were used (Figure 7B), indicating a saturating and potential toxic effect on vector production when increasing the amount of viruses containing shRNA.

A Lentiviral vector delivery system was also used to screen 10 shRNA sequences, i.e. N1 to N5 and N6 to N10, targeting NCL and NPM1 genes for their effects on NCL and NPM1 expression respectively.

Figure 8A showed that introducing shRNA sequences NPM-N6 to NPM-N10 (SEQ ID NOs: 4 to 8) to cells achieved a significant down-regulation of NPM1 expression when compared to two control cells that were either with scramble shRNA targeting non-mammal genes (scramble) or without any shRNA sequences (293T). shRNA NPM-N6 and NPM-N9 (Figure 8A, circled) showed the most gene knockdown among the 5 sequences tested and were thus selected for subsequent investigations. The reduction of NPM1 expression in NPM1-N9 and NPM1-N6 knockdown cells was sustained for over 80 days after culture in puromycin selection medium (Figure 8B) and also in the knockdown cells recovered from cryopreservation (Figure 8C).

The 4 shRNA sequences targeting NCL gene, NCL-N1 to NCL-N4 (SEQ ID NOs: 9 to 12) showed a partial reduction of NCL expression, among which NCL-N1 and NCL-N4 had a greater effect (Figure 9A, circled) and were thus selected for further study. The knockdown effect of NCL-N1 and NCL-N4 on NCL expression also sustained for over 80 days after cultured in puromycin selection medium (Figure 9B) and after cryopreservation (data not shown).

Figure 10 shows that down-regulation of NPM1 with shRNA NPM1-N9 led to up to 35 fold increase in vector genome titres of AAV2/GFP (Figure 10A) and up to 30 fold in AAV8/GFP (Figure 10B), indicating an important role of NPM1 protein in AAV production. The up-regulating effect of NPM1 on vector genome titres showed chronological changes for both AAV2/GFP (Figure 10A) and AAV8/GFP (Figure 10B), with a maximum effect of an up to 30 fold increase observed in the knockdown producer cells in culture for around 40 days, and was sustained in producer cells for up to 90 after gene knockdown.

Down regulation of NCL showed similar up-regulating effect on AAV2 vector production with up to 40 fold increases in AAV2 genome titres. However, there existed a significant variation, ranging from 2-40 fold, among different batches of producer cells (Figure 11A). Gene knockdown of NCL had a lesser up-regulating effect (up to 10 fold increase) on AAV5 and AAV8 genome titres (Figure 11B and 11C, respectively) compared to AAV2. No significant chronological changes were observed from NCL knockdown producer cells (data not shown).

Example 6 – CRISPR Knockdown of YB1

CRISPR genome editing was used to knockout or knockdown YB1 expression as an alternative to shRNA. The gRNA sequences used in this study were designed using the CRISPR/Cas9

programme (https://chopchop.rc.fas.harvard.edu/) targeting entire Homo sapiens chromosome 1 sequence (Accession number NC_000001.11) that includes YB1 gene sequence. Four pairs of gRNA sequences (A, B, C and D) (Table 4). were selected and a GeneArt CRISPR nuclease vector kit (Life technology, cat number A21174) was used to generate YB1 gRNA knockout producer cells.

Two batches (B1 and B2) of CRISPR gRNA knockout cells were produced for each of the four CRISPR gRNA sequences A, B, C or D. Single cell clones (C1 to C5) were further derived from the parental B1 and B2 gRNA producer cells for each gRNA pair. Parental 293T cells without any gRNA sequences (293T) were used as a control; Housekeeping gene GAPDH was further used for sample loading normalisation.

Table 4: YB1 CRISPR gRNA pairs

gRNA pair	First sequence (5' to 3')	Second sequence (5' to 3')	Location
A	ATCGGCGGCGCCTGCCGGCGGTTTT (SEQ ID NO: 33)	CGCCGGCAGGCGCCGCCGATCGGTG (SEQ ID NO: 34)	within exon 1 of YB1 gene
B	GTAATGGCTTTTGTAGGGTGGTTT (SEQ ID NO: 35)	CACCCTACAAAAGCCATTACCGGTG (SEQ ID NO: 36)	within exon 5 of YB1 gene
C	GGACCATACCTGCGGAATCGGTTTT (SEQ ID NO: 37)	CGATTCCGCAGGTATGGTCCCGGTG (SEQ ID NO: 38)	within intron 6 of YB1 gene
D	CAAAGACAGCCTAGAAGGAGTTTT (SEQ ID NO: 39)	TCCTCTCTAGGCTGTCTTTGCGGTG (SEQ ID NO: 40)	within exon 7 of YB1 gene

YB1 gene knockout was observed in a total of 10 single cell clones. Figure 12 shows the effect on YB1 expression using these three gRNA pairs. Lanes labelled 293 refer to the 293 parental cell line. B1 and B2 are bulk knockout cells generated before single cell cloning. C1 to C5 are single cell clones generated from the bulk knockout cells.

As is clear from Figure 12; gRNA pair A significantly inhibited YB1 expression in clonal cell line C1; gRNA pair B significantly inhibited YB1 expression in bulk knockout cells B2 and clonal cell lines C1 to C4, as well as observably inhibiting YB1 expression in bulk knockout cells B1; and gRNA pair C significantly inhibited YB1 expression in bulk knockout cells B2 and clonal cell lines C2 to C4, as well as observably inhibiting YB1 expression in clonal cell lines C1 and C5.

Example 7 – Molecular analysis of YB1 knockdown producer cells

In order to understand the molecular mechanism of YB1 influence on AAV production, AAV2 protein (Rep and Cap) expression and vector DNA production in YB1 knockdown cells was

systematically analysed. Figure 13A shows a 12- and 4-fold increase in rep gene expression in YB1 knockdown cells compared to that in scramble cells 24 and 72 hours after transient transfection, respectively, indicating a negative effect of native YB1 on rep gene expression. In contrast, the production of total AAV2 capsid proteins in YB1 knockdown cells ($1.29 \pm 0.2 \times 10^{14}$ capsids/10^8 cells) was ~7-fold lower than that in scramble cells ($8.87 \pm 0.1 \times 10^{14}$ capsids/10^8 cells) (Figure 13B), indicating that the presence of native YB1 was beneficial for cap gene expression. Furthermore, the amount of capsid proteins in the harvested vector samples was comparable between YB1 knockdown cells ($6.27 \pm 0.3 \times 10^{13}$ capsids/10^8 cells) and scramble ($6.77 \pm 0.2 \times 10^{13}$ capsids/10^8 cells) cells (Figure 13B), indicating that vector capsid particle formation may be independent of the amount of capsid proteins produced in the cells, and that there was a limit in the number of particles to be formed in cells.

DNA integration was systematically analysed using realtime qPCR targeting U6 sequence for YB1 shRNA integration, and targeting rep and CMV promoter sequences for AAV2 packaging plasmids pRep/Cap and phrGFP integration, respectively. There were $1.3 \pm 0.17 \times 10^8$ copies/10^8 cells of YB1 shRNA sequences detected in YB1 knockdown cells, equivalent to an average of one copy of YB1 shRNA per cell in pooled YB1 knockdown cells. Integration of AAV packaging plasmid pRep/Cap and phrGFP were comparable between scramble and YB1 knockdown cells, at ~2×10^7 copies of Rep/Cap and ~3×10^7 copies of phrGFP per 10^8 cells.

To analyse the influence of YB1 gene knockdown on the production of AAV2 vector DNA, the copies of (1) total vector DNA that contains both packaged and unpackaged vector DNA, (2) unpackaged vector DNA, and (3) packaged vector DNA in YB1 knockdown cells 72 h after transient transfection of AAV packaging plasmids was systematically quantified using qPCR targeting vector-specific CMV sequence. Total vector DNA was prepared by removing plasmid DNA with benzenase, removing cell membranes and nuclei after sample freeze-thawing, and disassembling AAV capsid with proteinase K to release packaged vector DNA from the capsids. Non-integrated vector plasmid DNA may also contribute to the total vector DNA copies in cytoplasm; however, considering the transfection and production procedure was performed in parallel and was identical for both YB1 and scramble cells, the amount of plasmid DNA in cytoplasm should likewise be comparable and would not significantly alter the calculation of relative total vector DNA production. Figure 13C shows that the copies of total vector DNA (+PK) were ~13-fold higher in YB1 knockdown producer cells ($1.93 \pm 0.08 \times 10^{13}$ copies/10^8 cells) than that in scramble cells ($1.46 \pm 0.5 \times 10^{12}$ copies/10^8 cells) indicating that YB1 gene knockdown promoted the vector DNA production.

Unpackaged AAV2 vector DNA was prepared in a similar way as that for total DNA samples except without proteinase K treatment (-PK) to release packaged vector DNA from AAV capsid. The copies of the unpackaged vector DNA were ~8 x higher in YB1 knockdown cells ($2.02 \pm 0.5 \times 10^{12}$ copies/10^8 cells) than that in scramble cells ($2.61 \pm 0.8 \times 10^{11}$ copies/10^8 cells) (unpackaged DNA, Figure 13C). Packaged AAV2 vector DNA, representing physical vector genome titres, was prepared by the addition of DNAse I treatment (+ DNAse I) to remove unpackaged vector DNA in cytoplasm followed by proteinase K treatment (+ PK) to release the packaged vector DNA from capsids. The copies of packaged vector DNA (+ DNAse I/+ PK, Figure 13C) were 4-fold higher in YB1 knockdown cells ($7.97 \pm 0.5 \times 10^{10}$ copies/10^8 cells) than that in scramble cells ($2.16 \pm 0.3 \times 10^{10}$ copies/10^8 cells) (Figure 13C). It should be noted that in this particular set of molecular studies, we observed a 4-fold (significant) increases in AAV2 vector genome titres in YB1 knockdown cells compared to that in scramble cells.

In summary, the significant difference observed among the fold changes in total vector DNA (13 x increase), unpackaged (8 x increase), and packaged vector DNA (4 x increase) underlined a potential for the improvement of AAV vector DNA packaging in YB1 production system. Moreover, taking into consideration that the amount of capsid proteins was comparable (Figure 13B) but the copies of packaged DNA (Figure 13C) was 4-fold higher in the same batch of harvested vector samples from YB1 knockdown cells compared to that from scramble cells, the results revealed a considerable advantage of YB1 gene knockdown in reducing the number of empty particles in AAV products.

Discussion

The present inventors have demonstrated for the first time an important role of YB1 in AAV vector production. Introducing the shRNA sequence Y4 that targets and down-regulates YB1 gene to AAV producer cells resulted in up to 50 and 10 fold increase in vector genome titres of AAV2 and AAV8, respectively. Molecular characterization of YB1 knockdown cells showed an ~12-fold increases in rep expression, an ~13-fold increase in vector DNA production, and an ~7-fold decrease in cap expression in YB1knockdown cells compared to scramble cells, uncovering a significant role of the YB1 gene in AAV biology.

YB1 is a DNA and RNA-binding protein involved in almost all DNA and mRNA-dependent processes. YB1 packs and stabilizes mRNA to mediate gene regulation at different levels. YB1 binds to both double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA), but has a far higher binding affinity to ssDNA. It has been suggested that YB1 prevents binding of activating proteins by

stabilising ssDNA in the region of promoters or enhances in a sequence-specific manner. In particular, YB1 has highest binding affinity to the ss-DNA motif GGGG(TT). Analysis of the AAV2 ssDNA genome showed that such a single-stranded GGGG(TT) motif is presented within the AAV2 ITR region from nucleotide 137 to 142 of the AAV2 genome (NCBI sequence NC_001401.2), indicating a potential AAV DNA binding site for the YB1 protein. ITR deletion mutagenesis showed that the 20 nucleotide D sequence (from nucleotide 126--146), which covers the single-stranded GGGG(TT) motif and immediately follows the 125 nucleotide long hairpin, is required for the encapsidation of the AAV DNA genome and has thus been proposed as packaging signal for AAV; in particular, the N-terminal region of AAV capsid proteins binds to the D sequence resulting in the encapsidation of AAV ssDNA into preassembled AAV capsid. Therefore, the capability of both YB1 and AAV capsids for binding to the ITR D sequence region may impose competition between YB1 and AAV capsids and compromise encapsidation of AAV genome.

Analysis of the genome of AAV serotype 2 (AAV2) by the present inventors has shown that this GGGG(TT) motif is present within the inverted terminal repeat (ITR) region from positions 137 to 142 of the AAV2 genome (NCBI sequence NC_001401, version NC_001401.2 GI:110645916). This indicates a potential binding site for YB1 to the AAV2 genome. The present inventors have generated chimeric forms of the AAV5 and AAV8 serotypes comprising the AAV2 ITR (but with the AAV5 and AAV8 capsid proteins respectively). Therefore, if the GGGG(TT) motif was the sole binding site for YB1, it would be expected that YB1 would have a similar effect on virus particle production for AAV2 and the chimeric forms of AAV5 and AAV8. However, the present inventors have demonstrated there was a significant difference in the AAV vector titres relative to the scramble controls among the AAV2, AAV5 and AAV8 vectors produced in YB1 knockdown cells. In particular, the present inventors found that there was a 50-fold increase in AAV2 vector titre in producer cells with YB1 knockdown, compared with a 10-fold increase in AAV8 titre and no significant increase in AAV5 titre. This suggests that there may also be some AAV capsid protein-specific element to the effect of YB1 knockdown on AAV vector production, as well as the DNA sequence-specific effect.

There are three YB1 domains, namely A/P, CSD and CTD, which are involved in protein-protein interaction. In particular, YB1 protein is known to interact with important regulatory proteins, such as p53, Akt kinase, hnRNP K and TATA-binding protein. YB1 is also known to play an important role in the replication of a number of viruses by binding to viral proteins, such as HIV TAT, the large T antigen of polymavirus, Hepatitis C Virus (HCV) protein NS3/4A and influenza ribonucleoprotein (RNP). Indeed, shRNA knockdown of YB1 has been shown to result in a reduction of HCV titres of up to 80%. In that study, it was found that knockdown of YB1 did not influence the

expression of viral proteins, nor the production and stability of viral RNA, indicating that the knockdown of YB1 disrupted the formation of the YB1-NS3/4A vRNA interactome required for recruiting core proteins for virus assembly.

The role of YB1 in adenovirus replication is particularly relevant to the understanding of the inventors' findings that down-regulation of YB1 significantly improved AAV production by as much as 50-fold. The interaction of adenoviral protein E1B with YB1 in adenovirus-infected cells has been shown to result in the accumulation of YB1 in nuclei, YB1 activation of the E2A gene, and subsequently the initiation of adenoviral DNA replication. Overexpression of YB1-regulated adenoviral E2 promoter in an E1-independent manner led not only to adenoviral DNA replication but also a 2–3-log increase in the production of infectious particles from E1-deleted adenovirus vectors. As a result, overexpression of YB1 has been further exploited in adenovirus-based vector development and virotherapy.

So far there has been no direct association of YB1 reported with AAV virus; however the role of adenovirus in the AAV life cycle has been well documented and this may facilitate understanding the mechanism behind the enhancement of YB1 knockdown on AAV vector production. The AAV production system used in the present Examples provide four Adenoviral elements, i.e. E1, E2A, E4 and VA genes that were expressed either stably in the producer cells or transiently from a plasmid with helper function for AAV. Open-reading frame 6 of the E4 region is important for the conversion of the single-stranded AAV genome into a double-stranded form which is the substrate for subsequent steps in DNA replication. Protein E2A plays a key role in viral DNA replication via binding to AAV viral DNA, promoting DNA elongation and displacement of the elongating strand from its template. Both YB1 and E2A are DNA binding proteins (DBP) but differ from each other by their cellular and viral origins respectively. YB1 and E2A share a comparable binding preference for single stranded DNA. It is possible that adenoviral E2A has a prime regulation property over cellular DBP in the AAV life cycle; therefore, down-regulation of YB1 would reduce the competition of YB1 binding to AAV DNA, resulting in the enhancement of E2A-AAV DNA interaction, the efficiency of AAV DNA replication and ultimately increase in AAV vector genome titres. This speculation could be supported by the observation that cells lacking AAV helper components including E2A could still produce small amount of AAV particles, indicating a low level of cellular helper function from abundant but less efficient cellular DBPs, e.g. YB1 in the AAV life cycle.

On the other hand, adenoviral proteins E1A-E1B and E2A play an important role in activating AAV2 p5 promoter, resulting in the transcription and expression of the AAV2 rep gene. It has previously been demonstrated that binding of YB1 to the vascular endothelial growth factor

promoter prevented the binding of other transcription factors and resulted in the inhibition of transcription and translation. It has also been shown that YB1 binding to the ssDNA region of a promoter resulted in the stabilization of ssDNA that also inhibited gene transcription and translation. Therefore, it is possible that down-regulation of YB1 promoted E2A binding to the AAV2p5 promoter that synergistically contributed to the significant increase in AAV Rep gene expression and vector titres observed in this experiments reported herein.

The present Examples show a serotype-specific role of YB1 in AAV production; in particular, knockdown of YB1 improved AAV2 and AAV8 production by 50 and 10 fold respectively but had no significant effect on AAV5 production. The three serotypes of AAV vectors investigated, i.e. AAV2, AAV5 and AAV8, have an identical ITR sequence that shares the potential YB1 binding motif GGGG(TT) and were produced in the presence of the same helper elements from Adenovirus. AAV2 and chimeric AAV8 vectors further share the same AAV2 rep gene sequence in the Rep/Cap packaging plasmids. In terms of differences among the three serotypes of AAV2, AAV5 and AAV8 vectors, the serotype-specific cap gene sequences contribute to one of them. AAV2and AAV8 capsid proteins share more than 82% homology in their primary sequence and a much similar overall topology in the structure of capsid proteins. The notable structural differences between AAV2 and AAV8 capsid proteins are located on the capsid surface and are known to be associated with the binding property of AAV2 and AAV8 to target cells rather than being involved in capsid assembly and genome packaging , further demonstrating the similarity between AAV2 and AAV8 in terms of capsid assembly. In contrast, AAV5 is one of the most divergent AAV serotypes, sharing only ~55% sequence homology to other serotypes, including AAV2 and AAV8. Unique structural features of AAV5 capsid proteins, including a smaller HI and VR-IV loop and larger VR-VII, are located in the VP region that controls the specificity of capsid assembly, genome packaging, and antigenic determinants, and may explain the observed difference in AAV2 and AAV8 vector production. The results disclosed herein also show that YB1 gene knockdown resulted in up to 12- and 13-fold increases in rep gene expression and vector DNA production, respectively, and a ~7-fold decrease in cap gene expression, underlying the molecular mechanism of YB1 influence on AAV vector production.

Another significant difference is that the AAV5 has rep gene shares only 58% homology with AAV2 rep gene in AAV2 and AAV8 vectors. Further sequence analysis showed that AAV2 and AAV5 use different promoters, i.e. p5 and p7 for rep transcription and translation of AAV2 and AAV5, respectively. It has been shown that due to the efficiency of p7 in rep gene expression, the AAV5 p7 promoter is less dependent in 293 cells on Ad5 elements when compared to the AAV2 p5. On the

other hand, Adenoviral proteins E1A-E1B and E2A play an important role in activating AAV2 p5 promoter, resulting in the transcription and expression of AAV2 rep gene. There have been a significant number of reports on the mechanism of YB1 regulation on cellular and viral promoters. For example, binding of YB1 to the VEGF promoter prevents the binding of other transcription factors and results in the inhibition of transcription and translation. It has also been shown that YB1 binding to the ssDNA region of a promoter resulted in the stabilisation of ssDNA that also inhibited gene transcription and translation. Therefore, it is possible that down regulation of YB1 promoted E2A binding to the AAV2p5 promoter that synergistically contributed to the significant increase in AAV2 and AAV8 titres which we observed.

Taking into account the potential roles of YB1 in DNA replication, AAV transcription and translation, it is possible that deregulation of YB1 resulted in not only the increase in AAV DNA replication via reducing YB1 competition with E2A for AAV2 ssDNA e.g. the ITR sequence, but also the increase in Rep and Cap expression under the control of the AAV2p5 promoter. In addition, the native AAV2p5 promoter in AAV2/2 vectors may perform more effectively compared to the chimeric AAV2/8 Rep/Cap in the expression of Rep and Cap proteins, caused the observed 50 and 10 fold increase in AAV2 and AAV8 production, respectively. In the case of AAV5 vectors, vector DNA replication may likewise be increased via a similar competition-based mechanism as for AAV2 and AAV8; however, the low level of AAV5 Rep/Cap expression under the control of the AAV5p7 promoter that is less dependent on combined YB1 and Adenoviral helper function, may have limited the excess AAV vector genome to be packaged into full AAV5 vectors, resulting in the accumulation of AAV vector DNA in producer cells.

In summary, the present inventors have identified using LC-MS/MS and validated using immunoblotting the association of YB1 with AAV vectors and, revealed for the first time the significant enhancement of YB1 gene knockdown on AAV vector production. The significant increase in AAV2 vector titres may be due to a significant increase in rep gene expression and vector DNA production in YB1 knockdown cells compared to scramble cells. Although there has been no direct involvement of YB1 in the AAV life cycle has been reported, it is speculated that YB1 exerts negative effects on AAV production, including the blockage of AAV vector DNA replication. This may be mediated by competition with E2A for binding to the ITR sequence and AAV2p5 promoter, inhibiting the transcription and expression of AAV viral proteins (such as AAV rep proteins) by binding to the AAV2p5 promoter and by preventing the binding of activating proteins, e.g. E2A. It is possible that effects of YB1 on AAV are adenoviral helper virus dependent.

CLAIMS

1 A transgenic producer cell line in which the expression of at least one of YB1, NPM1 and NCL is modulated compared with a control producer cell line.

2 The transgenic producer cell line of claim 1, wherein the expression of: (i) NPM1 and NCL; (ii) YB1 and NPM1; (iii) YB1 and NCL; or (iv) YB1, NPM1 and NCL; is modulated compared with a control producer cell line.

3 The transgenic producer cell line of claim 1 or 2, wherein the expression of at least one of YB1, NPM1 and NCL is reduced compared with a control producer cell line.

4 The transgenic producer cell line of claim 3, wherein the expression of YB1, NPM1 and/or NCL is reduced using CRISPR genome editing, a double stranded RNA (dsRNA), a small interfering RNA (siRNA), a small hairpin RNA (shRNA),a micro RNA or an antisense RNA.

5 The transgenic producer cell line of claim 4, wherein the expression of YB1, NPM1 and/or NCL is reduced using a shRNA, and preferably:

(a) YB1 expression is reduced using a shRNA comprising the nucleotide sequence SEQ ID NOs: 14 to 18 (Y1 to Y5);
(b) NPM1 expression is reduced using a shRNA comprising the nucleotide sequence SEQ ID NOs: 4 to 8 (NPM-N6 to NPM-N10); and/or
(c) NCL expression is reduced using a shRNA comprising the nucleotide sequence SEQ ID NOs: 9 to 13 (NCL-N1 to NCL-N5).

6 The transgenic producer cell line of claim 4, wherein the expression of YB1, NPM1 and/or NCL is reduced using CRISPR genome editing.

7 The transgenic producer cell line of claim 6, wherein the expression of YB1 is reduced using a gRNA pair selected from SEQ ID NOs: 33 and 34; SEQ ID NOs: 35 and 36; SEQ ID NOs: 37 and 38; and/or SEQ ID NOs: 39 and 40.

8 The transgenic producer cell line of any one of claims 1 to 7, wherein the expression of one or more additional genes and/or proteins listed in Table 2 is modulated compared with a control producer cell line.

9 The transgenic producer cell line of any one of claims 1 to 8, which is a human embryonic kidney 293T cell line.

10 A method for producing an adeno-associated viral (AAV) vector comprising culturing an adeno-associated virus in a producer cell line in which the expression of at least one of YB1, NPM1 and NCL is modulated.

11 The method of claim 10, wherein the expression of: (i) NPM1 and NCL; (ii) YB1 and NPM1; (iii) YB1 and NCL; or (iv) YB1, NPM1 and NCL ; in the producer cell line is modulated.

12 The method of claim 10 or 11, wherein the modulation of YB1, NPM1 and/or NCL expression is a reduction in the expression of YB1, NPM1 and/or NCL

13 The method of claim 12, wherein the expression of YB1, NPM1 and/or NCL is reduced using CRISPR genome editing, a double stranded RNA (dsRNA), a small interfering RNA (siRNA), a small hairpin RNA (shRNA),a micro RNA or an antisense RNA.

14 The method of claim 13, wherein the expression of YB1, NPM1 and/or NCL is reduced using a shRNA, and preferably:

(a) YB1 expression is reduced using a shRNA comprising the nucleotide sequence SEQ ID NOs: 14 to 18 (Y1 to Y5);
(b) NPM1 expression is reduced using a shRNA comprising the nucleotide sequence SEQ ID NOs: 4 to 8 (NPM-N6 to NPM-N10); and/or
(c) NCL expression is reduced using a shRNA comprising the nucleotide sequence SEQ ID NOs: 9 to 13(NCL-N1 to NCL-N5).

15 The method of claim 13, wherein the expression of YB1, NPM1 and/or NCL is reduced using CRISPR genome editing.

16 The method of claim 15, wherein the expression of YB1 is reduced using a gRNA pair selected from SEQ ID NOs: 33 and 34; SEQ ID NOs: 35 and 36; SEQ ID NOs: 37 and 38; and/or SEQ ID NOs: 39 and 40.

17 The method of any one of claims 10 to 16, wherein the expression of one or more of the additional genes and/or proteins listed in Table 2 is modulated in the producer cell line.

18 The method of any one of claims 10 to 17, wherein the titre of AAV vector is increased at least 2 fold compared with the titre of AAV vector produced by a control method.

19 The method of any one of claims 10 to 18, wherein the ratio of complete: empty AAV vector is increased by at least 20% compared with the ratio of complete: empty AAV vector produced by a control method.

20 The method of any one of claims 10 to 19, wherein the producer cell line is a human embryonic kidney 293T cell line.

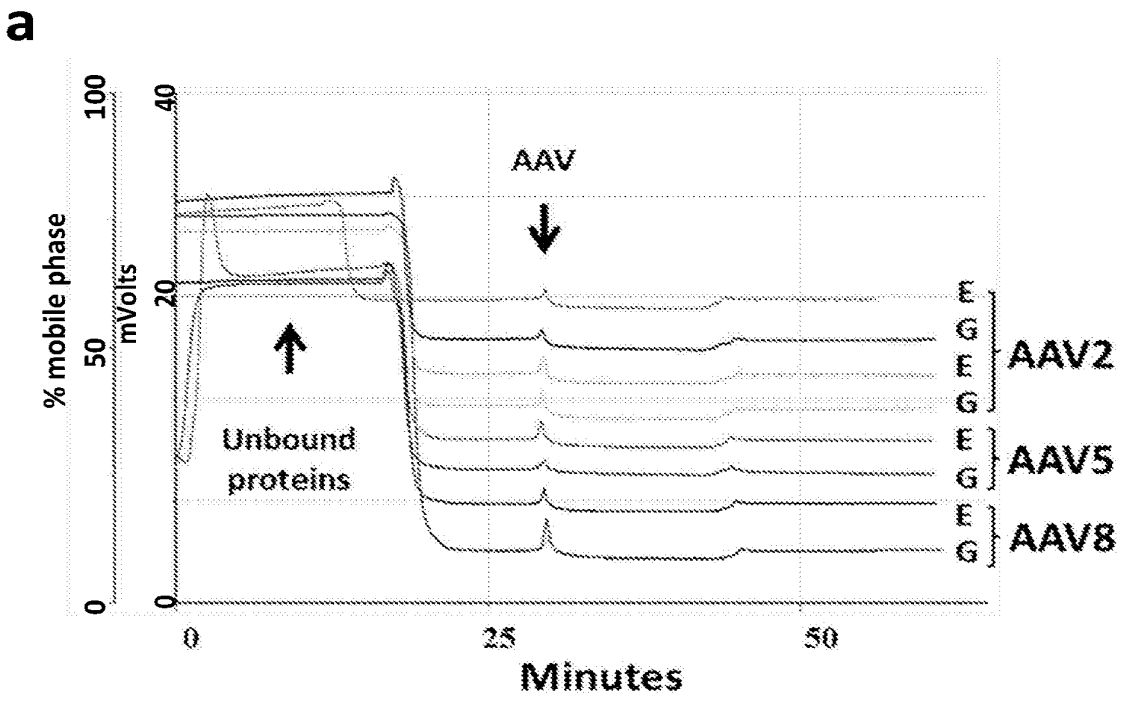
21 The method of any one of claims 10 to 20, wherein the serotype of the AAV vector is AAV2, AAV5 or AAV8.

22 A population of adeno-associated viral (AAV) vectors obtainable from the producer cell line of any one of claims 1 to 9, or by the method of any one of claims 10 to 21.

23 The population of claim 22, wherein the ratio of complete: empty AAV vectors in the population is increased by at least 20% compared the ratio of complete: empty AAV vector in a population produced by a control method.

24 The population of claim 22 or 23, wherein the AAV vectors are AAV2, AAV5 or AAV8 serotype vectors.

Figure 1


a
% mobile phase
100
50
0
mVolts
40
20
0
AAV
Unbound proteins
E
G
E
G
AAV2
E
G
AAV5
E
G
AAV8
0
25
50
Minutes

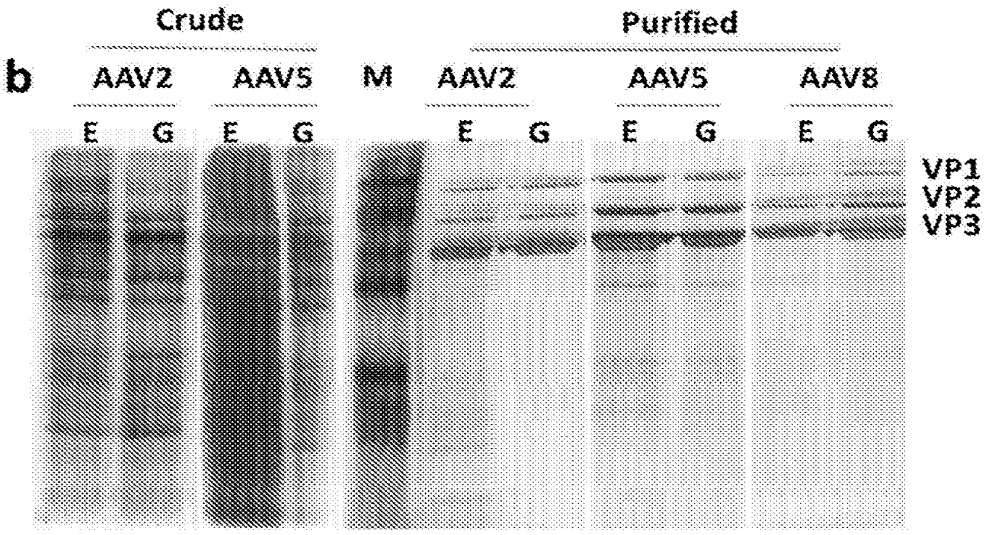

b
Crude
Purified
AAV2
AAV5
M
AAV2
AAV5
AAV8
E
G
E
G
E
G
E
G
E
G
VP1
VP2
VP3

Figure 2

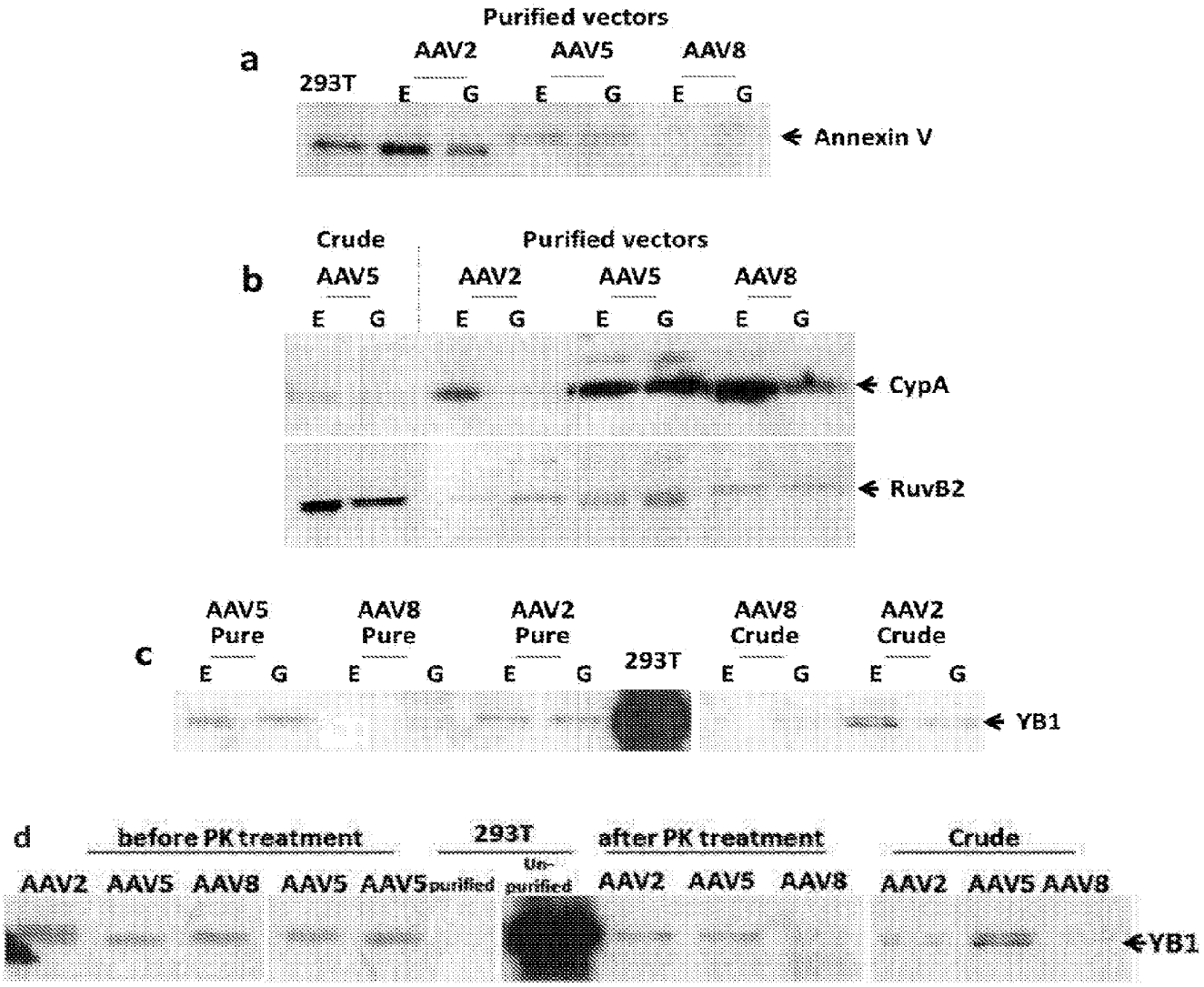

a
Purified vectors
AAV2
AAV5
AAV8
293T
E
G
E
G
E
G
Annexin V
b
Crude
Purified vectors
AAV5
AAV2
AAV5
AAV8
E
G
E
G
E
G
E
G
CypA
RuvB2
c
AAV5 Pure
AAV8 Pure
AAV2 Pure
293T
AAV8 Crude
AAV2 Crude
E
G
E
G
E
G
E
G
E
G
YB1
d
before PK treatment
293T
after PK treatment
Crude
AAV2
AAV5
AAV8
AAV5
AAV5
purified
Un-purified
AAV2
AAV5
AAV8
AAV2
AAV5
AAV8
YB1

Figure 3

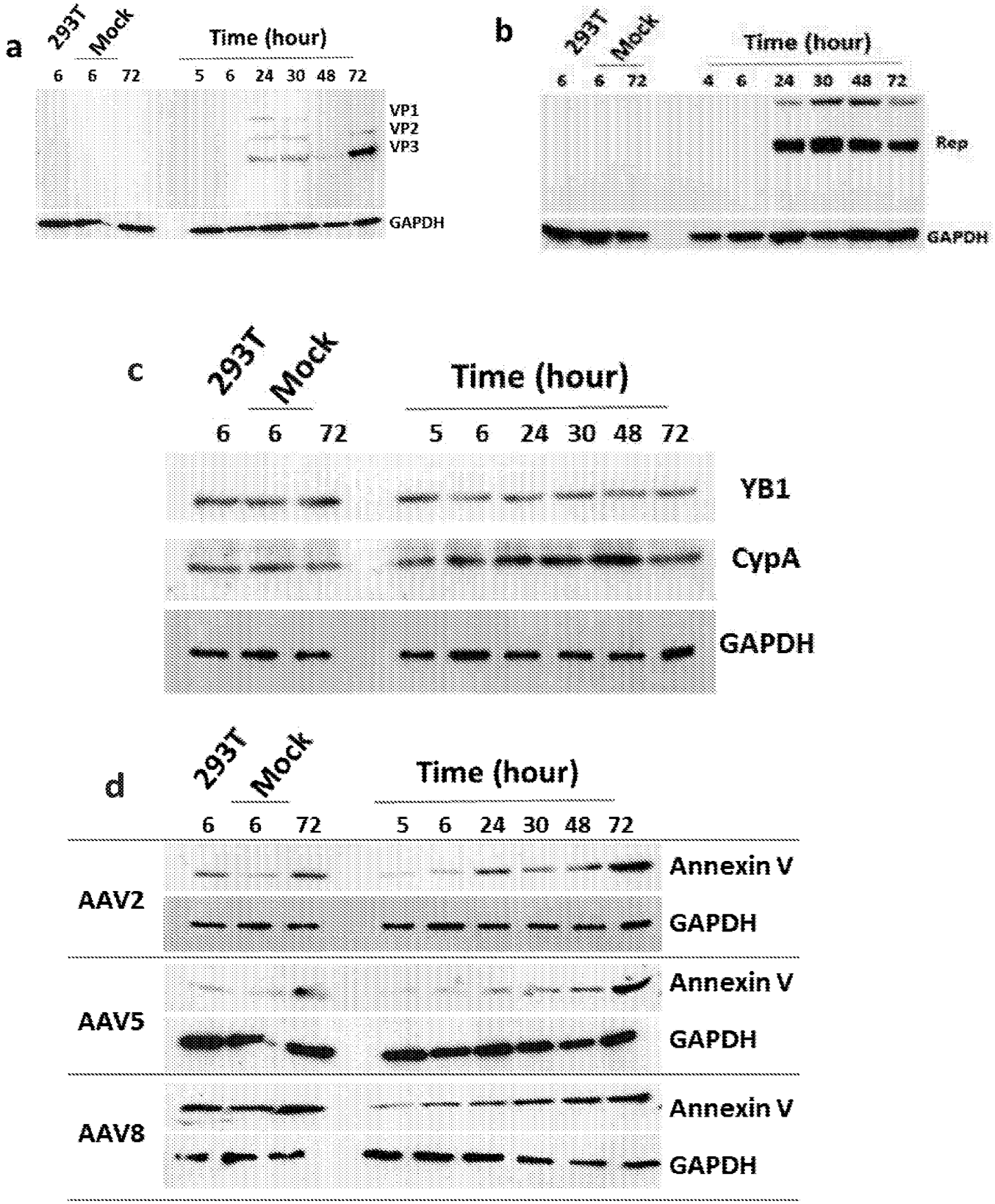

a
293T
Mock
Time (hour)
6 6 72 5 6 24 30 48 72
VP1
VP2
VP3
GAPDH
b
293T
Mock
Time (hour)
6 6 72 4 6 24 30 48 72
Rep
GAPDH
c
293T
Mock
Time (hour)
6 6 72 5 6 24 30 48 72
YB1
CypA
GAPDH
d
293T
Mock
Time (hour)
6 6 72 5 6 24 30 48 72
AAV2
Annexin V
GAPDH
AAV5
Annexin V
GAPDH
AAV8
Annexin V
GAPDH

Figure 4

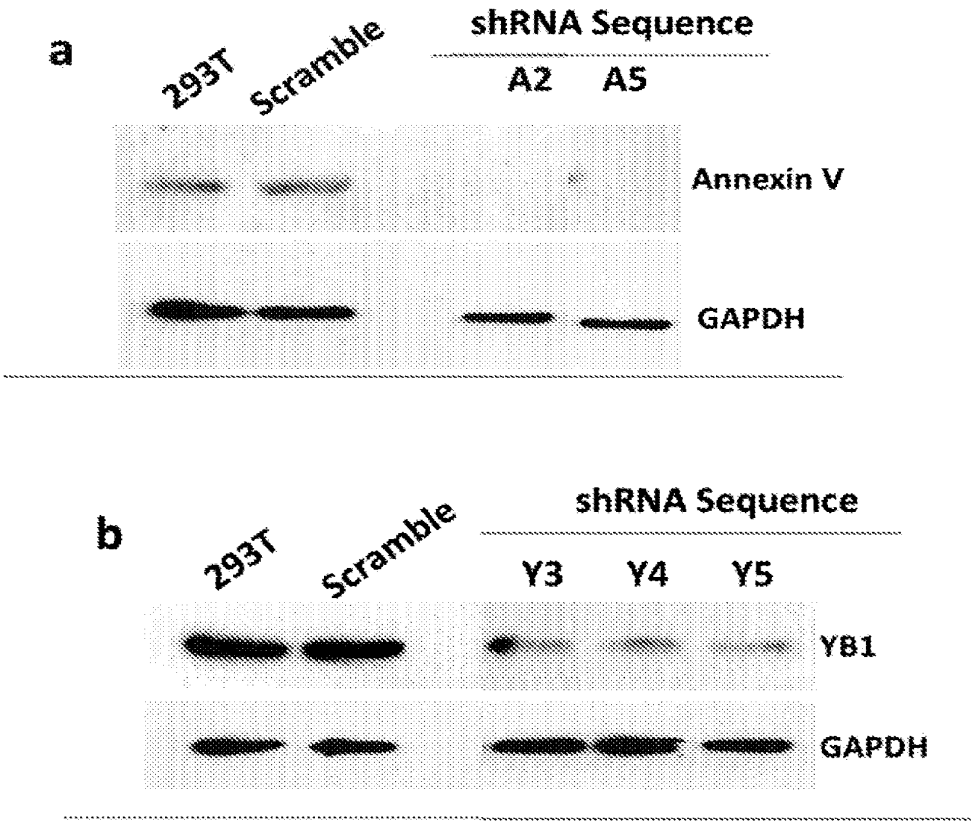

a
293T
Scramble
shRNA Sequence
A2
A5
Annexin V
GAPDH
b
293T
Scramble
shRNA Sequence
Y3
Y4
Y5
YB1
GAPDH

Figure 5

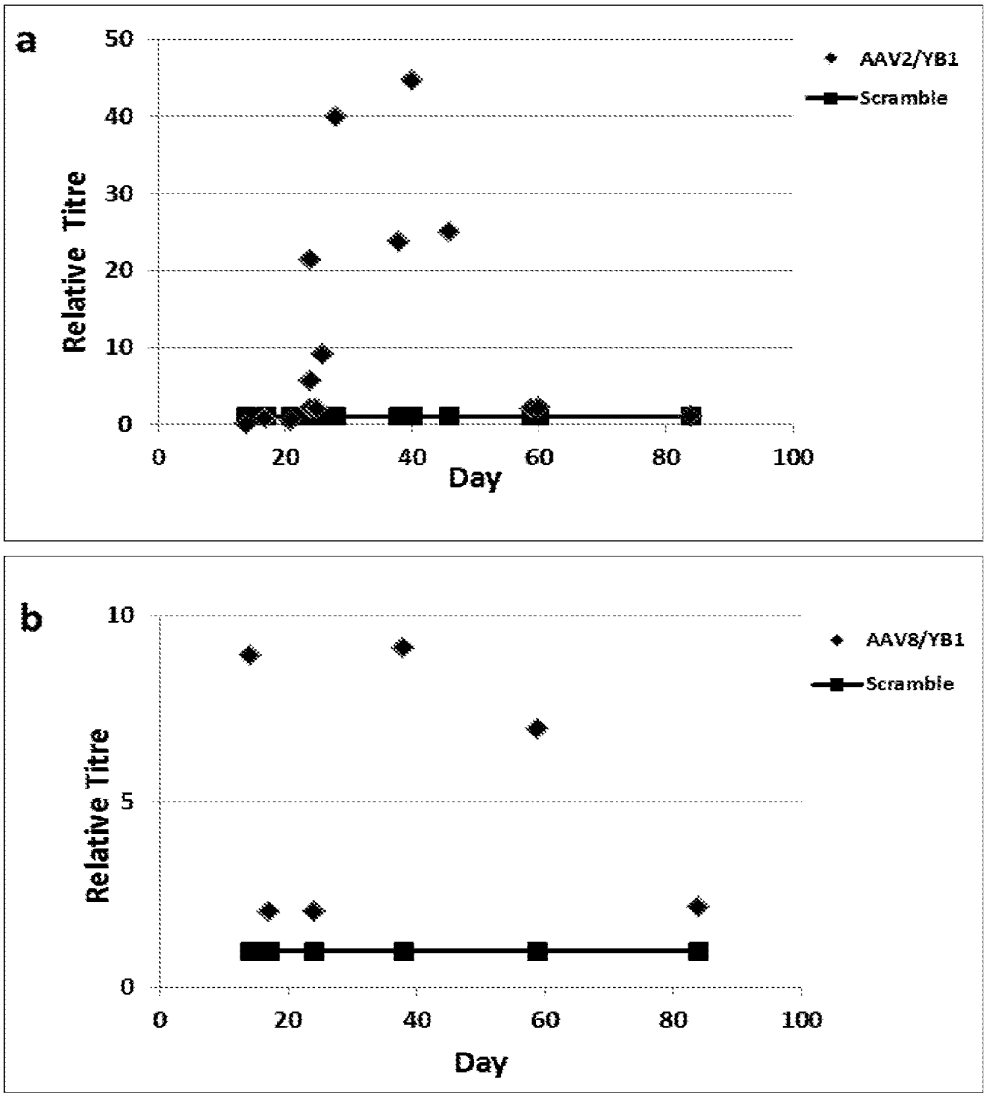


Figure 6

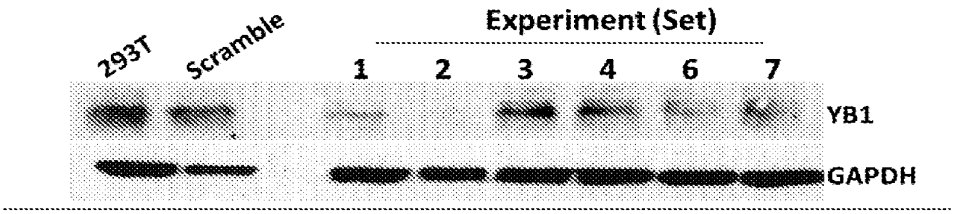


Figure 7

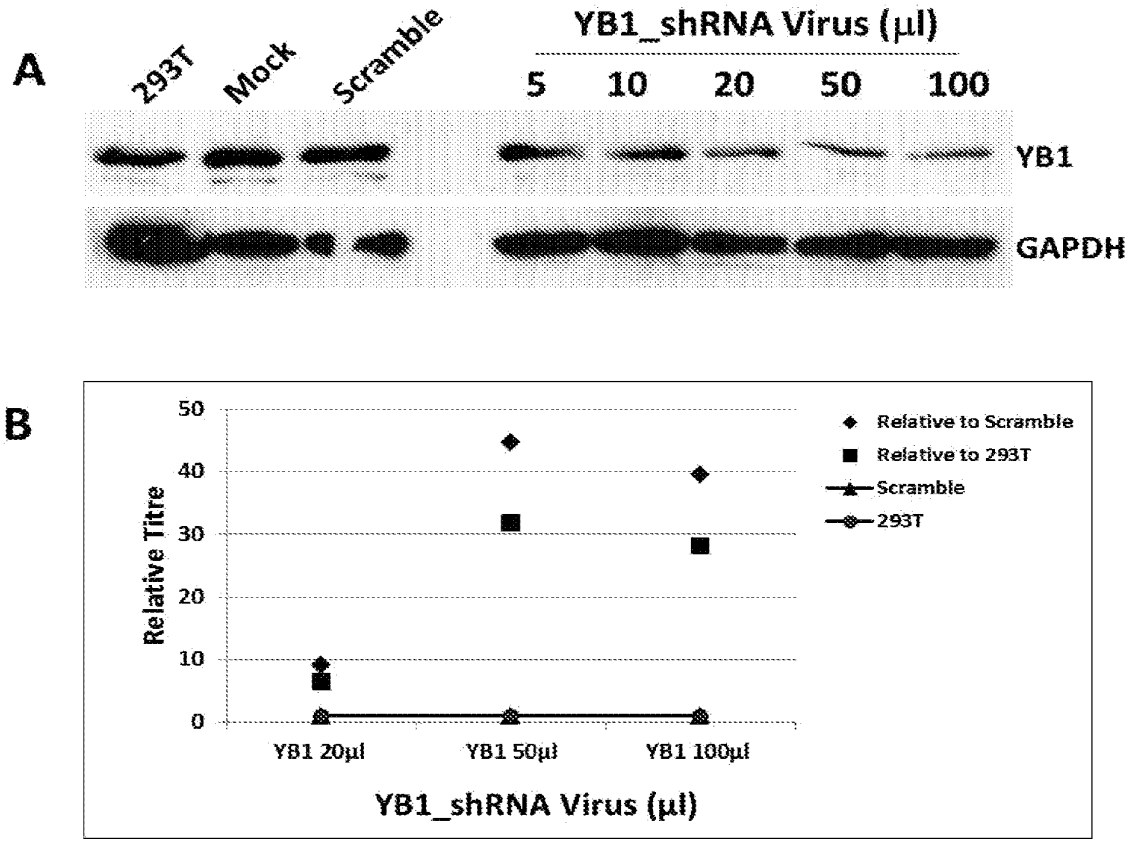

A
293T
Mock
Scramble
YB1_shRNA Virus (µl)
5
10
20
50
100
YB1
GAPDH
B
Relative Titre
50
40
30
20
10
0
YB1 20µl
YB1 50µl
YB1 100µl
YB1_shRNA Virus (µl)
Relative to Scramble
Relative to 293T
Scramble
293T

Figure 8

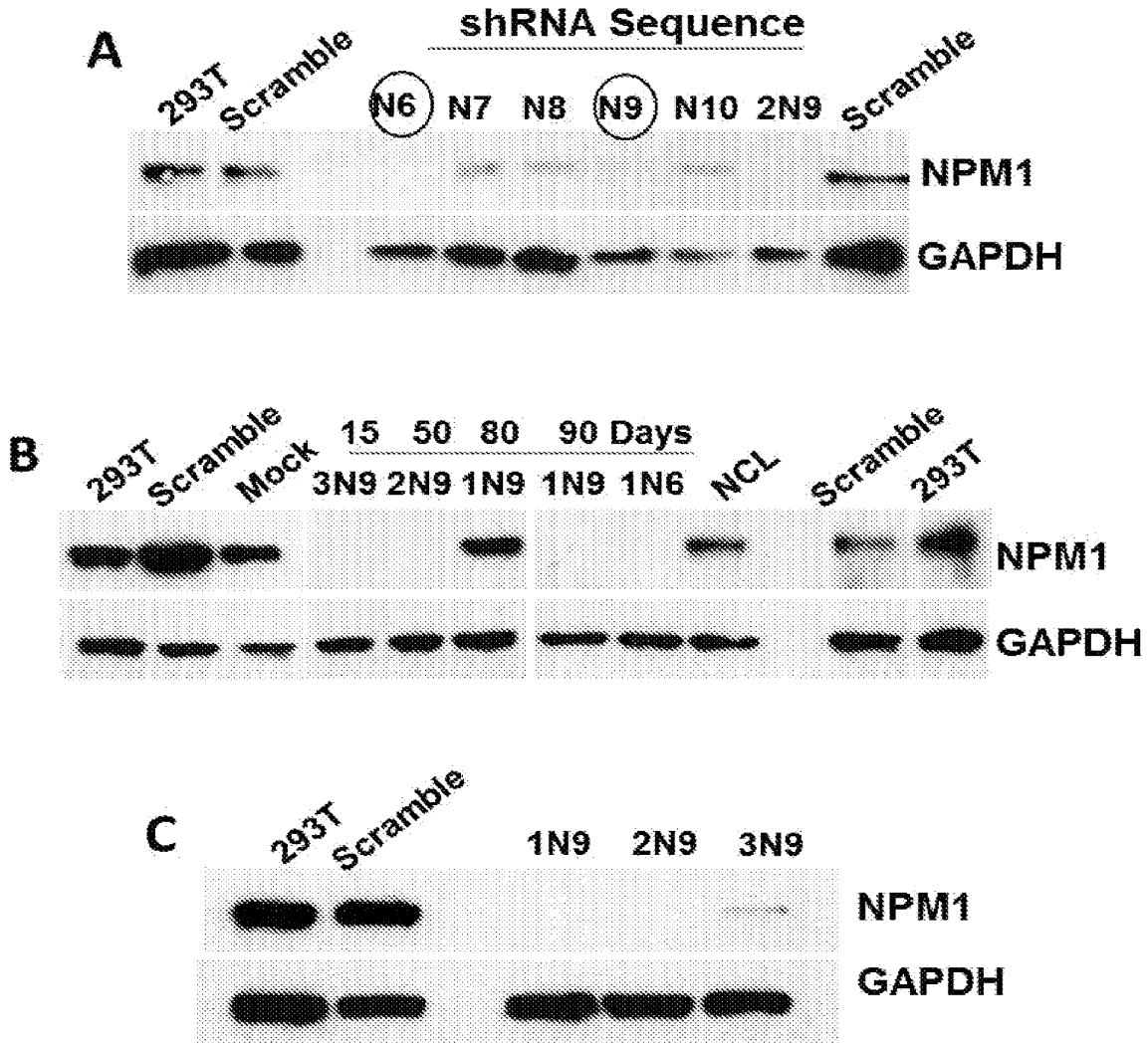

A
shRNA Sequence
293T
Scramble
N6
N7
N8
N9
N10
2N9
Scramble
NPM1
GAPDH
B
293T
Scramble
Mock
15
50
80
90 Days
3N9
2N9
1N9
1N9
1N6
NCL
Scramble
293T
NPM1
GAPDH
C
293T
Scramble
1N9
2N9
3N9
NPM1
GAPDH

Figure 9

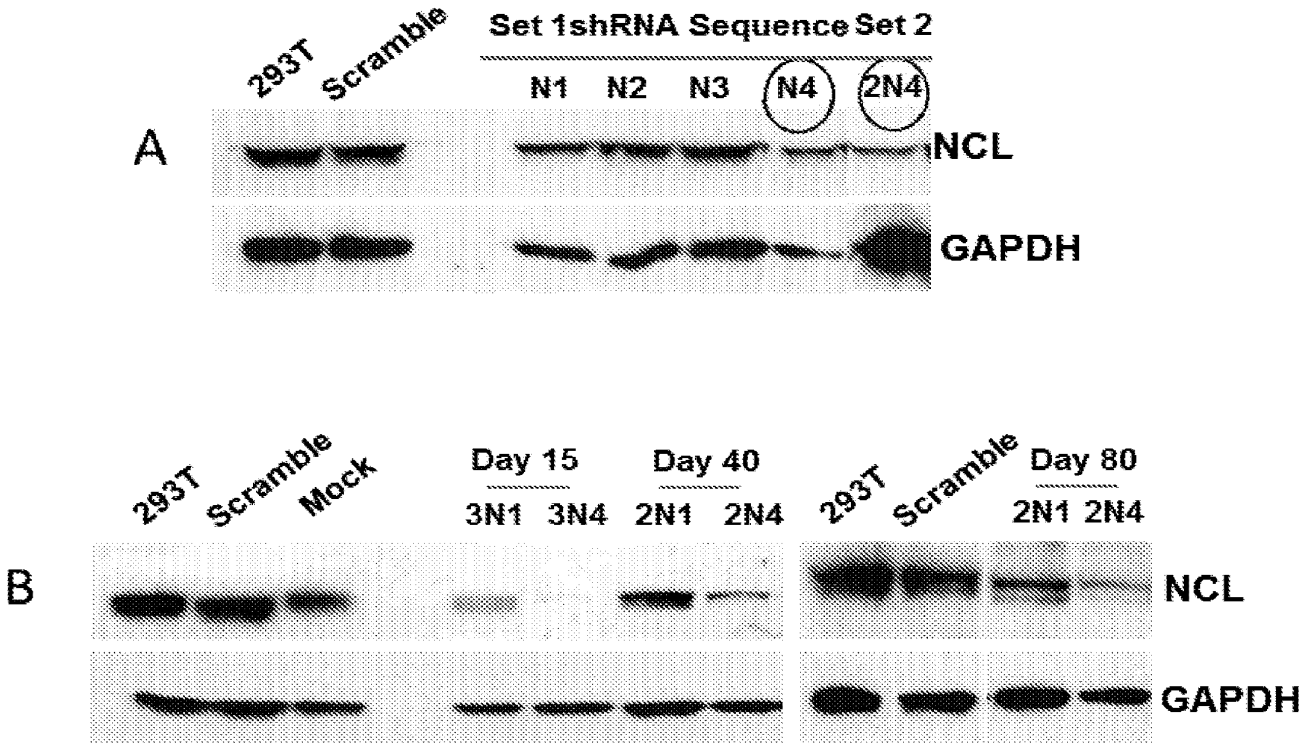

A
293T
Scramble
Set 1shRNA Sequence Set 2
N1
N2
N3
N4
2N4
NCL
GAPDH
B
293T
Scramble
Mock
Day 15
Day 40
3N1
3N4
2N1
2N4
293T
Scramble
Day 80
2N1
2N4
NCL
GAPDH

Figure 10

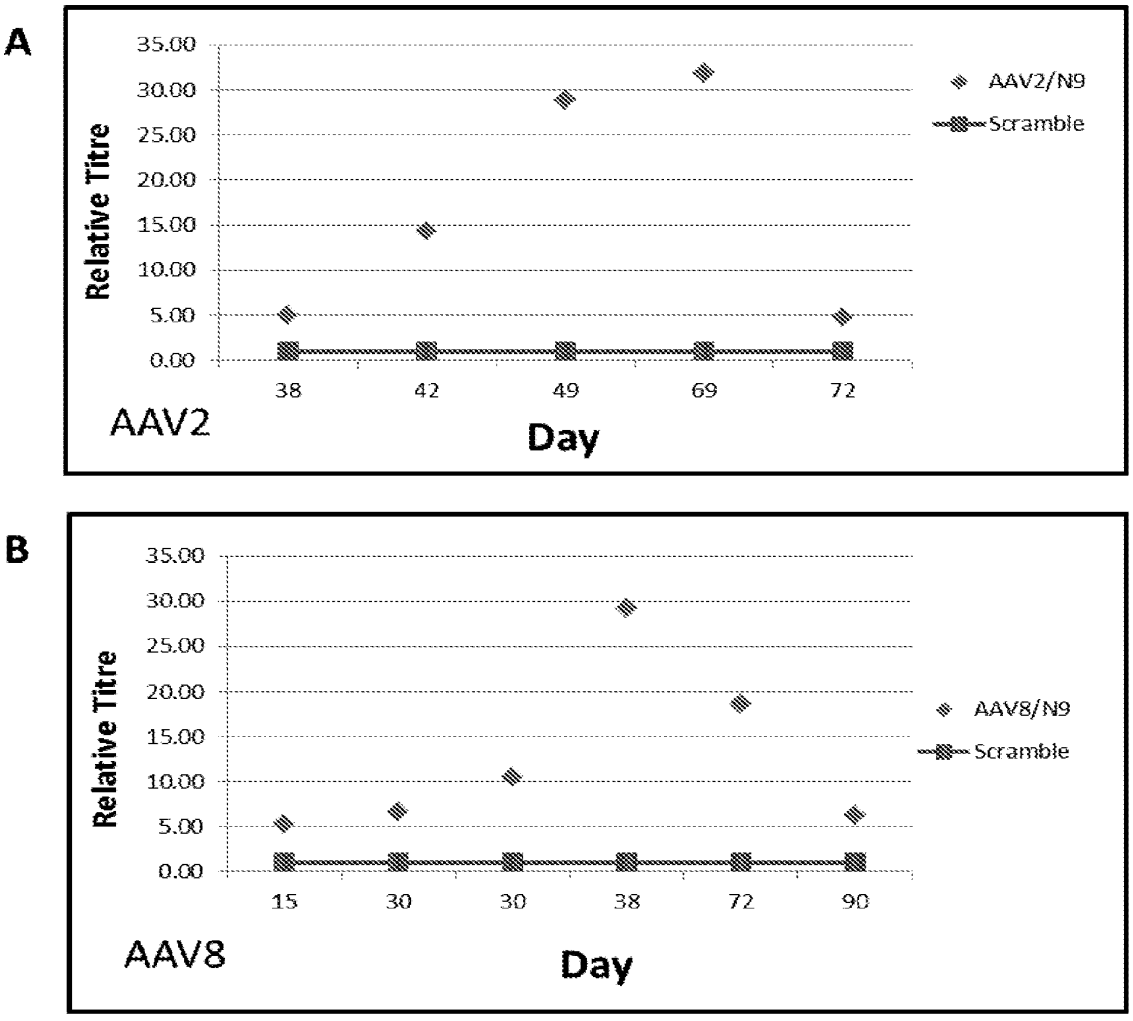

A
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Relative Titre
38
42
49
69
72
AAV2
Day
AAV2/N9
Scramble
B
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Relative Titre
15
30
30
38
72
90
AAV8
Day
AAV8/N9
Scramble

Figure 11

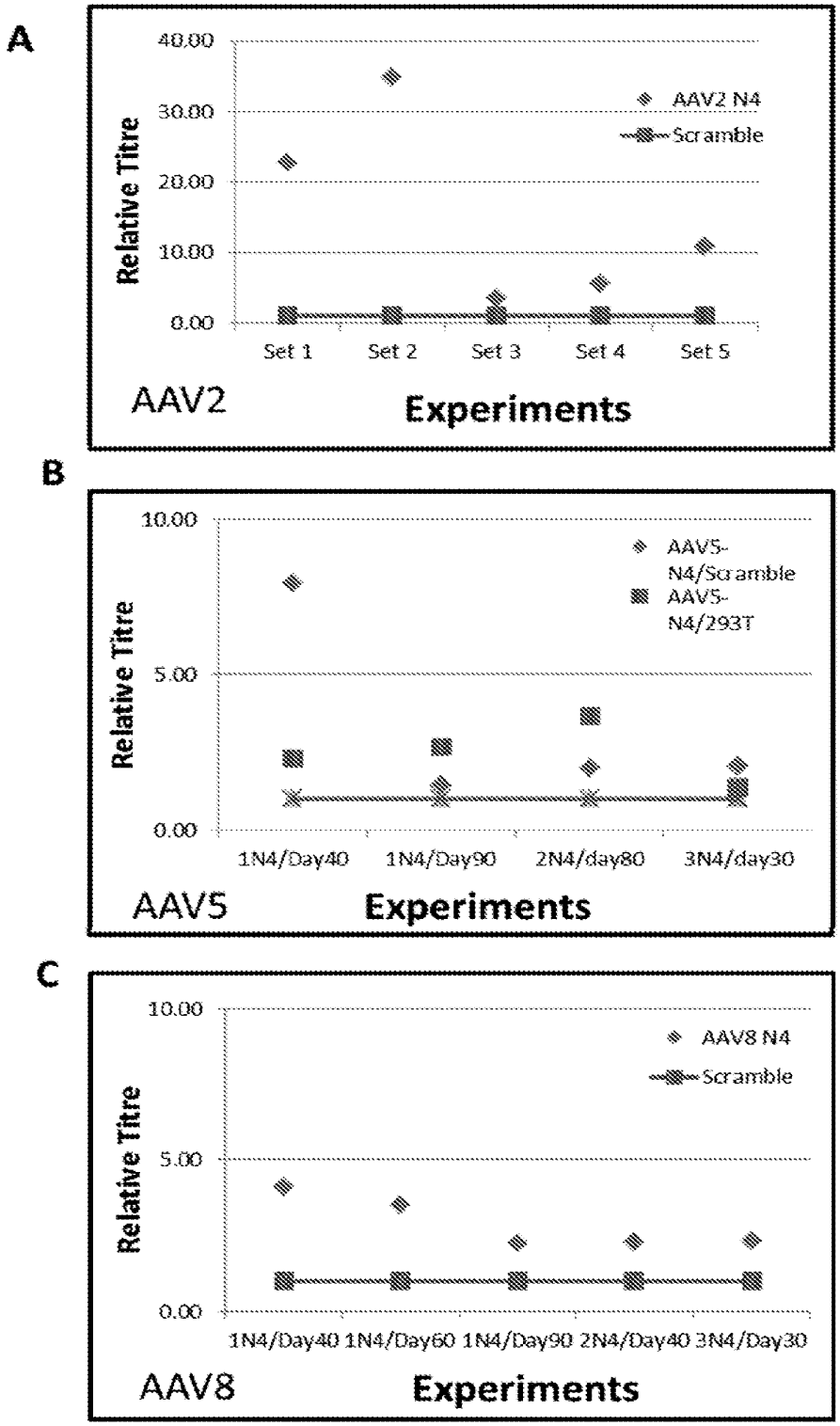

A
40.00
30.00
20.00
10.00
0.00
Relative Titre
AAV2 N4
Scramble
Set 1
Set 2
Set 3
Set 4
Set 5
AAV2
Experiments
B
10.00
5.00
0.00
Relative Titre
AAV5-N4/Scramble
AAV5-N4/293T
1N4/Day40
1N4/Day90
2N4/day80
3N4/day30
AAV5
Experiments
C
10.00
5.00
0.00
Relative Titre
AAV8 N4
Scramble
1N4/Day40
1N4/Day60
1N4/Day90
2N4/Day40
3N4/Day30
AAV8
Experiments

Figure 12

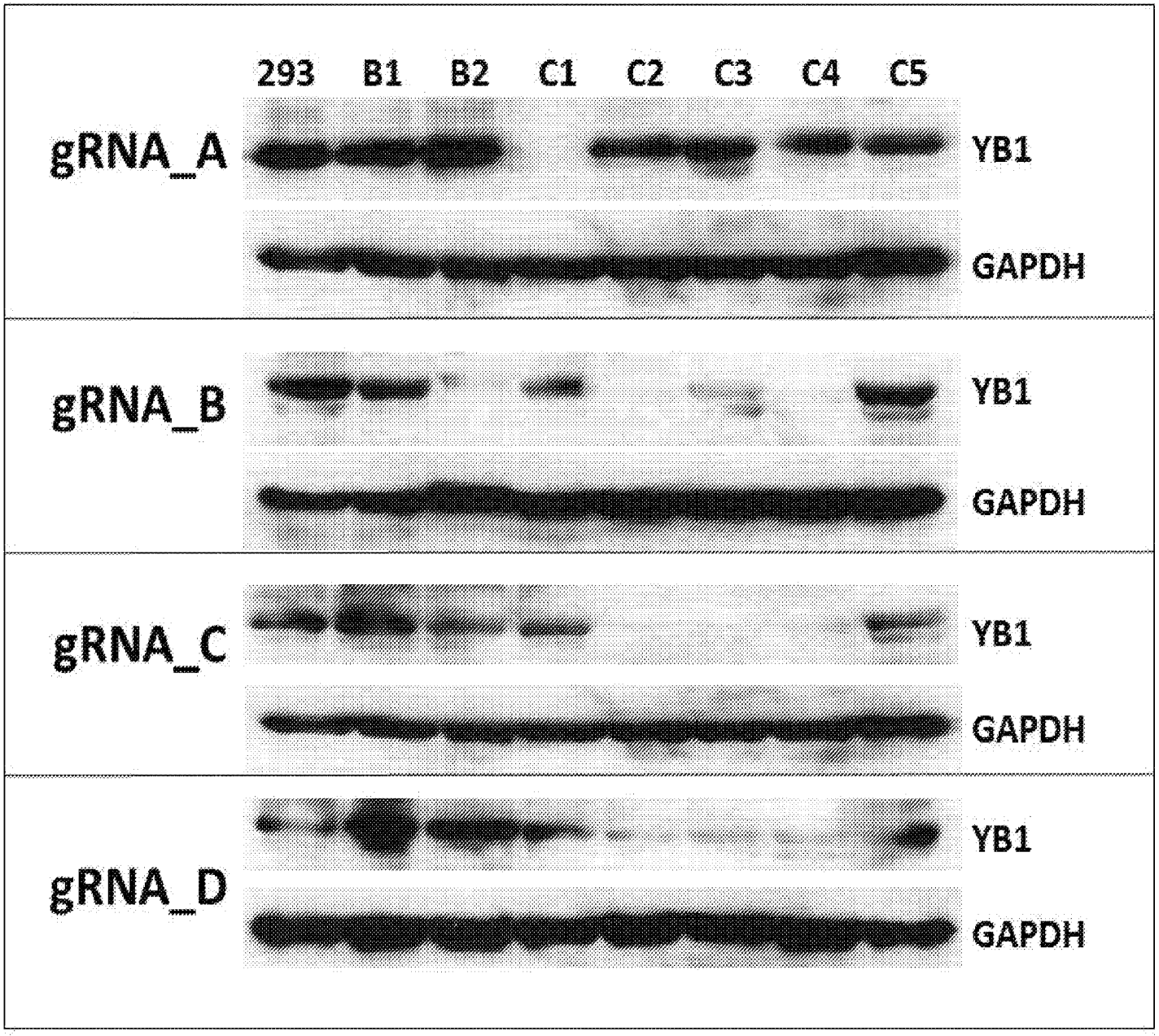
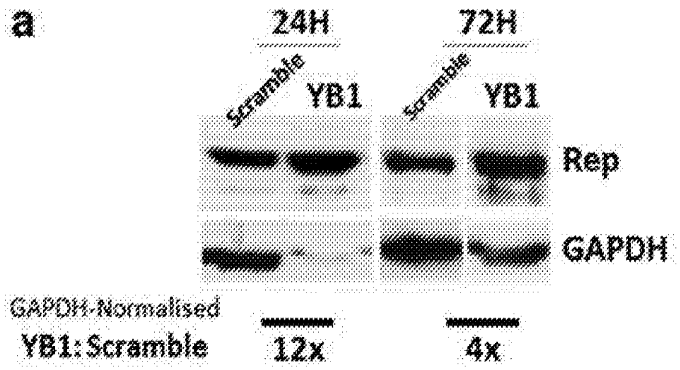

293
B1
B2
C1
C2
C3
C4
C5
gRNA_A
YB1
GAPDH
gRNA_B
YB1
GAPDH
gRNA_C
YB1
GAPDH
gRNA_D
YB1
GAPDH

Figure 13



	b ELISA Proteins (Capsid/10^8 cells)				c Real Time qPCR DNA (Copies/10^8 cells)					
n=3	Cell lysate S:Y		Vector S:Y		Total DNA (+PK) Y:S		Unpackaged DNA (-PK) Y:S		Packaged DNA (+DNAase I/+PK) Y:S	
Scramble	$8.87\pm0.1 \times 10^{14}$	7x	$6.77\pm0.2 \times 10^{13}$	1x	$1.46\pm0.5 \times 10^{12}$		$2.61\pm0.8 \times 10^{11}$		$2.16\pm0.3 \times 10^{10}$	
YB1	$1.29\pm0.2 \times 10^{14}$		$6.27\pm0.3 \times 10^{13}$		$1.93\pm0.08 \times 10^{13}$	13x	$2.02\pm0.5 \times 10^{12}$	8x	$7.97\pm0.5 \times 10^{10}$	4x

Figure 14

YB1 mRNA sequence (SEQ ID NO: 3)

```
   1 gggcttatcc cgcctgtccc gccattctcg ctagttcgat cggtagcggg agcggagagc
  61 ggaccccaga gagccctgag cagccccacc gccgccgccg gcctagttac catcacaccc
 121 cgggaggagc cgcagctgcc gcagccggcc ccagtcacca tcaccgcaac catgagcagc
 181 gaggccgaga cccagcagcc gcccgccgcc cccccccgcg ccccccgccct cagcgccgcc
 241 gacaccaagc ccggcactac gggcagcggc gcagggagcg gtggcccggg cggcctcaca
 301 tcggcggcgc ctgccggcgg ggacaagaag gtcatcgcaa cgaaggtttt gggaacagta
 361 aaatggttca atgtaaggaa cggatatggt ttcatcaaca ggaatgacac caaggaagat
 421 gtatttgtac accagactgc cataaagaag aataacccca ggaagtacct tcgcagtgta
 481 ggagatggag agactgtgga gtttgatgtt gttgaaggag aaaagggtgc ggaggcagca
 541 aatgttacag gtcctggtgg tgttccagtt caaggcagta aatatgcagc agaccgtaac
 601 cattatagac gctatccacg tcgtaggggt cctccacgca attaccagca aaattaccag
 661 aatagtgaga gtggggaaaa gaacgaggga tcggagagtg ctcccgaagg ccaggcccaa
 721 caacgccggc cctaccgcag gcgaaggttc ccaccttact acatgcggag accctatggg
 781 cgtcgaccac agtattccaa ccctcctgtg cagggagaag tgatggaggg tgctgacaac
 841 cagggtgcag gagaacaagg tagaccagtg aggcagaata tgtatcgggg atatagacca
 901 cgattccgca ggggccctcc tcgccaaaga cagcctagag aggacggcaa tgaagaagat
 961 aaagaaaatc aaggagatga gacccaaggt cagcagccac ctcaacgtcg gtaccgccgc
1021 aacttcaatt accgacgcag acgcccagaa aaccctaaac cacaagatgg caaagagaca
1081 aaagcagccg atccaccagc tgagaattcg tccgctcccg aggctgagca gggcggggct
1141 gagtaaatgc cggcttacca tctctaccat catccggttt agtcatccaa caagaagaaa
1201 tatgaaattc cagcaataag aaatgaacaa aagattggag ctgaagacct aaagtgcttg
1261 ctttttgccc gttgaccaga taaatagaac tatctgcatt atctatgcag catggggttt
1321 ttattatttt tacctaaaga cgtctctttt tggtaataac aaacgtgttt tttaaaaaag
1381 cctggttttt ctcaatacgc ctttaaaggt ttttaaattg tttcatatct ggtcaagttg
1441 agatttttaa gaacttcatt tttaatttgt aataaaagtt tacaacttga ttttttcaaa
1501 aaagtcaaca aactgcaagc acctgttaat aaaggtctta aataataaaa aaaaaaaaaa
1561 a
```

YB1 amino acid sequence (SEQ ID NO: 29)

```
1    MSSEAETQQPPAAPPAAPALSAADTKPGTTGSGAGSGGPGGLTSAAPAGGDKKVIATKVL  60
61   GTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKNNPRKYLRSVGDGETVEFDVVEGEKGA  120
121  EAANVTGPGGVPVQGSKYAADRNHYRRYPRRRGPPRNYQQNYQNSESGEKNEGSESAPEG  180
181  QAQQRRPYRRRRFPPYYMRRPYGRRPQYSNPPVQGEVMEGADNQGAGEQGRPVRQNMYRG  240
241  YRPRFRRGPPRQRQPREDGNEEDKENQGDETQGQQPPQRRYRRNFNYRRRRPENPKPQDG  300
301  KETKAADPPAENSSAPEAEQGGAE  324
```

Figure 15

NPM1 mRNA sequence (SEQ ID NO: 1)

```
   1 ggggcctggt gtgattccgt cctgcgcggt tgttctctgg agcagcgttc ttttatctcc
  61 gtccgccttc tctcctacct aagtgcgtgc cgccacccga tggaagattc gatggacatg
 121 gacatgagcc ccctgaggcc ccagaactat ctttteggtt gtgaactaaa ggccgacaaa
 181 gattatcact ttaaggtgga taatgatgaa aatgagcacc agttatcttt aagaacggtc
 241 agtttagggg ctggtgcaaa ggatgagttg cacattgttg aagcagaggc aatgaattac
 301 gaaggcagtc caattaaagt aacactggca actttgaaaa tgtctgtaca gccaacggtt
 361 tcccttgggg gctttgaaat aacaccacca gtggtcttaa ggttgaagtg tggttcaggg
 421 ccagtgcata ttagtggaca gcacttagta gctgtggagg aagatgcaga gtcagaagat
 481 gaagaggagg aggatgtgaa actcttaagt atatctggaa agcggtctgc ccctggaggt
 541 ggtagcaagg ttccacagaa aaaagtaaaa cttgctgctg atgaagatga tgacgatgat
 601 gatgaagagg atgatgatga agatgatgat ggtgatgatt ttgatgatga ggaagctgaa
 661 gaaaaagcgc cagtgaagaa atctatacga gatactccag ccaaaaatgc acaaaagtca
 721 aatcagaatg gaaaagactc aaaaccatca tcaacaccaa gatcaaaagg acaagaatcc
 781 ttcaagaaac aggaaaaaac tcctaaaaca ccaaaaggac ctagttctgt agaagacatt
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 901 ttcatcaatt gtgtgaagaa ttgcttccgg atgactgacc aagaggctat tcaagatctc
 961 tggcagtgga ggaagtctct ttaagaaaat agtttaaaca atttgttaaa aaatttccg
1021 tcttatttca tttctgtaac agttgatatc tggctgtcct ttttataatg cagagtgaga
1081 actttcccta ccgtgtttga taaatgttgt ccaggttcta ttgccaagaa tgtgttgtcc
1141 aaaatgccgt ttagttttta aagatggaac tccacccttt gcttggtttt aagtatgtat
1201 ggaatgttat gataggacat agtagtagcg gtggtcagac atggaaatgg tggggagaca
1261 aaaatataca tgtgaaataa aactcagtat tttaataaag tgaaaaaaaa aaaaaaaaaa
1321 aaaaaaaaaa a
```

NPM1 amino acid sequence (SEQ ID NO: 27)

```
1    MEDSMDMDMSPLRPQNYLFGCELKADKDYHFKVDNDENEHQLSLRTVSLGAGAKDELHIV  60
61   EAEAMNYEGSPIKVTLATLKMSVQPTVSLGGFEITPPVVLRLKCGSGPVHISGQHLVAVE  120
121  EDAESEDEEEEDVKLLSISGKRSAPGGGSKVPQKKVKLAADEDDDDDEEDDDEDDDGDD  180
181  FDDEEAEEKAPVKKSIRDTPAKNAQKSNQNGKDSKPSSTPRSKGQESFKKQEKTPKTPKG  240
241  PSSVEDIKAKMQASIEKGGSLPKVEAKFINCVKNCFRMTDQEAIQDLWQWRKSL  294
```

Figure 16

NCL mRNA sequence (SEQ ID NO: 2)

```
   1 attctgctgt agacatagag atgatgatca tagctgacta tgatgatgat ccccgcgag
  61 cctgaaagag gaaatgctct ggtttgctaa gcccgcgaat cgagtgagac ccacccacaa
 121 agctaaccgt ggaagtcact ggcggcctcc ttcgccctgc cagccgggga acccatccgg
 181 tggctctcga cctgctcccg ggccatctgg tgacactgac ttcgcagcca ccaccttaat
 241 tggcgcattc gacccaaata ataacctggg aacctgtggg cggtctaagg ccccgctctg
 301 cggtcgccct cccaggcccc tctccctggc cctgtgaggc cagaaagtta cttctccgag
 361 gccagttccc catgtctgag aaatatctcc caacttgagg ttctgtgggg taggggaggg
 421 ttcgtgactt tctcacagaa aacctcgtac agaccccgcc actgccttta ttaacagctc
 481 tcaggagact gcctgcagga ggggggtcgc tccggcccca tgctcgcggg caagcaggga
 541 taagctgtgc ctccaaaagg gccaacggga actccgcggt ccctgaactt ccggtgctgg
 601 aggactcctc gctccagggc caccaggagc cgcggcgtga gtgcgtgccg gaaccgaggg
 661 cggggtctct gaggaactcc aaggctgccc aagcctacgg acccagccac attggcgaac
 721 cggagaccgc ccgattccac cacccccgcg ctcccctcac agccggcgcc aaaaacgcca
 781 gtcccacgac gcaggccggg acccgcgcgc ccacggccca atcagcgcga ccttgcacaa
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 901 cgcctactcc ccgccccgcg ccgttcacgg ttagaggctc gcgattggct catggggacg
 961 gccgcgagct ttggttggtc ggcgcggagt cacgaggcgc cgtcgtcgcc tttccacagg
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1081 cgtgctccgg gatcttcagc acccgcggcc gccatcgccg tcgcttggct tcttctggac
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2221 tagtgaagat gaggaaatgt cagaagatga agaagatgat agcagtggag aagaggtaat
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```

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7621 attgcttata ttgaatttaa gacagaagct gatgcagaga aaacctttga agaaaagcag
7681 ggaacagaga tcgatgggcg atctatttcc ctgtactata ctggagagaa aggtcaaaat
7741 caagactata gaggtggaaa gaatagcact tggagtggta agaaattagg cttgttccaa
7801 ggttttcaga attggttgag ggaactcttc tagtctttgt atttcataag tttataaata
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8041 accatgtgcg ttttgaactc agatgattct aggaaaacag accagtcatc agcctatgta
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8221 tagatattac tgttgtgacc gaagctggga cactgtttca gagtcttagg aatgtggctc
8281 tctatttcga ggtgaatcaa aaactctggt tttaagcaac ctctcctaca gtgcaacaga
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8401 tggcaaatct aaagggtaag ataatacctt tgtatcatca gttataggcc tatatatgtc
8461 ttagaggtct aaggacgtaa ggtcatgtgt cctgtagaaa aaagctaaat aattttagcc
8521 tagtaaatga gtgtaaaata agtatattta ggtccaacct tgagagaagg gccttggcca
8581 gatcatgtga ccagtggtat agagagcatg tgcctggtaa attactctaa gcattaactg
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8701 aattcctgta ataaaaggga aattgagggc agagcaatca ggctggagtt gcaaggaccc
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9541 gagaattcac agaactagca ttattttacc ttctgtcttt acagaggtat atttagctgt
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10201 gacgaagttt gaatagcttc tgtccctctg ctttcccttt tccatttgaa agaaaggact

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10261 ctggggtttt tactgttacc tgatcaatga cagagccttc tgaggacatt ccaagacagt
10321 atacagtcct gtggtctcct tggaaatccg tctagttaac atttcaaggg caataccgtg
10381 ttggttttga ctggatattc atataaactt tttaaagagt tgagtgatag agctaaccct
10441 tatctgtaag ttttgaattt atattgtttc atcccatgta caaaaccatt ttttcctaca
10501 aatagtttgg gttttgttgt tgttactttt ttttttgttt ttgtttttt ttttttgcg
10561 ttcgtggggt tgtaaaagaa aagaaagcag aatgttttat catggttttt gcttcaccgc
10621 tttaggacaa attaaaagtc aactctggtg ccagacgtgt tacttcctaa agagtgtttc
10681 ccctggaatc tcactggaga gcatggcaaa gccagctctg ccacttgctt cacccatccc
10741 aatggaaatg gcttagtgcg tgtttccagt atcccagccc taactaactt ggttgaaatg
10801 ctggtgaggg gacctgctcc tgcagccctg gtgctgactt gaaggctgct gcagcttctc
10861 ctacttttag caggtctcga ggattatgtc tgaagaccac tctggaaaga ggtcgaggaa
10921 cagattagtc aggtttccta gg
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NCL amino acid sequence (SEQ ID NO: 28)

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1    MVKLAKAGKNQGDPKKMAPPPKEVEEDSEDEEMSEDEEDDSSGEEVVIPQKKGKKAAATS  60
61   AKKVVVSPTKKVAVATPAKKAAVTPGKKAAATPAKKTVTPAKAVTTPGKKGATPGKALVA  120
121  TPGKKGAAIPAKGAKNGKNAKKEDSDEEEDDDSEEDEEDDEDEDEDEDEIEPAAMKAAAA  180
181  APASEDEDDEDDEDDEDDDDDEEDDSEEEAMETTPAKGKKAAKVVPVKAKNVAEDEDEEE  240
241  DDEDEDDDDDEDDEDDDDEDDEEEEEEEEEEPVKEAPGKRKKEMAKQKAAPEAKKQKVEG  300
301  TEPTTAFNLFVGNLNFNKSAPELKTGISDVFAKNDLAVVDVRIGMTRKFGYVDFESAEDL  360
361  EKALELTGLKVFGNEIKLEKPKGKDSKKERDARTLLAKNLPYKVTQDELKEVFEDAAEIR  420
421  LVSKDGKSKGIAYIEFKTEADAEKTFEEKQGTEIDGRSISLYYTGEKGQNQDYRGGKNST  480
481  WSGESKTLVLSNLSYSATEETLQEVFEKATFIKVPQNQNGKSKGYAFIEFASFEDAKEAL  540
541  NSCNKREIEGRAIRLELQGPRGSPNARSQPSKTLFVKGLSEDTTEETLKESFDGSVRARI  600
601  VTDRETGSSKGFGFVDFNSEEDAKEAMEDGEIDGNKVTLDWAKPKGEGGFGGRGGGRGGF  660
661  GGRGGGRGGRGGFGGRGRGGFGGRGGFRGGRGGGGDHKPQGKKTKFE  707
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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2015/050240

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N7/02
ADD. C12N15/86

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, EMBL, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. S. JOHNSON ET AL: "Enhancement of Adeno-Associated Virus Infection by Mobilizing Capsids into and Out of the Nucleolus", JOURNAL OF VIROLOGY, vol. 83, no. 6, 24 December 2008 (2008-12-24), pages 2632-2644, XP055183712, ISSN: 0022-538X, DOI: 10.1128/JVI.02309-08 abstract page 2640, left-hand column, paragraph 1 - page 2641, left-hand column, paragraph 1 page 2643, left-hand column, paragraph 1 figures 6-8 ----- -/--	1-9

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 April 2015	30/04/2015

Name and mailing address of the ISA/	Authorized officer
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Form PCT/ISA/210 (second sheet) (April 2005)

Sarepta Exhibit 1002, page 2039

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2015/050240

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KALUDOV N ET AL: "Scalable purification of adeno-associated virus type 2, 4, or 5 using ion-exchange chromatography", HUMAN GENE THERAPY, vol. 13, no. 10, 1 July 2002 (2002-07-01), pages 1235-1243, XP002382732, ISSN: 1043-0342, DOI: 10.1089/10430340232013901 4 abstract page 1242, left-hand column, paragraph 2	22-24
X	WO 2004/113494 A2 (AVIGEN INC) 29 December 2004 (2004-12-29) claims 1-15	22-24
X,P	STIFANI SATKUNANATHAN ET AL: "Establishment of a Novel Cell Line for the Enhanced Production of Recombinant Adeno-Associated Virus Vectors for Gene Therapy", HUMAN GENE THERAPY, vol. 25, no. 11, 1 November 2014 (2014-11-01), pages 929-941, XP055183608, ISSN: 1043-0342, DOI: 10.1089/hum.2014.041 the whole document	1-24
A	K. NASH ET AL: "Identification of Cellular Proteins That Interact with the Adeno-Associated Virus Rep Protein", JOURNAL OF VIROLOGY, vol. 83, no. 1, 29 October 2008 (2008-10-29), pages 454-469, XP055183628, ISSN: 0022-538X, DOI: 10.1128/JVI.01939-08 abstract table 1	1-24

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Form PCT/ISA/210 (continuation of second sheet) (April 2005)

Sarepta Exhibit 1002, page 2040

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2015/050240

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☒ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

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2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2015/050240

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004113494 A2	29-12-2004	AT 490307 T	15-12-2010
		DK 2277996 T3	20-10-2014
		EP 1625210 A2	15-02-2006
		EP 2277996 A2	26-01-2011
		EP 2281877 A2	09-02-2011
		EP 2781596 A1	24-09-2014
		ES 2521682 T3	13-11-2014
		JP 4559429 B2	06-10-2010
		JP 5166477 B2	21-03-2013
		JP 2006528886 A	28-12-2006
		JP 2010155868 A	15-07-2010
		JP 2012235788 A	06-12-2012
		PT 1625210 E	15-03-2011
		PT 2277996 E	16-12-2014
		SI 2277996 T1	31-12-2014
		US 2005042740 A1	24-02-2005
		US 2007243615 A1	18-10-2007
		US 2012135515 A1	31-05-2012
		WO 2004113494 A2	29-12-2004

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Published:

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— *with sequence listing part of description (Rule 5.2(a))*

WO 2015/137802 A1

(54) **Title**: FURTHER IMPROVED AAV VECTORS PRODUCED IN INSECT CELLS

(57) **Abstract**: The present invention relates to the production of adeno-associated viral vectors in insect cells. The insect cells therefore comprise a first nucleotide sequence encoding the adeno-associated virus (AAV) capsid proteins, whereby the initiation codon for translation of the AAV VP1 capsid protein is a non-ATG, suboptimal initiation codon and wherein the coding sequence for one or more amino acid residues have been inserted between the suboptimal translation initiation codon and the codon encoding the amino acid residue that corresponds to the amino acid residue at position 2 of the wild type capsid amino acid sequence of which the first amino acid residue is alanine, glycine, valine, aspartic acid or glutamic acid. The insect cell further comprises a second nucleotide sequence comprising at least one AAV inverted terminal repeat (ITR) nucleotide sequence; a third nucleotide sequence comprising a Rep52 or a Rep40 coding sequence operably linked to expression control sequences for expression in an insect cell; and, a fourth nucleotide sequence comprising a Rep78 or a Rep68 coding sequence operably linked to expression control sequences for expression in an insect cell. The invention further relates to adeno-associated viral vectors with an altered ratio of the viral capsid proteins.

Further improved AAV vectors produced in insect cells

Field of the invention

The present invention relates to the production of adeno-associated virus in insect cells and to adeno-associated virus that provides improved infectivity.

Background of the invention

Adeno-associated virus (AAV) may be considered as one of the most promising viral vectors for human gene therapy. AAV has the ability to efficiently infect dividing as well as non-dividing human cells, the AAV viral genome integrates into a single chromosomal site in the host cell's genome, and most importantly, even though AAV is present in many humans it has never been associated with any disease. In view of these advantages, recombinant adeno-associated virus (rAAV) is being evaluated in gene therapy clinical trials for hemophilia B, malignant melanoma, cystic fibrosis, and other diseases. Numerous clinical trials and recent approval of a first gene therapy medicine in Europe, Alipogene tiparvovec (Glybera®, uniQure), holds a promise for AAV to become main stay of clinical practice.

In general, there are two main types of production systems for recombinant AAV. On the one hand there are conventional production systems in mammalian cell types (such as 293 cells, COS cells, HeLa cells, KB cells) and on the other hand more recently, production systems using insect cells have been developed.

The mammalian production system suffers from several drawbacks, of which the most important one for therapeutic use is the limited number of rAAV particles generated per cell (order of 10^4 particles (reviewed in Clark, 2002, Kidney Int. 61(Suppl. 1): 9–15). For a clinical study, more than 10^{15} particles of rAAV may be required. To produce this number of rAAV particles, transfection and culture with approximately 10^{11} cultured human 293 cells, the equivalent of 5,000 175-cm^2 flasks of cells, would be required., which means transfecting up to 10^{11} 293 cells. Therefore, large scale production of rAAV using mammalian cell culture systems to obtain material for clinical trials has already proven to be problematic, production at commercial scale may not even be feasible. Furthermore there is always the risk, that a vector for clinical use that is produced in a mammalian cell culture will be

contaminated with undesirable, perhaps pathogenic, material present in the mammalian host cell.

To overcome these problems of mammalian productions systems, an AAV production system has been developed using insect cells (Urabe et al., 2002, Hum. Gene Ther. 13: 1935–1943; US 20030148506 and US 20040197895). For production of AAV in insect cells some modifications were necessary in order to achieve the correct stoichiometry of the three AAV capsid proteins (VP1, VP2 and VP3), which relies on a combination of alternate usage of two splice acceptor sites and the suboptimal utilization of an ACG initiation codon for VP2 that is not accurately reproduced by insect cells. To mimic the correct stoichiometry of the capsid proteins in insect cells Urabe et al. (2002, *supra*) use a construct that is transcribed into a single polycistronic messenger that is able to express all three VP proteins without requiring splicing and wherein the most upstream initiator codon is replaced by the suboptimal initiator codon ACG.

WO2007/046703 discloses the further improvement of the infectivity of baculovirus-produced rAAV vectors based production by optimisation of the stoichiometry of AAV capsid proteins in insect cells.

Kohlbrenner et al. (2005, Mol. Ther. 12: 1217-25) reported that the baculovirus construct for expression of the two Rep protein, as used by Urabe et al., suffers from an inherent instability. By splitting the palindromic orientation of the two Rep genes in Urabe's original vector and designing two separate baculovirus vectors for expressing Rep52 and Rep78, Kohlbrenner et al. (2005, *supra*) increased the passaging stability of the vector. However, despite the consistent expression of Rep78 and Rep52 from the two independent baculovirus-Rep constructs in insect cells over at least 5 passages, rAAV vector yield is 5 to 10-fold lower as compared to the original baculovirus-Rep construct designed by Urabe et al. (2002, *supra*).

WO2009/014445 provides an alternative for improving the stability during of baculovirus-based rAAV vector production by using repeated coding sequences with differential codon biases.

Urabe et al. (J. Virol., 2006, 80(4):1874-1885) report that AAV5 particles produced in the baculovirus system using ACG as initiation codon of the VP1 capsid protein have a poor infectivity and that - in contrast to AAV2 with VP1 expressed from an ACG initiation codon - mutating the +4 position to a G-residue in the AAV5 VP1

coding sequence did not improve infectivity. Urabe et al. addressed this problem by constructing chimeric AAV2/5 VP1 proteins, wherein a N-terminal portion of at least 49 amino acids of AAV5 VP1 is replaced with the corresponding part of AAV2 VP1 in order to improve the infectivity of the virions. There is thus still a need in the art for an AAV5 VP1 expressed from an ACG initiation codon that retains infectivity without extensive modifications.

The present inventors have, however, found that AAV vectors, in particular AAV5 vectors, such as non-chimeric AAV5 vectors which have been modified according to Urabe (Urabe et al., 2002, Hum. Gene Ther. 13: 1935–1943), WO2007/046703 or WO2009/014445, produced in the baculovirus system show a reduced infectivity in *in vitro* and *in vivo* studies in mice as compared to e.g. corresponding AAV vectors produced in conventional mammalian 293 cells. Hence, there is still a need for a baculovirus-based production system for rAAV vectors with improved infectivity.

Description of the invention

Brief description of the invention

In a first aspect, the present invention relates to a nucleic acid molecule having a nucleotide sequence comprising an open reading frame, wherein the reading frame in 5' to 3'order comprises, or consists of: :

(i) a first codon, which is a suboptimal translation initiation codon selected from the group consisting of CTG, ACG, TTG and GTG;

(ii) a second codon encoding an amino acid residue selected from the group consisting of alanine, glycine, valine, aspartic acid and glutamic acid;

(iii) optionally, one or more codons encoding additional amino acid residues following the second codon; and,

(iv) a sequence encoding adeno-associated virus (AAV) capsid proteins, whereby the sequence lacks only the VP1 translation initiation codon.

In a preferred embodiment, the AAV capsid proteins are AAV serotype 5, AAV serotype 8, or AAV serotype 9 capsid proteins, more preferably the AAV capsid proteins have an amino acid sequence selected from the group consisting of: SEQ ID NO: 22, 28, 30, 71 and 73.

Alternatively or in combination with any previous embodiments, in a further preferred embodiment the second codon encodes alanine.

Alternatively or in combination with any previous embodiments, in a further preferred embodiment the second codon is selected from the group consisting of GCT, GCC, GCA, GCG and GGU, preferably wherein the codon is GCT.

In a second aspect, the present invention relates to a nucleic acid construct comprising a nucleic acid molecule according to the invention, wherein the nucleotide sequence of the reading frame encoding the adeno-associated virus (AAV) capsid proteins is operably linked to expression control sequences for expression in an insect cell.

Alternatively or in combination with any previous embodiments, in a further preferred embodiment the nucleotide sequence of the reading frame is operably linked to a promoter selected from the group consisting of: polyhedron promoter, p10 promoter, 4xHsp27 EcRE+minimal Hsp70 promoter, deltaE1 promoter, E1 promoter. In a preferred embodiment of the present invention, the construct is an insect-compatible vector, preferably a baculoviral vector.

Alternatively or in combination with any previous embodiments, the nucleic acid molecule comprises an open reading frame selected from the group consisting of: SEQ ID NO: 51, 69, 42, 47, 48 and 50, preferably SEQ ID NO:51 or SEQ ID NO:69, more preferably SEQ ID NO:51.

In a third aspect, the present invention relates to an insect cell comprising a nucleic acid construct according to the invention.

Alternatively or in combination with any previous embodiments, in a further preferred embodiment the insect cell further comprises: (a)a second nucleotide sequence comprising at least one AAV inverted terminal repeat (ITR) nucleotide sequence; (b) a third nucleotide sequence comprising a Rep78 or a Rep68 coding sequence operably linked to expression control sequences for expression in an insect cell; (c) optionally, a fourth nucleotide sequence comprising a Rep52 or a Rep40 coding sequence operably linked to expression control sequences for expression in an insect cell.

Alternatively or in combination with any previous embodiments, in a further preferred embodiment the insect cell comprises: (a) a first nucleic acid construct according to the invention, whereby the first nucleic acid construct further comprises

the third and fourth nucleotide sequences as defined above; and, (b) a second nucleic acid construct comprising the second nucleotide sequence as defined above, wherein the second nucleic acid construct preferably is an insect cell-compatible vector, more preferably a baculoviral vector.

Alternatively or in combination with any previous embodiments, in a further preferred embodiment the second nucleotide sequence further comprises at least one nucleotide sequence encoding a gene product of interest (for expression in a mammalian cell) and whereby the at least one nucleotide sequence encoding a gene product of interest becomes incorporated into the genome of an AAV serotype 5 produced in the insect cell.

Alternatively or in combination with any previous embodiments, in a further preferred embodiment the second nucleotide sequence comprises two AAV ITR nucleotide sequences and wherein the at least one nucleotide sequence encoding a gene product of interest is located between the two AAV ITR nucleotide sequences.

Alternatively or in combination with any previous embodiments, in a further preferred embodiment the first nucleotide sequence, second nucleotide sequence, third nucleotide sequence and optionally fourth nucleotide sequence are stably integrated in the genome of the insect cell.

In a fourth aspect, the present invention relates to an AAV virion, comprising in its genome at least one nucleotide sequence encoding a gene product of interest, whereby the at least one nucleotide sequence preferably is not a native AAV nucleotide sequence, and wherein the AAV VP1 capsid protein comprises, or consists of, from N terminus to C terminus:

(i) a first amino acid residue, which is encoded by a translation initiation codon, preferably by a suboptimal translation initiation codon selected from the group consisting of CTG, ACG, TTG and GTG;

(ii) a second amino acid residue selected from the group consisting of alanine, glycine, valine, aspartic acid and glutamic acid;

(iii) optionally, one or more additional amino acid residues following the second amino acid residue; and,

(iv) an amino acid sequence of the AAV VP1 capsid protein, whereby the sequence lacks only the amino acid residue that is encoded by the VP1 translation initiation codon.

Preferably, an AAV virion according to the invention comprises a gene product of interest that encodes a Factor IX or a Factor VIII protein.

In a fifth aspect, the present invention relates to a method for producing an AAV in an insect cell, comprising the steps of: (a) culturing an insect cell according to the invention under conditions such that AAV is produced; and optionally (b) recovery of the AAV.

Definitions

As used herein, the term "operably linked" refers to a linkage of polynucleotide (or polypeptide) elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a transcription regulatory sequence is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein encoding regions, contiguous and in reading frame.

"Expression control sequence" refers to a nucleic acid sequence that regulates the expression of a nucleotide sequence to which it is operably linked. An expression control sequence is "operably linked" to a nucleotide sequence when the expression control sequence controls and regulates the transcription and/or the translation of the nucleotide sequence. Thus, an expression control sequence can include promoters, enhancers, internal ribosome entry sites (IRES), transcription terminators, a start codon in front of a protein-encoding gene, splicing signal for introns, and stop codons. The term "expression control sequence" is intended to include, at a minimum, a sequence whose presence are designed to influence expression, and can also include additional advantageous components. For example, leader sequences and fusion partner sequences are expression control sequences. The term can also include the design of the nucleic acid sequence such that undesirable, potential initiation codons in and out of frame, are removed from the sequence. It can also include the design of the nucleic acid sequence such that undesirable potential splice sites are removed. It includes sequences or polyadenylation sequences (pA) which direct the addition of a polyA tail, i.e., a string of adenine residues at the 3'-end of a mRNA, sequences referred to as polyA sequences. It also can be designed to enhance mRNA stability. Expression control sequences which affect the transcription and translation stability, e.g., promoters, as well as

sequences which effect the translation, e.g., Kozak sequences, are known in insect cells. Expression control sequences can be of such nature as to modulate the nucleotide sequence to which it is operably linked such that lower expression levels or higher expression levels are achieved.

As used herein, the term "promoter" or "transcription regulatory sequence" refers to a nucleic acid fragment that functions to control the transcription of one or more coding sequences, and is located upstream with respect to the direction of transcription of the transcription initiation site of the coding sequence, and is structurally identified by the presence of a binding site for DNA-dependent RNA polymerase, transcription initiation sites and any other DNA sequences, including, but not limited to transcription factor binding sites, repressor and activator protein binding sites, and any other sequences of nucleotides known to one of skill in the art to act directly or indirectly to regulate the amount of transcription from the promoter. A "constitutive" promoter is a promoter that is active in most tissues under most physiological and developmental conditions. An "inducible" promoter is a promoter that is physiologically or developmentally regulated, e.g. by the application of a chemical inducer. A "tissue specific" promoter is only active in specific types of tissues or cells.

The terms "substantially identical", "substantial identity" or "essentially similar" or "essential similarity" means that two peptide or two nucleotide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default parameters, share at least a certain percentage of sequence identity as defined elsewhere herein. GAP uses the Needleman and Wunsch global alignment algorithm to align two sequences over their entire length, maximizing the number of matches and minimizes the number of gaps. Generally, the GAP default parameters are used, with a gap creation penalty = 50 (nucleotides) / 8 (proteins) and gap extension penalty = 3 (nucleotides) / 2 (proteins). For nucleotides the default scoring matrix used is nwsgapdna and for proteins the default scoring matrix is Blosum62 (Henikoff & Henikoff, 1992, PNAS 89, 915-919). It is clear than when RNA sequences are said to be essentially similar or have a certain degree of sequence identity with DNA sequences, thymine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence. Sequence alignments and scores for percentage sequence identity may be determined using computer programs, such as the GCG Wisconsin Package, Version 10.3, available from Accelrys Inc., 9685 Scranton Road, San Diego, CA 92121-3752

USA or the open-source software Emboss for Windows (current version 2.7.1-07). Alternatively percent similarity or identity may be determined by searching against databases such as FASTA, BLAST, etc.

Nucleotide sequences encoding parvoviral Rep proteins of the invention may also be defined by their capability to hybridise with the nucleotide sequence of SEQ ID NO.1, respectively, under moderate, or preferably under stringent hybridisation conditions. Stringent hybridisation conditions are herein defined as conditions that allow a nucleic acid sequence of at least about 25, preferably about 50 nucleotides, 75 or 100 and most preferably of about 200 or more nucleotides, to hybridise at a temperature of about 65°C in a solution comprising about 1 M salt, preferably 6 x SSC or any other solution having a comparable ionic strength, and washing at 65°C in a solution comprising about 0.1 M salt, or less, preferably 0.2 x SSC or any other solution having a comparable ionic strength. Preferably, the hybridisation is performed overnight, i.e. at least for 10 hours and preferably washing is performed for at least one hour with at least two changes of the washing solution. These conditions will usually allow the specific hybridisation of sequences having about 90% or more sequence identity.

Moderate conditions are herein defined as conditions that allow a nucleic acid sequences of at least 50 nucleotides, preferably of about 200 or more nucleotides, to hybridise at a temperature of about 45°C in a solution comprising about 1 M salt, preferably 6 x SSC or any other solution having a comparable ionic strength, and washing at room temperature in a solution comprising about 1 M salt, preferably 6 x SSC or any other solution having a comparable ionic strength. Preferably, the hybridisation is performed overnight, i.e. at least for 10 hours, and preferably washing is performed for at least one hour with at least two changes of the washing solution. These conditions will usually allow the specific hybridisation of sequences having up to 50% sequence identity. The person skilled in the art will be able to modify these hybridisation conditions in order to specifically identify sequences varying in identity between 50% and 90%.

Detailed description of the invention

The present invention relates to the use of animal parvoviruses, in particular dependoviruses such as infectious human or simian AAV, and the components thereof

(e.g., an animal parvovirus genome) for use as vectors for introduction and/or expression of nucleic acids in mammalian cells. In particular, the invention relates to improvements in infectivity of such parvoviral vectors when produced in insect cells.

Viruses of the Parvoviridae family are small DNA animal viruses. Parvoviridae may be divided between two subfamilies: the Parvovirinae, which infect vertebrates, and the Densovirinae, which infect insects. Members of the subfamily Parvovirinae are herein referred to as the parvoviruses and include the genus Dependovirus. As may be deduced from the name of their genus, members of the Dependovirus are unique in that they usually require coinfection with a helper virus such as adenovirus or herpes virus for productive infection in cell culture. The genus Dependovirus includes AAV, which normally infects humans (e.g., serotypes 1, 2, 3A, 3B, 4, 5, 6, 7, 8, 9, 10 , 11, 12, 13) or primates (e.g., serotypes 1 and 4), and related viruses that infect other warm-blooded animals (e.g., bovine, canine, equine, and ovine adeno-associated viruses). Further information on parvoviruses and other members of the Parvoviridae is described in Kenneth I. Berns, "Parvoviridae: The Viruses and Their Replication," Chapter 69 in Fields Virology (3d Ed. 1996). For convenience the present invention is further exemplified and described herein by reference to AAV. It is however understood that the invention is not limited to AAV but may equally be applied to other parvoviruses.

The genomic organization of all known AAV serotypes is very similar. The genome of AAV is a linear, single-stranded DNA molecule that is less than about 5,000 nucleotides (nt) in length. Inverted terminal repeats (ITRs) flank the unique coding nucleotide sequences for the non-structural replication (Rep) proteins and the structural (VP) proteins. The VP proteins (VP1, -2 and -3) form the capsid. The terminal 145 nt are self-complementary and are organized so that an energetically stable intramolecular duplex forming a T-shaped hairpin may be formed. These hairpin structures function as an origin for viral DNA replication, serving as primers for the cellular DNA polymerase complex. Following wtAAV infection in mammalian cells the Rep genes (i.e. Rep78 and Rep52) are expressed from the P5 promoter and the P19 promoter, respectively and both Rep proteins have a function in the replication of the viral genome. A splicing event in the Rep ORF results in the expression of actually four Rep proteins (i.e. Rep78, Rep68, Rep52 and Rep40). However, it has been shown that the unspliced mRNA, encoding Rep78 and Rep52 proteins, in mammalian cells are sufficient for AAV vector production. Also in insect cells the Rep78 and Rep52

proteins suffice for AAV vector production. The three capsid proteins, VP1, VP2 and VP3 are expressed from a single VP reading frame from the p40 promoter. wtAAV infection in mammalian cells relies for the capsid proteins production on a combination of alternate usage of two splice acceptor sites and the suboptimal utilization of an ACG initiation codon for VP2. This is however not accurately reproduced in insect cells, thus requiring further features to obtain the correct stoichiometry of the AAV capsid proteins.

In a first aspect the invention relates to a nucleic acid molecule having a nucleotide sequence comprising an open reading frame encoding adeno-associated virus (AAV) capsid proteins. Preferably, the reading frame encoding the capsid proteins is modified, compared to a wild type open reading frame encoding AAV capsid proteins, by at least: (i) replacement of the ATG initiation codon for a suboptimal translation initiation codon selected from the group consisting of CTG, ACG, TTG and GTG; and (ii) the insertion of codons for one or more amino acid residues inserted between the suboptimal translation initiation codon and the codon encoding the amino acid residue that corresponds to the amino acid residue at position 2 of a capsid protein amino acid sequence, preferably the amino acid residue at position 2 of a wild type capsid protein amino acid sequence. It is understood that position 2 of a (wild type) capsid protein amino acid sequence preferably refers to position 2 of the amino acid sequence of a (wild type) AAV VP1 capsid protein. Preferably, the suboptimal translation initiation codon is immediately followed at its 3'-end by a codon for an amino acid residue selected from the group consisting of alanine, glycine, valine, aspartic acid and glutamic acid.

Alternatively, in this aspect the invention relates to a nucleic acid molecule having a nucleotide sequence comprising an open reading frame, wherein the open reading frame in 5' to 3'order comprises, or consists of:

(i) a first codon, which is a suboptimal translation initiation codon selected from the group consisting of CTG, ACG, TTG and GTG;

(ii) a second codon selected from the group consisting of alanine, glycine, valine, aspartic acid and glutamic acid;

(iii) optionally, one or more codons for additional amino acid residues following the second codon; and,

(iv) a sequence encoding AAV capsid proteins, whereby the sequence lacks the VP1 translation initiation codon, preferably whereby the sequence lacks only the VP1 translation initiation codon or, alternatively said, whereby the sequence lacks no more than the VP1 translation initiation codon.

Thus, in (iv) the sequence preferably comprises, or consists of: the remainder of an open reading frame encoding AAV capsid proteins whereby the remainder starts at the position corresponding to the second amino acid position in a wild type open reading frame encoding the capsid proteins.

A nucleic acid molecule having a nucleotide sequence comprising an open reading frame encoding adeno-associated virus (AAV) capsid proteins is herein understood to comprise nucleotide sequences encoding, preferably all three, VP1, VP2, and VP3 capsid proteins of animal parvoviruses.

The phrase "starts with a suboptimal translation initiation codon selected from the group consisting of CTG, ACG, TTG and GTG" or "first codon, which is a suboptimal translation initiation codon selected from the group consisting of CTG, ACG, TTG and GTG" is herein understood to mean that the initiation codon of the open reading frame encoding the adeno-associated virus (AAV) capsid proteins at the position encoding the amino terminus of the VP1 capsid protein is a suboptimal translation initiation codon selected from the group consisting of CTG, ACG, TTG and GTG.

Suboptimal is herein understood to mean that the codon is less efficient in the initiation of translation in an otherwise identical context as compared to the normal ATG codon. Preferably the initiation codon for translation of the AAV VP1 capsid protein is selected from ACG, TTG, GTG, and CTG, more preferably the initiation codon for translation of the AAV VP1 capsid protein is selected from CTG and ACG and most preferably the initiation codon for translation of the AAV VP1 capsid protein is CTG. The animal parvovirus preferably is a dependovirus, more preferably a human or simian adeno-associated virus (AAV).

In a particularly preferred embodiment, the suboptimal initiation codon of VP1 is CTG, one additional codon is introduced immediately adjacent to the suboptimal initiation codon at its 3'end, the additional codon coding for alanine. Preferably the capsid proteins are AAV5 capsid proteins. This results in improved potency of the AAV5 virions. The term "potency" is herein used to mean the ability of a vector to drive the expression of its genetic material.

The open reading frame further comprises a second codon encoding an amino acid residue selected from the group consisting of alanine, glycine, valine, aspartic acid and glutamic acid, preferably encoding alanine. More preferably, the second codon is selected from the group consisting of GCT, GCC, GCA, GCG and GGU, preferably wherein the codon is GCT. The open reading frame optionally comprises one or more codons encoding further additional amino acid residues following the second codon, for example codons for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 additional amino acids, but preferably less than 60, 50, 40, 35, 30, 25, 20, 19, 18, 17, 16, 15 or 14 additional amino acid residues. As will be readily understood, the codons encoding the additional amino acid residues are to be in frame with the open reading frame of the capsid proteins.

In an embodiment, if the open reading frame is compared with a wild-type capsid protein, the open reading frame encoding the capsid proteins further comprises codons that encode for one or more amino acid residues inserted between the suboptimal translation initiation codon of VP1 and the codon that encodes for the amino acid residue immediately adjacent to the initiation codon on its 3' end in the corresponding wild-type capsid protein. For example, the open reading frame comprises codons for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 additional amino acid residues as compared to the corresponding wild-type capsid protein. Preferably, the open reading frame comprises codons for less than 60, 50, 40, 35, 30, 25, 20, 19, 18, 17, 16, 15 or 14 additional amino acid residues as compared to the corresponding wild-type capsid protein. As will be readily understood, the codons encoding the additional amino acid residues are to be in frame with the open reading frame of the capsid proteins. Of these codons that encode the additional amino acid residues as compared to the corresponding wild-type capsid proteins, the first codon, i.e. the codon that is immediately adjacent to the suboptimal translation initiation codon at its 3' end, encodes for an amino acid residue selected from the group consisting of alanine, glycine, valine, aspartic acid and glutamic acid. Thus, if there is only one additional codon between the translation initiation codon and the codon that encodes for the amino acid residue that corresponds to residue 2 of the wild-type sequence, that additional codon encodes an amino acid residue selected from the group consisting of alanine, glycine, valine, aspartic acid and glutamic acid. If there are more than one additional codon between the translation initiation codon and the codon that encodes

for amino acid residue 2 of the wild-type sequence, then the codon immediately following the translation initiation codon encodes an amino acid residue selected from the group consisting of alanine, glycine, valine, aspartic acid and glutamic acid. Preferably, the additional amino acid residue immediately following the suboptimal translation initiation codon (i.e. at its 3' end) is alanine, glycine or valine, more preferably alanine. In other words, in a preferred embodiment of the present invention, the codon immediately following the suboptimal translation initiation codon encodes alanine.

In a preferred embodiment of the present invention, the codon immediately following the suboptimal translation initiation codon, i.e. the second codon, is selected from the group consisting of GCT, GCC, GCA, GCG, GGU, GGC, GGA, GGG, GUU, GUC, GUA, GUG, GAU, GAC, GAA and GAG, preferably from the group consisting of GCT, GCC, GCA, GCG and GGU, even more preferably the codon is GCT.

The sequence encoding AAV capsid proteins in step (iv) can be a capsid sequence as found in nature such as for example of AAV1 – AAV13 of which the nucleotide and amino acid sequences are shown in SEQ ID NO: 13 – 38 and SEQ ID NO: 70 - 73. Hence, the sequence encoding AAV capsid proteins in step (iv) can for example be a capsid sequence selected from the group consisting of: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12 and AAV13. Alternatively, the sequence is man-made, for example, the sequence may be a hybrid form or may be codon optimized, such as for example by codon usage of *AcmNPv* or *Spodoptera frugiperda*. For example, the capsid sequence may be composed of the VP2 and VP3 sequences of AAV1 whereas the remainder of the VP1 sequence is of AAV5. A preferred capsid protein is AAV5, preferably as provided in SEQ ID NO: 22, AAV8, preferably as provided in SEQ ID NO: 28 or AAV9, preferably as provided in SEQ ID NO: 30, SEQ ID NO 71 or SEQ ID NO:73. Thus, in a preferred embodiment, the AAV capsid proteins are AAV serotype 5, AAV serotype 8, or AAV serotype 9 capsid proteins that have been modified according to the invention. If the capsid protein is AAV9, it is preferred that the capsid protein has a sequence such as for example disclosed in WO 03/052052 or in WO 05/033321 or as provided in SEQ ID NO: 29, 30, 70, 71, 72, 73 or 74. More preferably, if the capsid protein is AAV9 the capsid protein has a sequence as provided in SEQ ID NO: 72 and 73. More preferably, the AAV capsid proteins are AAV serotype 5 capsid proteins that

have been modified according to the invention. It is understood that the exact molecular weights of the capsid proteins, as well as the exact positions of the translation initiation codons may differ between different parvoviruses. However, the skilled person will know how to identify the corresponding position in nucleotide sequence from other parvoviruses than AAV-5. Alternatively, the sequence encoding AAV capsid proteins is a man-made sequence, for example as a result of directed evolution experiments. This can include generation of capsid libraries via DNA shuffling, error prone PCR, bioinformatic rational design, site saturated mutagenesis. Resulting capsids are based on the existing serotypes but contain various amino acid or nucleotide changes that improve the features of such capsids. The resulting capsids can be a combination of various parts of existing serotypes, "shuffled capsids" or contain completely novel changes, *i.e.* additions, deletions or substitutions of one or more amino acids or nucleotides, organized in groups or spread over the whole length of gene or protein. See for example Schaffer and Maheshri; Proceedings of the 26th Annual International Conference of the IEEE EMBS San Francisco, CA, USA ; September 1-5, 2004, pages 3520-3523; Asuri et al. (2012) Molecular Therapy 20(2):329-3389; Lisowski et al. (2014) Nature 506(7488):382-386, herein incorporated by reference.

In a preferred embodiment of the invention, the open reading frame encoding VP3 capsid protein starts with non-canonical translation initiation codon selected from the group consisting of: ACG, ATT, ATA, AGA, AGG, AAA, CTG, CTT, CTC, CTA, CGA, CGC, TTG, TAG and GTG. Preferably, the non-canonical translation initiation codon is selected from the group consisting of GTG, CTG, ACG, TTG, more preferably the non-canonical translation initiation codon is CTG.

A preferred nucleotide sequence of the invention for the expression of the AAV capsid proteins is a nucleotide sequence comprising an expression control sequence comprising a VP2 initiator context. A VP2 initiator context is herein understood to mean a number of nucleotides preceding the non-canonical translational imitation start of VP2. In a preferred embodiment, the VP initiator context is a nine nucleotide sequence of SEQ. ID NO: 3 or a nucleotide sequence substantially homologous to SEQ. ID NO: 3, upstream of the suboptimal translation initiation codon of the nucleotide sequence encoding the AAV VP1 capsid protein, preferably immediately upstream of the suboptimal translation initiation codon, i.e. immediately adjacent to the suboptimal translation initiation codon at its 5' end. A sequence with substantial identity to the

nucleotide sequence of SEQ. ID NO: 3 and that will help increase expression of VP1 is e.g. a sequence which has at least 60%, 70%, 80% or 90% identity, preferably 100% identity, to the nine nucleotide sequence of SEQ ID NO: 3.

A further preferred nucleotide sequence of the invention for the expression of the AAV capsid proteins is a nucleotide sequence comprising an expression control sequence comprising a Kozak consensus sequence around the initiation codon of the nucleotide sequence encoding the AAV VP1 capsid protein. The Kozak consensus sequence is herein defined as GCCRCC(NNN)G (SEQ. ID NO: 4), wherein R is a purine (i.e. A or G) and wherein (NNN) stands for any of the suboptimal initiation codons as defined herein above. Preferably, in the Kozak consensus sequence in the nucleotide sequence of the invention, the R is a G. The nucleotide sequence of the invention for the expression of the AAV capsid proteins comprising a Kozak consensus sequence is thus preferably selected from GCCACC(ACG)G (SEQ ID NO: 5), GCCGCC(ACG)G (SEQ ID NO: 6), GCCACC(TTG)G (SEQ ID NO: 7), GCCGCC(TTG)G (SEQ ID NO: 8), GCCACC(GTG)G (SEQ ID NO: 9), GCCGCC(GTG)G (SEQ ID NO: 10), GCCACC(CTG)G (SEQ ID NO: 11) and GCCGCC(CTG)G (SEQ ID NO: 12), more preferably the nucleotide sequence comprising the Kozak consensus sequence is selected from GCCACC(CTG)G (SEQ ID NO: 11) and GCCGCC(CTG)G (SEQ ID NO: 12), most preferably, the nucleotide sequence comprising the Kozak consensus sequence is GCCGCC(CTG)G (SEQ ID NO: 12). The nucleotides in brackets herein indicate the position of the initiation codon of the VP1 protein.

The nucleotide sequence of the invention for expression of the AAV capsid proteins further preferably comprises at least one modification of the nucleotide sequence encoding AAV VP1 capsid protein selected from among a G at nucleotide position 12, an A at nucleotide position 21, and a C at nucleotide position 24, wherein the nucleotide positions correspond to the nucleotide positions of the wild-type nucleotide sequences, for example as shown in SEQ ID NO:21. A "potential/possible false start site" or "potential/possible false translation initiation codon" is herein understood to mean an in-frame ATG codon located in the coding sequence of the capsid protein(s). Elimination of possible false start sites for translation of VP1 of other serotypes will be well understood by an artisan of skill in the art, as will be the elimination of putative splice sites that may be recognized in insect cells. For example,

the modification of the nucleotide at position 12 is not required for recombinant AAV5, since the nucleotide T is not giving rise to a false ATG codon. For example, the further modification of the nucleotide sequence for AAV5 can be as presented in SEQ ID NO:39. The various modifications of the wild-type AAV sequences for proper expression in insect cells is achieved by application of well-known genetic engineering techniques such as described e.g. in Sambrook and Russell (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, New York. Various further modifications of VP coding regions are known to the skilled artisan which could either increase yield of VP and virion or have other desired effects, such as altered tropism or reduce antigenicity of the virion. These modifications are within the scope of the present invention.

In a preferred embodiment, the nucleic acid molecule according to the present invention comprises or consists of an open reading frame selected from the group consisting of: SEQ ID NO: 51, 69, 41, 42, 43, 44, 45, 46, 47, 48, 50 and 52, more preferably the nucleic acid molecule according to the present invention comprises or consists of an open reading frame selected from the group consisting of: SEQ ID NO: 51, 69, 42, 43, 47, 48 and 50, and even more preferably it comprises or consists of SEQ ID NO: 69 or 51, and still more preferably it comprises or consists of SEQ ID NO:51.

Preferably the nucleotide sequence of the invention encoding the AAV capsid proteins is operably linked to expression control sequences for expression in an insect cell. Thus, in a second aspect, the present invention relates to a nucleic acid construct comprising a nucleic acid molecule according to the invention, wherein the nucleotide sequence of the open reading frame encoding the adeno-associated virus (AAV) capsid proteins is operably linked to expression control sequences for expression in an insect cell. These expression control sequences will at least include a promoter that is active in insect cells. Techniques known to one skilled in the art for expressing foreign genes in insect host cells can be used to practice the invention. Methodology for molecular engineering and expression of polypeptides in insect cells is described, for example, in Summers and Smith. 1986. A Manual of Methods for Baculovirus Vectors and Insect Culture Procedures, Texas Agricultural Experimental Station Bull. No. 7555, College Station, Tex.; Luckow. 1991. In Prokop et al., Cloning and Expression of Heterologous Genes in Insect Cells with Baculovirus Vectors' Recombinant DNA Technology and Applications, 97-152; King, L. A. and R. D. Possee, 1992, The baculovirus expression

system, Chapman and Hall, United Kingdom; O'Reilly, D. R., L. K. Miller, V. A. Luckow, 1992, Baculovirus Expression Vectors: A Laboratory Manual, New York; W. H. Freeman and Richardson, C. D., 1995, Baculovirus Expression Protocols, Methods in Molecular Biology, volume 39; US 4,745,051; US2003148506; and WO 03/074714. A particularly suitable promoter for transcription of the nucleotide sequence of the invention encoding of the AAV capsid proteins is e.g. the polyhedron (polH) promoter, such as the polH promoter provided in SEQ ID NO:53 and the short polH promoter provided in SEQ ID NO:54. However, other promoters that are active in insect cells are known in the art, e.g. a polyhedrin (polH) promoter, p10 promoter, p35 promoter, 4xHsp27 EcRE+minimal Hsp70 promoter, deltaE1 promoter, E1 promoter or IE-1 promoter and further promoters described in the above references.

Preferably the nucleic acid construct for expression of the AAV capsid proteins in insect cells is an insect cell-compatible vector. An "insect cell-compatible vector" or "vector" is understood to a nucleic acid molecule capable of productive transformation or transfection of an insect or insect cell. Exemplary biological vectors include plasmids, linear nucleic acid molecules, and recombinant viruses. Any vector can be employed as long as it is insect cell-compatible. The vector may integrate into the insect cells genome but the presence of the vector in the insect cell need not be permanent and transient episomal vectors are also included. The vectors can be introduced by any means known, for example by chemical treatment of the cells, electroporation, or infection. In a preferred embodiment, the vector is a baculovirus, a viral vector, or a plasmid. In a more preferred embodiment, the vector is a baculovirus, i.e. the construct is a baculoviral vector. Baculoviral vectors and methods for their use are described in the above cited references on molecular engineering of insect cells.

In a preferred embodiment, the nucleic acid molecule comprised in the nucleic acid construct according to the present invention, comprises or consists of an open reading frame selected from the group consisting of: SEQ ID NO: 51, 69, 42, 43, 47, 48 and 50, more preferably it comprises or consists of SEQ ID NO:51 or SEQ ID NO:69, even more preferably it comprises or consists of SEQ ID NO:51.

In a third aspect the invention relates to an insect cell comprising a nucleic acid construct of the invention as defined above. Any insect cell which allows for replication of AAV and which can be maintained in culture can be used in accordance with the present invention. For example, the cell line used can be from Spodoptera

frugiperda, drosophila cell lines, or mosquito cell lines, e.g., Aedes albopictus derived cell lines. Preferred insect cells or cell lines are cells from the insect species which are susceptible to baculovirus infection, including e.g. expresSF+®, Drosophila Schneider 2 (S2) Cells, Se301, SeIZD2109, SeUCR1, Sf9, Sf900+, Sf21, BTI-TN-5B1-4, MG-1, Tn368, HzAm1, Ha2302, Hz2E5 and High Five from Invitrogen.

A preferred insect cell according to the invention further comprises: (a) a second nucleotide sequence comprising at least one AAV inverted terminal repeat (ITR) nucleotide sequence; (b) a third nucleotide sequence comprising a Rep52 or a Rep40 coding sequence operably linked to expression control sequences for expression in an insect cell; and, (c) a fourth nucleotide sequence comprising a Rep78 or a Rep68 coding sequence operably linked to expression control sequences for expression in an insect cell.

In the context of the invention "at least one AAV ITR nucleotide sequence" is understood to mean a palindromic sequence, comprising mostly complementary, symmetrically arranged sequences also referred to as "A," "B," and "C" regions. The ITR functions as an origin of replication, a site having a "cis" role in replication, i.e., being a recognition site for trans acting replication proteins (e.g., Rep 78 or Rep68) which recognize the palindrome and specific sequences internal to the palindrome. One exception to the symmetry of the ITR sequence is the "D" region of the ITR. It is unique (not having a complement within one ITR). Nicking of single-stranded DNA occurs at the junction between the A and D regions. It is the region where new DNA synthesis initiates. The D region normally sits to one side of the palindrome and provides directionality to the nucleic acid replication step. An AAV replicating in a mammalian cell typically has two ITR sequences. It is, however, possible to engineer an ITR so that binding sites are on both strands of the A regions and D regions are located symmetrically, one on each side of the palindrome. On a double-stranded circular DNA template (e.g., a plasmid), the Rep78- or Rep68-assisted nucleic acid replication then proceeds in both directions and a single ITR suffices for AAV replication of a circular vector. Thus, one ITR nucleotide sequence can be used in the context of the present invention. Preferably, however, two or another even number of regular ITRs are used. Most preferably, two ITR sequences are used. In view of the safety of viral vectors it may be desirable to construct a viral vector that is unable to further propagate after initial introduction into a cell. Such a safety mechanism for

limiting undesirable vector propagation in a recipient may be provided by using rAAV with a chimeric ITR as described in US2003148506. In a preferred embodiment, the nucleotide sequence encoding the parvoviral VP1, VP2 and VP3 capsid proteins comprises at least one in frame insertion of a sequence coding for an immune evasion repeat, such as described in WO 2009/154452. This results in formation of a so-called self-complementary or monomeric duplex parvoviral virion, which has the advantage that it shows a reduced immune response. In a preferred embodiment, the sequence encoding the parvoviral VP1, VP2 and VP3 capsid proteins comprises a monomeric duplex or self complementary genome. For the preparation of a monomeric duplex AAV vector, AAV Rep proteins and AAV capsid proteins are expressed in insect cells according to the present invention and in the presence of a vector genome comprising at least one AAV ITR, wherein Rep52 and/or Rep40 protein expression is increased relative to Rep78 and/or Rep68 protein expression. Monomeric duplex AAV vectors, can also be prepared by expressing in insect cells AAV Rep proteins and AAV Cap proteins in the presence of a vector genome construct flanked by at least one AAV ITR, wherein the nicking activity of Rep78 and/or Rep 60 is reduced relative to the helicase/encapsidation activity of Rep52 and/or Rep 40, as for example described in WO2011/122950.

The number of vectors or nucleic acid constructs employed is not limiting in the invention. For example, one, two, three, four, five, six, or more vectors can be employed to produce AAV in insect cells in accordance with the present invention. If six vectors are employed, one vector encodes AAV VP 1, another vector encodes AAV VP2, yet another vector encodes AAV VP3, still yet another vector encodes Rep52 or Rep40, while Rep78 or Rep 68 is encoded by another vector and a final vector comprises at least one AAV ITR. Additional vectors might be employed to express, for example, Rep52 and Rep40, and Rep78 and Rep 68. If fewer than six vectors are used, the vectors can comprise various combinations of the at least one AAV ITR and the VP1, VP2, VP3, Rep52/Rep40, and Rep78/Rep68 coding sequences. Preferably, two vectors or three vectors are used, with two vectors being more preferred as described above. If two vectors are used, preferably the insect cell comprises: (a) a first nucleic acid construct for expression of the AAV capsid proteins as defined above, which construct further comprises the third and fourth nucleotide sequences as defined in (b) and (c) above, the third nucleotide sequence comprising a Rep52 or a Rep40 coding

sequence operably linked to at least one expression control sequence for expression in an insect cell, and the fourth nucleotide sequence comprising a Rep78 or a Rep68 coding sequence operably linked to at least one expression control sequence for expression in an insect cell; and (b) a second nucleic acid construct comprising the second nucleotide sequence as defined in (a) above, comprising at least one AAV ITR nucleotide sequence. If three vectors are used, preferably the same configuration as used for two vectors is used except that separate vectors are used for expression of the capsid proteins and for expression of the Rep52, Rep40 Rep78 and Rep68 proteins. The sequences on each vector can be in any order relative to each other. For example, if one vector comprises ITRs and an ORF comprising nucleotide sequences encoding VP capsid proteins, the VP ORF can be located on the vector such that, upon replication of the DNA between ITR sequences, the VP ORF is replicated or not replicated. For another example, the Rep coding sequences and/or the ORF comprising nucleotide sequences encoding VP capsid proteins can be in any order on a vector. In is understood that also the second, third and further nucleic acid construct(s) preferably are an insect cell-compatible vectors, preferably a baculoviral vectors as described above. Alternatively, in the insect cell of the invention, one or more of the first nucleotide sequence, second nucleotide sequence, third nucleotide sequence, and fourth nucleotide sequence and optional further nucleotide sequences may be stably integrated in the genome of the insect cell. One of ordinary skill in the art knows how to stably introduce a nucleotide sequence into the insect genome and how to identify a cell having such a nucleotide sequence in the genome. The incorporation into the genome may be aided by, for example, the use of a vector comprising nucleotide sequences highly homologous to regions of the insect genome. The use of specific sequences, such as transposons, is another way to introduce a nucleotide sequence into a genome.

Thus, in a preferred embodiment, an insect cell according to the invention comprises: (a) a first nucleic acid construct according to the invention, whereby the first nucleic acid construct further comprises the third and fourth nucleotide sequences as defined above; and, (b) a second nucleic acid construct comprising the second nucleotide sequence as defined above, wherein the second nucleic acid construct preferably is an insect cell-compatible vector, more preferably a baculoviral vector.

The a preferred embodiment of the invention, the second nucleotide sequence present in the insect cells of the invention, i.e. the sequence comprising at least one

AAV ITR, further comprises at least one nucleotide sequence encoding a gene product of interest (preferably for expression in a mammalian cell), whereby preferably the at least one nucleotide sequence encoding a gene product of interest becomes incorporated into the genome of an AAV produced in the insect cell. Preferably, at least one nucleotide sequence encoding a gene product of interest is a sequence for expression in a mammalian cell. Preferably, the second nucleotide sequence comprises two AAV ITR nucleotide sequences and wherein the at least one nucleotide sequence encoding a gene product of interest is located between the two AAV ITR nucleotide sequences. Preferably, the nucleotide sequence encoding a gene product of interest (for expression in the mammalian cell) will be incorporated into the AAV genome produced in the insect cell if it is located between two regular ITRs, or is located on either side of an ITR engineered with two D regions. Thus, in a preferred embodiment, the invention provides an insect cell according the invention, wherein the second nucleotide sequence comprises two AAV ITR nucleotide sequences and wherein the at least one nucleotide sequence encoding a gene product of interest is located between the two AAV ITR nucleotide sequences.

Typically, the gene product of interest, including ITRs, is 5,000 nucleotides (nt) or less in length. In another embodiment an oversize DNA, i.e. more than 5,000 nt in length, can be expressed *in vitro* or *in vivo* by using AAV vector described by the present invention. An oversized DNA is here understood as a DNA exceeding the maximum AAV packaging limit of 5kbp. Therefore, the generation of AAV vectors able to produce recombinant proteins that are usually encoded by larger genomes than 5.0 kb is also feasible. For instance, the present inventors have generated rAAV5 vectors containing partially, uni-directionally packaged fragments of hFVIII in insect cells. The total size of vector genome encompassing at least 5.6kb packaged into two populations of FVIII fragment-containing AAV5 particles. These variant AAV5-FVIII vectors were shown to be actively secreting FVIII. This was confirmed in vitro, where the AAV vector comprising a gene product of interest encoding Factor VIII after infection of Huh7 cells resulted in production of active FVIII protein. Similarly, tail vein delivery of rAAV.FVIII in mice resulted in production of active FVIII protein. The molecular analysis of the encapsidation products unequivocally showed that the 5.6kbp FVIII expression cassette is not entirely encapsidated in AAV particle. Without wishing to be bound by any theory, we hypothesize that + and – DNA strands of the

encapsidated molecules revealed missing 5' ends. This is consistent with a previously reported unidirectional (starting at 3' end) packaging mechanism operating according to "head-full principia" with 4.7-4,9kbp limit (see for example Wu et al. [2010] Molecular Therapy 18(1):80-86; Dong et al. [2010] Molecular Therapy 18(1):87-92; Kapranov et al. [2012] Human Gene Therapy 23:46-55; and in particular Lai et al. [2010] Molecular Therapy 18(1):75-79;. Although only approximately 5 kb of the whole 5.6 kb vector genome was encapsidated, the vector was potent and lead to expression of active FVIII. We have shown that the correct template for production of FVIII was assembled in the target cell based on partial complementation of + and – DNA strains followed by second strand synthesis.

The second nucleotide sequence defined herein above may thus comprise a nucleotide sequence encoding at least one "gene product of interest" for expression in a mammalian cell, located such that it will be incorporated into an AAV genome replicated in the insect cell. Any nucleotide sequence can be incorporated for later expression in a mammalian cell transfected with the AAV produced in accordance with the present invention, as long as the constructs remain within the packaging capacity of the AAV virion. The nucleotide sequence may e.g. encode a protein it may express an RNAi agent, i.e. an RNA molecule that is capable of RNA interference such as e.g. a shRNA (short hairpin RNA) or an siRNA (short interfering RNA). "siRNA" means a small interfering RNA that is a short-length double-stranded RNA that are not toxic in mammalian cells (Elbashir et al., 2001, Nature 411: 494-98; Caplen et al., 2001, Proc. Natl. Acad. Sci. USA 98: 9742-47). In a preferred embodiment, the second nucleotide sequence may comprise two nucleotide sequences and each encodes one gene product of interest for expression in a mammalian cell. Each of the two nucleotide sequences encoding a product of interest is located such that it will be incorporated into a rAAV genome replicated in the insect cell.

The product of interest for expression in a mammalian cell may be a therapeutic gene product. A therapeutic gene product can be a polypeptide, or an RNA molecule (siRNA), or other gene product that, when expressed in a target cell, provides a desired therapeutic effect such as e.g. ablation of an undesired activity, e.g. the ablation of an infected cell, or the complementation of a genetic defect, e.g. causing a deficiency in an enzymatic activity. Examples of therapeutic polypeptide gene products include CFTR, Factor IX, Lipoprotein lipase (LPL, preferably LPL S447X; see WO 01/00220),

Apolipoprotein A1, Uridine Diphosphate Glucuronosyltransferase (UGT), Retinitis Pigmentosa GTPase Regulator Interacting Protein (RP-GRIP), cytokines or interleukins like e.g. IL-10, dystrophin, PBGD, NaGLU, Treg167, Treg289, EPO, IGF, IFN, GDNF, FOXP3, Factor VIII, VEGF, AGXT and insulin. Alternatively, or in addition as a second gene product, second nucleotide sequence defined herein above may comprise a nucleotide sequence encoding a polypeptide that serve as marker proteins to assess cell transformation and expression. Suitable marker proteins for this purpose are e.g. the fluorescent protein GFP, and the selectable marker genes HSV thymidine kinase (for selection on HAT medium), bacterial hygromycin B phosphotransferase (for selection on hygromycin B), Tn5 aminoglycoside phosphotransferase (for selection on G418), and dihydrofolate reductase (DHFR) (for selection on methotrexate), CD20, the low affinity nerve growth factor gene. Sources for obtaining these marker genes and methods for their use are provided in Sambrook and Russel (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, New York. Furthermore, second nucleotide sequence defined herein above may comprise a nucleotide sequence encoding a polypeptide that may serve as a fail-safe mechanism that allows to cure a subject from cells transduced with the rAAV of the invention, if deemed necessary. Such a nucleotide sequence, often referred to as a suicide gene, encodes a protein that is capable of converting a prodrug into a toxic substance that is capable of killing the transgenic cells in which the protein is expressed. Suitable examples of such suicide genes include e.g. the *E.coli* cytosine deaminase gene or one of the thymidine kinase genes from Herpes Simplex Virus, Cytomegalovirus and Varicella-Zoster virus, in which case ganciclovir may be used as prodrug to kill the transgenic cells in the subject (see e.g. Clair et al., 1987, Antimicrob. Agents Chemother. 31: 844-849).

In another embodiment the gene product of interest can be an AAV protein. In particular, a Rep protein, such as Rep78 or Rep68, or a functional fragment thereof. A nucleotide sequence encoding a Rep78 and/or a Rep68, if present on the rAAV genome of the invention and expressed in a mammalian cell transduced with the rAAV of the invention, allows for integration of the rAAV into the genome of the transduced mammalian cell. Expression of Rep78 and/or Rep68 in an rAAV-transduced or infected mammalian cell can provide an advantage for certain uses of the rAAV, by allowing

long term or permanent expression of any other gene product of interest introduced in the cell by the rAAV.

In the rAAV vectors of the invention the at least one nucleotide sequence(s) encoding a gene product of interest for expression in a mammalian cell, preferably is/are operably linked to at least one mammalian cell-compatible expression control sequence, e.g., a promoter. Many such promoters are known in the art (see Sambrook and Russel, 2001, *supra*). Constitutive promoters that are broadly expressed in many cell-types, such as the CMV promoter may be used. However, more preferred will be promoters that are inducible, tissue-specific, cell-type-specific, or cell cycle-specific. For example, for liver-specific expression a promoter may be selected from an α1-anti-trypsin promoter, a thyroid hormone-binding globulin promoter, an albumin promoter, LPS (thyroxine-binding globlin) promoter, HCR-ApoCII hybrid promoter, HCR-hAAT hybrid promoter and an apolipoprotein E promoter, LP1, HLP, minimal TTR promoter, FVIII promoter, hyperon enhancer, ealb-hAAT. Other examples include the E2F promoter for tumor-selective, and, in particular, neurological cell tumor-selective expression (Parr et al., 1997, Nat. Med. 3:1145-9) or the IL-2 promoter for use in mononuclear blood cells (Hagenbaugh et al., 1997, J Exp Med; 185: 2101-10).

AAV is able to infect a number of mammalian cells. See, e.g., Tratschin et al., Mol. Cell Biol., 5(11):3251-3260 (1985) and Grimm et al., Hum. Gene Ther., 10(15):2445-2450 (1999). However, AAV transduction of human synovial fibroblasts is significantly more efficient than in similar murine cells, Jennings et al., Arthritis Res, 3:1 (2001), and the cellular tropicity of AAV differs among serotypes. See, e.g., Davidson et al., Proc. Natl. Acad. Sci. USA, 97(7):3428-3432 (2000) (discussing differences among AAV2, AAV4, and AAV5 with respect to mammalian CNS cell tropism and transduction efficiency).

AAV sequences that may be used in the present invention for the production of AAV in insect cells can be derived from the genome of any AAV serotype. Generally, the AAV serotypes have genomic sequences of significant homology at the amino acid and the nucleic acid levels, provide an identical set of genetic functions, produce virions which are essentially physically and functionally equivalent, and replicate and assemble by practically identical mechanisms. For the genomic sequence of the various AAV serotypes and an overview of the genomic similarities see e.g. GenBank Accession number U89790; GenBank Accession number J01901; GenBank Accession

number AF043303; GenBank Accession number AF085716; Chlorini et al. (1997, J. Vir. 71: 6823-33); Srivastava et al. (1983, J. Vir. 45:555-64); Chlorini et al. (1999, J. Vir. 73:1309-1319); Rutledge et al. (1998, J. Vir. 72:309-319); and Wu et al. (2000, J. Vir. 74: 8635-47). Human or simian adeno-associated virus (AAV) serotypes are preferred sources of AAV nucleotide sequences for use in the context of the present invention, more preferably AAV serotypes which normally infects humans (e.g., serotypes 1, 2, 3A, 3B, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13) or primates (e.g., serotypes 1 and 4).

Preferably the AAV ITR sequences for use in the context of the present invention are derived from AAV1, AAV2, AAV5 and/or AAV4. Likewise, the Rep52, Rep40, Rep78 and/or Rep68 coding sequences are preferably derived from AAV1, AAV2, and/or AAV4. The sequences coding for the VP1, VP2, and VP3 capsid proteins for use in the context of the present invention may be taken from any of the known 42 serotypes, more preferably from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8 or AAV9 or newly developed AAV-like particles obtained by e.g. capsid shuffling techniques and AAV capsid libraries. In a preferred embodiment, the sequences coding for the VP1, VP2, and VP3 capsid proteins are from AAV5 or AAV8, more preferably from AAV5.

AAV Rep and ITR sequences are particularly conserved among most serotypes. The Rep78 proteins of various AAV serotypes are e.g. more than 89% identical and the total nucleotide sequence identity at the genome level between AAV2, AAV3A, AAV3B, and AAV6 is around 82% (Bantel-Schaal et al., 1999, J. Virol., 73(2):939-947). Moreover, the Rep sequences and ITRs of many AAV serotypes are known to efficiently cross-complement (i.e., functionally substitute) corresponding sequences from other serotypes in production of AAV particles in mammalian cells. US2003148506 reports that AAV Rep and ITR sequences also efficiently cross-complement other AAV Rep and ITR sequences in insect cells.

The AAV VP proteins are known to determine the cellular tropicity of the AAV virion. The VP protein-encoding sequences are significantly less conserved than Rep proteins and genes among different AAV serotypes. The ability Rep and ITR sequences to cross-complement corresponding sequences of other serotypes allows for the production of pseudotyped AAV particles comprising the capsid proteins of a serotype

(e.g., AAV3) and the Rep and/or ITR sequences of another AAV serotype (e.g., AAV2). Such pseudotyped AAV particles are a part of the present invention.

Modified "AAV" sequences also can be used in the context of the present invention, e.g. for the production of rAAV vectors in insect cells. Such modified sequences e.g. include sequences having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more nucleotide and/or amino acid sequence identity (e.g., a sequence having about 75-99% nucleotide sequence identity) to an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8 or AAV9 ITR, Rep, or VP can be used in place of wild-type AAV ITR, Rep, or VP sequences.

Although similar to other AAV serotypes in many respects, AAV5 differs from other human and simian AAV serotypes more than other known human and simian serotypes. In view thereof, the production of AAV5 can differ from production of other serotypes in insect cells. Where methods of the invention are employed produce rAAV5, it is preferred that one or more vectors comprising, collectively in the case of more than one vector, a nucleotide sequence comprising an AAV5 ITR, a nucleotide sequence comprises an AAV5 Rep52 and/or Rep40 coding sequence, and a nucleotide sequence comprises an AAV5 Rep78 and/or Rep68 coding sequence. Such ITR and Rep sequences can be modified as desired to obtain efficient production of rAAV5 or pseudotyped rAAV5 vectors in insect cells. E.g., the start codon of the Rep sequences can be modified.

In a preferred embodiment, the first nucleotide sequence, second nucleotide sequence, third nucleotide sequence and optionally fourth nucleotide sequence are stably integrated in the genome of the insect cell.

In a further aspect the invention relates to an AAV virion. Preferably, the AAV virion comprises in its genome at least one nucleotide sequence encoding a gene product of interest, whereby the at least one nucleotide sequence preferably is not a native AAV nucleotide sequence, and wherein the AAV VP1 capsid protein comprises or consists of, from the N-terminal end to the C-terminal end:

(i) a first amino acid residue, which is encoded by a translation initiation codon, preferably by a suboptimal translation initiation codon selected from the group consisting of CTG, ACG, TTG and GTG;

(ii) a second amino acid residue selected from the group consisting of alanine, glycine, valine, aspartic acid and glutamic acid;

(iii) optionally, one or more additional amino acid residues following the second amino acid residue; and,

(iv) an amino acid sequence of the AAV VP1 capsid protein, whereby the sequence lacks the amino acid residue that is encoded by the VP1 translation initiation codon. Preferably whereby the sequence lacks only the amino acid residue encoded by the VP1 translation initiation codon or, alternatively said, whereby the sequence lacks no more than the amino acid residue encoded by VP1 translation initiation codon.

Preferably, the amino acid sequence of the AAV VP1 capsid protein lacking only the amino acid residue encoded by the VP1 translation initiation codon is a naturally occurring amino acid sequence of a AAV VP1 capsid protein only lacking the amino acid residue encoded by the naturally occurring VP1 translation initiation codon. The first amino acid residue, which is encoded by a suboptimal translation initiation codon, typically is a methionine residue.

Alternatively, in this aspect the invention relates to an AAV virion, wherein the AAV virion comprises in its genome at least one nucleotide sequence encoding a gene product of interest, whereby the at least one nucleotide sequence preferably is not a native AAV nucleotide sequence, and wherein the AAV VP1 capsid has one or more additional amino acid residues inserted between the initiation codon and the amino acid residue that corresponds to the amino acid residue at position 2 of the wild type capsid protein, wherein the additional amino acid residue immediately following the initiation codon is selected from the group consisting of alanine, glycine, valine, aspartic acid and glutamic acid.

Preferably, in a virion according to the invention the stoichiometry of the AAV VP1, VP2, and VP3 capsid proteins is as follows; the amount of VP1: (a) is at least 100, 105, 110, 120, 150, 200 or 400% of the amount of VP2; or (b) is at least 8, 10, 10.5, 11, 12, 15, 20 or 40% of the amount of VP3; or (c) is at least as defined in both (a) and (b). Preferably, the amount of VP1, VP2 and VP3 is determined using an antibody recognizing an epitope that is common to each of VP1, VP2 and VP3. Various immunoassays are available in the art that will allow quantify the relative amounts of VP1, VP2 and/or VP3 (see e.g. Using Antibodies, E. Harlow and D. Lane, 1999, Cold

Spring Harbor Laboratory Press, New York). A suitable antibody recognizing an epitope that is common to each of the three capsid proteins is e.g. the mouse anti-Cap B1 antibody (as is commercially available from Progen, Germany).

A preferred AAV according to the invention is a virion comprising in its genome at least one nucleotide sequence encoding a gene product of interest, whereby the at least one nucleotide sequence preferably is not a native AAV nucleotide sequence, and whereby the AAV virion comprises a VP1 capsid protein that comprises a methionine, a threonine, a leucine or a valine at amino acid position 1. A more preferred AAV virion according to the invention has the ratio's of capsid proteins as defined above and comprises a VP1 capsid protein comprises a leucine or a valine at amino acid position 1. Even more preferred is an AAV virion that is obtainable from an insect cell as defined above in e.g. a method as defined herein below. Still more preferred is an AAV virion that comprises a threonine or a leucine at position 1 of the VP1 capsid protein, even more preferably a threonine residue.

An advantage of the AAV virions of the invention is their improved infectivity. Without wishing to be bound by any theory, it seems that in particular the infectivity increases with an increase of the amount of VP1 protein in the capsid in relation to the amounts of VP2 and/or VP3 in the capsid. The infectivity of an AAV virion is herein understood to mean the efficiency of transduction of the transgene comprised in the virion, as may be deduced from the expression rate of the transgene and the amount or activity of the product expressed from the transgene.

Preferably, an AAV virion of the invention comprises a gene product of interest that encodes a polypeptide gene product selected from the group consisting of: CFTR, Factor IX, Lipoprotein lipase (LPL, preferably LPL S447X; see WO 01/00220), Apolipoprotein A1, Uridine Diphosphate Glucuronosyltransferase (UGT), Retinitis Pigmentosa GTPase Regulator Interacting Protein (RP-GRIP), cytokines or interleukins like e.g. IL-10, dystrophin, PBGD, NaGLU, Treg167, Treg289, EPO, IGF, IFN, GDNF, FOXP3, Factor VIII, VEGF, AGXT and insulin. More preferably, the gene product of interest encodes a Factor IX or a Factor VIII protein.

In another aspect the invention thus relates to a method for producing an AAV in an insect cell. Preferably the method comprises the steps of: (a) culturing an insect cell as defined in herein above under conditions such that AAV is produced; and, optionally, (b) recovery of the AAV. Growing conditions for insect cells in culture, and

production of heterologous products in insect cells in culture are well-known in the art and described e.g. in the above cited references on molecular engineering of insects cells.

Preferably the method further comprises the step of affinity-purification of the AAV using an anti-AAV antibody, preferably an immobilized antibody. The anti-AAV antibody preferably is an monoclonal antibody. A particularly suitable antibody is a single chain camelid antibody or a fragment thereof as e.g. obtainable from camels or llamas (see e.g. Muyldermans, 2001, Biotechnol. 74: 277-302). The antibody for affinity-purification of AAV preferably is an antibody that specifically binds an epitope on a AAV capsid protein, whereby preferably the epitope is an epitope that is present on capsid protein of more than one AAV serotype. E.g. the antibody may be raised or selected on the basis of specific binding to AAV2 capsid but at the same time also it may also specifically bind to AAV1, AAV3 and AAV5 capsids.

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Description of the figures

Figure 1: Various mutant capsids harbouring reporter transgene SEAP were purified and resolved on an NuPage gel. Three capsid proteins, VP1 (87 kDa), VP2 (72 kDa) and VP3 (62 kDa) are shown.

Figure 2: *In vitro* potency assay with various AAV5 capsid mutants carrying *seap* expression cassette in Hela cells. The activity of the reporter gene is measured indirectly as emission of light and is expressed in RLU (relative light units). NTC = negative control.

Figure 3: *In vitro* potency assay with various AAV5 capsid mutants carrying *seap* expression cassette in Huh7 cells. The activity of the reporter gene is measured indirectly as emission of light and is expressed in RLU (relative light units). NTC = negative control .

Figure 4: In vivo potency assay of various capsid mutants carrying seap expression cassette in C57BL/6 mice. The activity of the reporter gene is measured indirectly as emission of light and is expressed in RLU (relative light units).

Figure 5: In vivo potency assay of various AAV5 capsid mutants carrying FIX expression cassette in C57BL/6 mice. FIX expression was monitored in mice upon administration of two different vectors *i.e.* capsid variant 160 and 765. Both capsids carry FIX expression cassette. FIX is measured in plasma at week 1, 2 and 4 post injections by means of specific ELISA. IU/ml represents international units of FIX protein found in 1 ml of plasma. PBS = phosphate buffered saline.

Figure 6: Mutant capsids harbouring reporter transgene SEAP were purified and resolved on an NuPage gel to show the three capsid proteins VP1, VP2 and VP3. Three clones of construct 43 are shown.

Figure 7: *In vitro* potency assay with various AAV5 capsid mutants carrying seap expression cassette in HeLa cells (A) and in Huh7 cells (B). The activity of the reporter gene is measured indirectly as emission of light and is expressed in RLU (relative light units) (a.u.: arbitrary units).

Examples

1. Introduction

The initial baculovirus system for production of rAAV was described by Urabe *et al* (Urabe et al. [2002] Human Gene Therapy 13(16):1935-1943) and consists of three baculoviruses, namely Bac-Rep, Bac-cap and Bac-vec, co-infection of which into insect cells *e.g.* SF9 resulted in generation of rAAV. The properties of such produced rAAV, *i.e.* physical and molecular characteristic including potency, did not differ significantly from the rAAV generated in mammalian cells (Urabe [2002] *supra*). In order to accomplish efficient generation of rAAV vectors in insect cells the AAV proteins needed for the process had to be expressed at appropriate levels. This required a number of adaptations of operons encoding for Rep and Cap proteins. Wild type AAV expresses large Rep78 to small Rep52 from two distinct promoters p5 and p19 respectively and splicing of the two messengers results in generation of Rep68 and Rep52 variants. This operon organization results in limited expression of Rep78 and relatively higher expression of Rep52. In order to mimic the low 78 to 52 ratio Urabe and colleagues constructed a DNA cassette in which expression of Rep78 was driven by the partially deleted promoter for the immediate-early 1 gene (*ΔIE-1*) whereas Rep52 expression was controlled by a strong polyhedrin promoter (*polh*). The spliced variants of large and small Reps were not observed in insect cells which likely relates to the difference in splicing processes between mammalian and insect cells. Another technical challenge to be overcome was related to the expression of the three major viral proteins (VP's). Wild type AAV expresses VP1, 2 and 3 from p40 promoter. Arising messenger RNA is spliced into two species: one responsible for VP1 expression whereas the second expresses both VP2 and VP3 via a "leaky ribosomal scanning mechanism" where the protein is initiated from non-canonical start *i.e.* ACG, is occasionally missed by the ribosome complex which than proceeds further until it finds the canonical start of VP3. Due to the differences in splicing machinery between vertebrate and insect cells the above described mechanism did not result in generation of proper capsids in insect cells. Urabe *et al.,* decided to introduce a modification of translational start of VP1 which was similar to these found in the VP2 in such a way that the translational start of VP1 was changed to ACG and the initiation context, which consists of 9 nucleotides preceeding VP1, was changed to those preceeding VP2. These genetic alterations resulted in expression of the three VPs in the correct stoichiometry

that could properly assemble into capsids from a single polycistronic mRNA. The transgene cassette on the other hand was similar to what was previously described for mammalian based systems, flanked by ITRs as the only *in trans* required elements for replication and packaging.

With the growing number of newly discovered AAV serotypes that hold different desired properties, there is a need for generation of these capsids in the BEV system. Although a successful production of AAV2 in the insect cells has been shown, not all serotypes perform equally well in the system adapted for AAV2. it seems that adapting a new serotype for optimum production and potency is not a trivial task and will require a tailor made approach. Previous attempts to adapt the rAAV5 sequence for production by BEVS in insect cells met a limited success, resulting in low incorporation of VP1 to the capsid (Kohlbrenner et al. (2005) Molecular Therapy 12 (6):1217-1225; Urabe et al. (2006) Journal of Virology 80(4):1874-1885). To circumvent this problem, Urabe et al. generated a chimeric type 2/5 virus which contains the N-terminal 136 amino acid residues from AAV type 2 and the remainder sequence from AAV serotype 5. Such virus was reported to produce well and to display similar potency to that of the wild type AAV5 (Urabe et al. (2006) *supra*). However, the resulting virion was a chimera and it does not represent the "true" rAAV5 serotype.

In order to generate genuine rAAV5 in insect cells with improved infectivity and/or potency, we designed several *capsid protein* 5 mutants. It seems important for the infectivity that the stoichiometry of the three viral proteins is balanced. For example, as previously reported we noticed that the lack of VP1 synthesis drastically influences the potency of the vector. Furthermore, we observed that the potency of the vectors was negatively correlated with the high incorporation of VP3 as compared to VP1 and VP2. Viral preparations with an excessive amount of VP3 were poor in transducing cells *in vitro* and *in vivo*. Finally we have constructed a genuine (or "true") rAAV5 capsid which displays superior potency to the chimeric rAAV5 generated by Urabe *et al* (2006, *supra)*. This new capsid was found to have balanced VP stoichiometry, and similar or superior potency as compared to the chimeric AAV2/5.

2. Methods

2.1. Generation of rAAV5 vectors

rAAV5 batches were generated by co-infecting expresSF+® insect cell line (Protein Sciences Corporation) with three different baculoviruses, which comprised expression cassettes for the capsid (rAAV5 variant library), replicase and transgene (Seap or Factor IX) under the control of a CMV and LP1 promoter, respectively. Capsid expression cassettes were under the control of a polyhedron promoter. Rep expression cassettes were as described in WO 2009/14445 (BAC.VD183) and under control of a deltaE1 and polyhedron promoter driving expression of Rep78 and Rep52, respectively. ExpresSF+® cells were infected at a 5:1:1 (Rep:Cap:Transgene) volumetric ratio using freshly amplified baculovirus stocks. After a 72 hour incubation at 28°C, cells were lysed with 10x lysis buffer (1,5M NaCl, 0,5M Tris-HCl, 1mM $MgCl_2$, 1% Triton X-100, pH= 8.5) for 1 hour at 28°C,. Genomic DNA was digested by Benzonase treatment for 1 hour at 37°C. Cell debris was removed by centrifugation for 15 minutes at 1900xg after which the supernatant containing the rAAV5 particles was stored at 4°C. Vector titers were determined in this so-called crude cell lysate with a specific Q-PCR directed against the promoter region of the transgene. Briefly, affinity purified vectors were analysed by Q-PCR. AAVs were treated with DNAse at 37°C to degrade extrageneous DNA. AAV DNA was then released from the particles by 1M NaOH treatment. Following a short heat treatment (30 minutes at 37°C) the alkaline environment was neutralized with an equal volume of 1M HCl. The neutralized samples contained the AAV DNA that was used in the Taqman Q-PCR. Q-PCR was performed according to standard procedures using primers and probes listed in Table 1 below.

2.2. Purification of rAAV5 vectors

rAAV5 particles were purified from crude lysates by a batch binding protocol using AVB sepharose (affinity resin, GE healthcare). rAAV5 crude cell lysates were added to washed (with 0.2M HPO_4 pH=7.5 buffer) resin. Subsequently, samples were incubated for 2 hours at room temperature under gentle mixing. Following the incubation the resin was washed in 0.2M HPO_4 pH=7.5 buffer and bound vectors were eluted by the addition of 0.2M Glycine pH=2.5. The pH of the eluted vectors was immediately neutralized by the addition of 0.5M Tris-HCl pH=8.5. Purified rAAV5 batches were stored at -20°C. Purified vectors were titered by a specific Q-PCR.

In order to generate higher vector amounts for *in vivo* study a modified purification protocol was used. Briefly, following the harvest, the clarified lysate was passed over a 0.22 µm filter (Millipak 60, 0.22 µm). Next, vector particles were affinity purified by means of a 8 ml AVB sepharose column (GE Healthcare) on a AKTA explorer (FPLC chromatography system, GE healthcare). Bound rAAV5 particles were eluted from the column with 0.2M Glycine pH=2.5. The eluate was immediately neutralized by 60 mM Tris HCl pH= 7.5. The buffer of the neutralized eluates was exchanged to PBS 5% Sucrose with the help of 100 KDa ultrafiltration (Millipore) filter. The final product was then filtered on a 0.22 µm filter (Millex GP), aliquoted and stored at -20°C until further use. Following the purification virus titers were determined with a specific Q-PCR.

Table 1. TAQMAN Q-PCR primers

primers used for detection of Seap transgene		Description	SEQ ID NO:
pr59	AATGGGCGGTAGGCGTGTA	CMV promotor fwd	55
pr60	AGGCGATCTGACGGTTCACTAA	CMV promotor rev	56
pb12	TGGGAGGTCTATATAAGCAG	CMV promotor probe Fam-MGB	57
primers used for detection of Factor IX transgene			
pr1103	CAAGTATGGCATCTACACCAAAGTCT	FIX fwd	58
pr1104	GCAATAGCATCACAAATTTCACAAA	FIX rev	59
pb25	TGTGAACTGGATCAAGGAGAAGACCAAGC	FIX probe Fam-Tamra	60

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2.3. VP protein composition of rAAV5 variants

VP protein composition of purified rAAV5 variants was determined on Bis-tris polyacrylamide gels (Nupage, Life technologies) stained with Sypro Ruby. Briefly, 15 µl of purified rAAV5 was mixed with 5 µl 4x LDS loading buffer (Life technologies) and loaded on a Bis-Tris polyacrylamide gel. The samples were electrophoretically separated for 2 hours at 100 Volts. Following electrophoresis the proteins were fixed for 30 minutes with 10% NaAC/7% EtOH and stained with Sypro Ruby (Life technologies) for 2 hours. VP proteins were then visualized under UV light on an ImageQuant system (GE Healthcare).

2.4. In vitro potency

To investigate *in vitro* potency of the different serotype 5 capsid variants, two continuous cell lines were used. Here, $1x10^5$ Hela and Huh7were infected with rAAV5 variants at various multiplicity of infection. The experiments were performed in a 24-well plate with approximately 80% confluency at 1e5 cells/well. In both experiments wild type adenovirus was used at a multiplicity of infection of 30. This addition of wild type adenovirus is only applied in *in vitro* potency tests, in order to accelerate the process of second strand synthesis to within about 24 hours, thereby allowing the assay to be performed in a relatively shorter period of time and avoiding the need of cell passages. 48 hours after the start of the infection Seap expression was measured in the supernatant using the Seap reporter assay kit (Roche). Luminescence was measured on a Spectramax L luminometer (Molecular devices) at 470 nm with an integration time of 1 second.

2.5. In vivo potency

To investigate *in vivo* potency of the different serotype 5 capsid variants, two different experiments were performed. Briefly, the potency of rAAV5 vectors constructs 159-164 harbouring Seap reporter gene was investigated in C57BL/6 mice. Different vectors were injected intramuscularly in mice at a dose of $5x10^{12}$ gc/kg. Groups consisted of 5 mice each, 7 groups in total including a PBS group. Mice plasma was obtained 2, 4 and 6 weeks after the injection after which the mice were sacrificed. Seap activity was measured in the plasma using the Seap reporter assay kit from Roche.

Luminescence was measured on a Spectramax L luminometer (Molecular devices) at 470 nm with an integration time of 1 second.

Next, the *in vivo* potency of variant AAV5(765) was compared to that of AAV5(160) and AAV5(92). AAV5(92) was a kind gift received from laboratory of dr. Kotin (Urabe et al, 2006) C57BL/6 mice were injected intravenously at doses of $2x10^{12}$ gc/kg and $2x10^{13}$ gc/kg with 765 or 160 both harbouring FIX as a reporter gene. In total seven groups of five mice each were injected including a PBS group. Plasma was collected 1, 2 and 4 weeks following injection after which the mice were sacrificed. Factor IX protein present in the plasma was measured with a factor IX specific ELISA (VisuLize FIX antigen kit, Kordia). Optical density was measured at 450 nm on a Versamax ELISA plate reader (Molecular devices).

3. Results

3.1. Generation of rAAV5 in BEVS

AAV is a mammalian virus that uses its host's machinery to express its genes, among which a *cap* gene. The mechanism by which a correct stoichiometry of VP1:VP2:VP3 is achieved in a mammalian host are not present or are not optimal in insect cells. Therefore, Urabe *et al.*, developed a strategy of genetic adjustments to organization of *cap* polycistronic mRNA which resulted in production of three VP's of AAV2 in insect cells at the correct stoichiometry (Urabe et al. (2002) *supra*). The attempts to establish similar methods to produce rAAV5 in BEVS proved to be unsuccessful to achieve sufficient infectious particles. Without wishing to be bound by any theory, this seems to be caused by a low incorporation of VP1 into the capsids (Urabe et al. (2006)_*supra*). Thereby, Urabe *et al.*, building on the previous success with the type 2 serotype, replaced the N-terminal portion of the type 5 VP1 with that of the type 2, to produce infectious AAV5 particles (Urabe et al. (2006) *supra*). Although successful, the chimeric AAV2/5 chimeric capsid does not comprise *bona fide* type 5 particles and as such may have altered properties as compared to AAV5, which could represent the combination of the two capsids rather than those from the type 5.

In order to allow for AAV5 virion production in insect cells with an improved infectivity and potency, in the present invention a series of genetic alteration to *cap5* expression cassette of AAV5 were made (Table 2). As previously noted (Urabe et al. (2006) *supra*) the wild type *cap5* gene (here clone number 763) did not support

generation of rAAV. Lack of recognition of native AAV splicing signals in insect cells most likely resulted in low expression of separate VP's and lack of vector production. Due to the fact that eukaryotic ribosomes read mRNA unidirectional from 5' to 3', the first translation initiation start (here VP1) of polycistronic *cap5* mRNA is detrimental for expression of all three proteins. The wild type initiation start is composed of ATG, a so-called strong translation initiation codon, that does not allow for ribosomal read through and thereby blocks the expression of other two VPs, which leads to lack of rAAV production. Due to the fact that wild type AAV uses ribosomal read through to express VP2 (non-canonical translation initiation start, ACG) and VP3 (ATG), lead us to investigate the translational start of VP1 and its immediate surroundings to alter the expression and/or assembly of three VP's.

It has been reported before that the nucleotide context of the translational start have an influence on the strength of the translational initiation (Kozak (1987) Nucleic Acid Research 15(20):8125 – 8148; WO2007/046703). The preferred nucleotides seem to be A at the position (-3) and G at the position (+4) with AUG counting +1, +2 and +3 respectively (Kozak *supra*; WO2007/046703). Table 2 details the specific changes that were introduced to the translational initiation start, its upstream and downstream context to tune the expression of three VPs. We have investigated the upstream initiation context that originally surrounds VP2 translational start; various non-canonical start codons (ACG, CTG, TTG, GTG), various mutagenic changes to the +2 wild type triplet and insertion between the +1 initiation triplet and the +2 wild type triplet. The expression cassettes encompassing combination of these features were used for generation of rAAV.

Table 2. Description of AAV5 capsid variants. A number of different mutations surrounding the translational start of VP1 were generatec improve the stoichiometry of three VPs expressed in insect cells. Nucleotides and amino residues changed as compared to the wild type serotyp capsid sequence are indicated in bold.

Bac.VD No.	VP2 initiator context - upstream	Start codon	Amino acid addition(s)	5' part of capsid sequence	SEQ ID NO:
AAV5 wild type	-	ATG	-	TCT TTT GTT GAT CAC CCT CCA GAT TGG T..... S F V D H P P D W	39
Changes surrounding the VP1 translation initiation start					
159	CCTGTTAAG	ACG	-	TCT TTT GTT GAT CAC CC**A** CC**C** GAT TGG T..... S F V D H P P D W	41
160	CCTGTTAAG	ACG	**GCT A**	TCT TTT GTT GAT CAC CC**A** CC**C** GAT TGG T..... S F V D H P P D W	42
161	CCTGTTAAG	ACG	-	**G**CT TTT GTT GAT CAC CC**A** CC**C** GAT TGG T..... **A** F V D H P P D W	43
162	CCTGTTAAG	CTG	-	**A**CT TTT GTT GAT CAC CC**A** CC**C** GAT TGG T..... **T** F V D H P P D W	44
163	CCTGTTAAG	CTG	**ACT T**	**AGC** TTT GTT GAT CAC CC**A** CC**C** GAT TGG T..... S F V D H P P D W	45
164	CCTGTTAAG	CTG	-	**AG**T TTT GTT GAT CAC CC**A** CC**C** GAT TGG T..... S F V D H P P D W	46
761	CCTGTTAAG	ACG	**GCT A**	TCT TTT GTT GAT CAC CC**A** CC**C** GAT TGG T..... S F V D H P P D W	47
762	-	ACG	**GCT A**	TCT TTT GTT GAT CAC CC**A** CC**C** GAT TGG T..... S F V D H P P D W	48
763 (wild type AAV5)	-	ATG	-	TCT TTT GTT GAT CAC CCT CCA GAT TGG T..... S F V D H P P D W	49
764	-	TTG	**GCT A**	TCT TTT GTT GAT CAC CC**A** CC**C** GAT TGG T..... S F V D H P P D W	50

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765	-	CTG	**GCT A**	TCT TTT GTT GAT CAC CC**A** CC**C** GAT TGG T..... S F V D H P P D W	51
766	-	GTG	**GCT A**	TCT TTT GTT GAT CAC CC**A** CC**C** GAT TGG T..... S F V D H P P D W	52
43	CCTGTTAAG	CTG	**GCT A**	TCT TTT GTT GAT CAC CC**A** CC**C** GAT TGG T..... S F V D H P P D W	69

Bac.VD No's 159 – 164 and 43 are operably linked to a polH promoter (SEQ ID NO: 53)

Bac.VD No's 761 – 766 are operably linked to a short polH promoter (SEQ ID NO: 54)

3.2. Small nucleotide changes surrounding the translation initiation start of VP1 have profound effects on the potency of the vector

Baculovirus constructs harbouring all variants of *cap5* expression cassettes listed in table 2 were successfully generated. Subsequently, these baculovirus constructs in combination with baculoviruses harbouring Rep(s) and transgene (reporter gene *e.g.* SEAP or FIX) were used for generation of rAAV. Some of the tested constructs irrespectively of multiple attempts did not support generation of rAAV production. This included wild type AAV5 (construct 763) and some of the constructs harbouring non-canonical starts, TTG (construct 764), GTG (construct 766). All the other constructs listed in table 2 resulted in successful generation of rAAV.

The three viral proteins (VPs) of successfully produced rAAV type 5 variants were isolated. The stoichiometry of the three VPs was investigated by electrophoretic separation (SDS-PAGE) of purified vectors (Figures 1 and 6). It appears that the small modifications introduced to the expression cassette of *cap5* gene have a profound influence on the expression and/or assembly of the three VP proteins which is reflected in the composition of the capsids. We have noted that the adaptation of serotype 5 capsid to the insect cells by introducing non-canonical start codon (ACG) and the nine nucleotide upstream context CCTGTTAAG, which was reported by Urabe *et al.*, as a modification allowing for insect cell production of serotype 2, resulted in low incorporation of VP1 (low VP1/VP2 ratio) and incorporation of excessive levels of VP3 into the capsid (high VP3/VP1 ratio) resulting in aberrant stoichiometry of the three VPs (Figure 1, construct 159). Similarly, modification of nucleotide +4 to constitute G and to resemble closer canonical Kozak sequence, which resulted in exchange of serine at position +2 for alanine (construct 161), resulted in low incorporation of VP1 and high incorporation of VP3 (low VP1/VP2 high VP3/VP1). Use of different non-canonical codon CTG in combination with upstream CCTGTTAAG and downstream modification, *i.e.* change +4 nucleotide to A (construct 162), or +4-5 to AG (construct 164) or insertion of ACT as a second triplet with the modification of original +2 triplet to AGC (construct 163) did not improve VP1 incorporation to the capsid resulting in low VP1/VP2 high VP3/VP1. One of the constructs that showed a VP1/VP2 ratio close to 1 was construct 160 which encompasses direct upstream insertion of CCTGTTAAG, non-canonical ACG and insertion of an additional alanine in position +2 encoded by GCT as compared to the

wild-type sequence, although the incorporation of VP3 was still in excess (equal VP1/VP2 high VP2/VP1). Subsequently, the promoter sequence in the construct 160 was mutated such that it resembles more precisely the wild type polyhedrin promoter. This generated the mutant 761. The VP2 initiation context was removed creating mutant 762. In both cases (761 and 762) there was a slight negative influence on the stoichiometry of the virus (lower VP1 incorporation) as compared to construct 160 (Figure 1). Next, translation initiation start site of VP1 in construct 160 (to preserve the beneficial GCT directly downstream from the translation start codon) was altered to wild type ATG (mutant 763), TTG (mutant 764), CTG (mutant 765), GTG (mutant 766). All but the 765 mutant resulted in lack of detectable production of rAAV. Interestingly, combination of CTG as a non-canonical VP1 initiation start and addition of GCT triplet (encoding extra alanine) immediately following the translational start (765) resulted in higher incorporation of VP1 than VP2 and strong attenuation of VP3 ultimately resulting in balanced wild type AAV like VP stoichiometry (high VP1/VP2 moderate VP3/VP1). Finally, construct 43, which is like construct 160 with CTG as VP1 initiation codon instead of ACG, resulted in VP1 production with an almost native VP ratio (Figure 6).

3.3. Superfluous expression of VP3 is responsible for a low potency of true type 5 AAV mutants in BEVS.

In order to study the potency of the library of serotype 5 capsids, i.e., the ability of the vector to drive the expression of its genetic material, that have different VP stoichiometry *in vitro* and *in vivo* study where performed. Two different continuous cell lines were used *i.e.* Hela (Figure 2 and Figure 7A) and Huh7 (Figure 3 and Figure 7B). In both cases the set of mutants which showed incorporation of VP1 below that of VP2 and excessive incorporation of VP3 (constructs 159, 161-164) showed very reduced potency (Figure 2 - 3). The potency of the vector was much improved by balancing VP1 and VP2 incorporation (construct 160). Shortening of the promoter (construct 761) and removal of the initiator constructs (construct 762) had a negative effect on the vector potency. The most potent vector, construct 765 (Figure 2 - 3) showed VP1 to VP2 ratio in favour of the former and significantly decreased VP3 incorporation. Finally, the polH promoter (not shortened) in combination with the initiator construct, the CTG initiation codon and additional GCT triplet (encoding extra alanine) (construct

43) showed a good potency, albeit somewhat less than the potency of construct 765 (Figure 7A and B).

A subset of mutants (constructs 159-164) was tested *in vivo* (C57BL/6 mice) for potency. The vectors carried a reporter gene SEAP. Mice were injected with capsid variants at a dose 5e12 gc/kg and monitored in time. In line with *in vitro* observation, variant that showed the best potency out of the tested set (160) also had VP1/VP2 in equimolar amounts (Figure 4).

3.4. Insect cell produced genuine AAV5 (765) performs superior to the chimeric type 2/5 mutant in vivo

In order to investigate the potency of the AAV5 (765) *in vivo* three vector batches were prepared. These included the chimeric type 2/5 (92) (Urabe et al. (2006) *supra*), the genuine type AAV5 that contains excessive amounts of VP3 (160) and the best *in vitro* performing genuine type 5 AAV with wild type stoichiometry of VP's (765). All batches were produced under the same conditions using baculovirus constructs harbouring Rep proteins and FIX expression cassette (as described in WO 2006/36502). In order to compare the potency of the three vector preparations black 6 mice were injected with two different doses of the vectors, *i.e.* low dose 2e12 gc/kg and a high dose 2e13 gc/kg. In total seven groups including the vehicle group consisting of 5 animals each, were included in the experiment. Following the start of the experiment, blood was collected at week 1, 2 and 4. The expression of FIX was monitored in the blood by means of specific ELISA. The results corroborated the previous *in vitro* findings were newly generated 765 mutant displayed significant improved potency over 160 construct. Interestingly, 765 construct was also significantly better that the type 2/5 chimera (construct 92) published by Urabe et al. (2006) (*supra*) (Figure 5). Unpaired t test was used to investigate the differences between 765 vs. 160 and 765 vs. 92. In all cases *i.e.* week 1,2 and 4 there was a statistical significant difference with a p value $<0,05$.

4. Discussion

Generation of rAAV in insect cells requires a number of adjustments in the genetic organization of the *cap* gene. In mammalian cells AAV expresses its VP proteins from a single open reading frame by utilizing alternative splicing and the poorly utilized

ACG initiator start for VP2. This results in a VP1:VP2:VP3 stoichiometry of 1:1:10. In insect cell these mechanisms failed to produce AAV vectors with a correct VP stoichiometry (Urabe et al. (2002) *supra*). This is a known problem which has previously been circumvented by Urabe *et al.,* to generate rAAV2 serotype by changing the VP1 initiator triplet to ACG and by mutating the 9 nucleotides upstream from the translation initiation start site. These changes resulted in production of all three rAAV2 VP's in a correct stoichiometry. Similar genetic alteration in rAAV5 expression cassette resulted in low VP1 production and low potency of produced virus. Building upon the success of the genetic adaptation to rAAV2, Urabe *et al.* decided to: make a series of six domain swap mutants where, rAAV5 received various length of N-terminal portion of VP1 from AAV2 (ranging from 7 amino acids up until 136 amino acids). This approach resulted in the production of a chimeric rAAV5 that showed a correct stoichiometry of VP's. Moreover, the domain swap mutants, resulted in a potency that was similar or superior to that of rAAV5 produced in 293T cells (Urabe et al. (2006) *supra*). Although, Urabe *et al.,* demonstrated that chimeric rAAV5 can be generated in insect cells the obtained vector does not comprise *bona fide* AAV5 particles and as such may differ in various aspects such as susceptibility to pre-existing neutralizing antibodies, intracellular trafficking, bio-distribution and/or targeting from the true AAV5 serotype. At the same time the Urabe *et al.,* reported that the attempts to produce infectious genuine rAAV5 failed due to low synthesis of VP1 polypeptide (Urabe et al. (2006) *supra*).

Here we have constructed a library of cap5 mutants objected at understanding the determinants underlying low potency of genuine rAAV5 produced in insect cells. First, we have examined a mutant (159) that incorporated a number of adaptations which were previously used for successful generation of rAAV2 in insect cells (Urabe et al. (2002) *supra*). This mutant contains 9 nucleotide upstream VP2 initiator context placed upstream of VP1 translational start and non-canonical translation initiation start ACG. These 9 nucleotides were previously used by Urabe *et al.,* to express serotype 2 gene in insect cells (Urabe et al. (2002) *supra*). This particular sequence naturally flanks non-canonical start codon (ACG) of VP2. Next, the wild type ATG was change to either ACG or CTG and in order to provide optimal downstream context from the start codon various mutations were introduced. Most of the mutants showed aberrant VPs stoichiometry with low incorporation of VP1 and excessive presence of VP3 (low

VP1/VP2 and high VP3/VP1 ratio). The ratio VP1/VP2 was much improved in the genetic design 160, which still however showed excessive incorporation of VP3 into the vector particles. Finally, one of the genetic designs *i.e.* 765 showed high incorporation of VP1 (high VP1/VP2 ratio) and reduced incorporation of VP3 as compared to other tested variants (balanced VP3/VP2 ratio).

The low ratio of VP1/VP2 proteins has been postulated before to be responsible for the low vector potency (Hermonat et al. (1984) Journal of Virology 51(2):329-339; Tratschin et al. (1984) Journal of Virology 51(3):611-619). Unique VP1 part of AAV is buried inside the capsid and becomes exposed during intracellular trafficking of virus to the nucleus. It first becomes exposed as a response to lowering pH in the lumen of endosome. Free N-terminal part of VP1 contains phospholipase domain which upon exposure to the outside of capsid becomes available to hydrolase specifically the 2-acyl ester (*sn-2*) bond of phospholipid substrates, resulting in release of lysophospholipids and free fatty acid allowing, in turn, endosomal escape of AAV. Unique portion of VP1 contains nucleus localization signals (clusters of basic amino acids) and was implicated in nucleus targeting of AAV. Finally, some authors suggest that unique portion of VP1 may play a role in virus uncoating in the nucleus. Low VP1/VP2 ratio and excessive incorporation of VP3 into viral particles (high VP3/VP1 ratio) may result in either 1) decreased incorporation of VP1 into the assembled particles on average or 2) generation of two particle populations A) correctly assembled particles (having close to wild type stoichiometry 1:1:10 , *i.e.* 5 VP1 molecules per vector particle) B) VP3/VP2 only particles. In both situations (1 and 2) such vector preparation may have altered potency. The excessive amounts of VP3 proteins (as compared to VP1 or VP2) present in the vector preparation likely results in impaired trafficking of the vector to the nucleus due to disturbed endosomal escape. In order to test the hypothesis that the VP stoichiometry is detrimental for vector potency and to generate more potent vector the library of mutants of serotype 5 capsid was tested *in vitro* and *in vivo*.

It appeared that the VP's stoichiometry correlated well with the potency of the vector. As shown before (Hermonat et al. (1984) *supra*; Tratschin et al. (1984) *supra*; WO2007046703A2) low VP1/VP2 ratio has strong influence on the potency of the virus. The mutants 159, 161-164 all have shown low VP1/VP2 ration and drastically reduced potency. Improved ratio between VP1/VP2 had significant impact on the potency of the vector (160). Interestingly further improvement in the VP1/VP2 ratio

and decreasing the incorporation of VP3 to vector particles (decreasing VP3/VP1 ratio) resulted in generation of improved vector 43 and of the most potent vector (construct 765) among the tested set. This data clearly indicate that the molecular make-up of the vector particle is detrimental for its potency. Improving incorporation of VP1 and at the same time decreasing that of VP3 seems to give the best results in terms of the vector potency. The influence of low VP1/VP2 ratio of particles generated in BEVS has been reported previously to have negative impact on vector potency. The ratio of VP2/VP3 was not considered so far, mainly due to the fact that its genetic design for production in BEVS is the same as in the wild type AAV virus. Thereby, it is not expected that it lead to altered VP2/VP3 ratio. However, with all but one mutant presented here, we observed excessive incorporation of VP3 into the vector particles (high VP3/VP1 ratio) indicating that alteration of VP1 translational start surroundings has strong effects on expression of VP2 and VP3. Only the mutant 765 showed balance stoichiometry with high VP1/VP2 ratio and decreased incorporation of VP3, which resulted in increased potency as compared to other tested variants. Furthermore, the potency of 765 variant was compared *in vivo* (mice) to AAV5 like vector produced in BEVS (construct 92). The 92 construct is chimera of AAV serotype 5 with the N-terminal 136 amino acid portion of serotype 2 (Urabe et al. (2006) *supra*). Although construct 92 does not comprise a true AAV5 it is the only alternative currently available for generation of AAV5 like particles in BEVS. The 765 construct showed statistically significant superiority to the 92 construct.

We hypothesize that the strong influence on expression of downstream VP2 and VP3 by mutagenic changes of VP1 translational reason is related to the translational process itself. Translation is unidirectional in eukaryotes and starts with mRNA 5'. Ribosomes, once engaged with mRNA, proceed until they find translational ATG start in appropriate context to initiate protein synthesis. Sometimes a week initiation starts *e.g.* ACG or CTG, if surrounded by appropriate nucleotide context may initiate protein synthesis in a non-canonical manner. This mechanism is called leaky ribosomal scanning. The strength of the leaky ribosomal scanning at VP1 will determine the portion of ribosomes "leakage" to VP2 and VP3 and strength of protein expression from the latter two. In turn the expressions of all three components will determine their presence in the final assembled capsid.

Claims

1. A nucleic acid molecule having a nucleotide sequence comprising an open reading frame, wherein the reading frame in 5' to 3'order comprises: :

(i) a first codon, which is a suboptimal translation initiation codon selected from the group consisting of CTG, ACG, TTG and GTG;

(ii) a second codon encoding an amino acid residue selected from the group consisting of alanine, glycine, valine, aspartic acid and glutamic acid;

(iii) optionally, one or more codons encoding additional amino acid residues following the second codon; and,

(iv) a sequence encoding adeno-associated virus (AAV) capsid proteins, whereby the sequence lacks only the VP1 translation initiation codon.

2. A nucleic acid molecule according to claim 1, wherein the AAV capsid proteins are AAV serotype 5, AAV serotype 8, or AAV serotype 9 capsid proteins, preferably wherein the capsid proteins have an amino acid sequence selected from the group consisting of: SEQ ID NO: 22, 28, 30, 71 and 73.

3. A nucleic acid molecule according to claim 1 or claim 2, wherein the second codon encodes alanine.

4. A nucleic acid molecule according to claim 1 or claim 2, wherein the second codon is selected from the group consisting of GCT, GCC, GCA, GCG and GGU, preferably wherein the codon is GCT.

5. A nucleic acid construct comprising a nucleic acid molecule according to any one of claims 1 – 4, wherein the nucleotide sequence of the reading frame encoding the adeno-associated virus (AAV) capsid proteins is operably linked to expression control sequences for expression in an insect cell.

6. A nucleic acid construct according to claim 5, wherein the nucleotide sequence of the reading frame is operably linked to a promoter selected from the group consisting

of: polyhedron promoter, p10 promoter, 4xHsp27 EcRE+minimal Hsp70 promoter, deltaE1 promoter, E1 promoter.

7. A nucleic acid construct according to claim 5 or claim 6, wherein the construct is an insect-compatible vector, preferably a baculoviral vector.

8 A nucleic acid construct according to any one of claims 5 – 7, wherein the nucleic acid molecule comprises an open reading frame selected from the group consisting of: SEQ ID NO: 51, 69, 42, 47, 48 and 50, preferably SEQ ID NO:51.

9. An insect cell comprising a nucleic acid construct according to any one of claims 5 – 8.

10. An insect cell according to claim 9, wherein the insect cell further comprises:
(a) a second nucleotide sequence comprising at least one AAV inverted terminal repeat (ITR) nucleotide sequence;
(b) a third nucleotide sequence comprising a Rep78 or a Rep68 coding sequence operably linked to expression control sequences for expression in an insect cell;
(c) optionally, a fourth nucleotide sequence comprising a Rep52 or a Rep40 coding sequence operably linked to expression control sequences for expression in an insect cell.

11. An insect cell according to claim 10, wherein the insect cell comprises:
(a) a first nucleic acid construct according to any one of claims 5 – 8, whereby the first nucleic acid construct further comprises the third and fourth nucleotide sequences as defined in (b) and (c) of claim 10; and,
(b) a second nucleic acid construct comprising the second nucleotide sequence as defined in (a) of claim 10, wherein the second nucleic acid construct preferably is an insect cell-compatible vector, more preferably a baculoviral vector.

12. An insect cell according to any one of claims 10 – 11, wherein the second nucleotide sequence further comprises at least one nucleotide sequence encoding a gene product of interest (for expression in a mammalian cell) and whereby the at least one

nucleotide sequence encoding a gene product of interest becomes incorporated into the genome of an AAV serotype 5 produced in the insect cell, preferably wherein the second nucleotide sequence comprises two AAV ITR nucleotide sequences and wherein the at least one nucleotide sequence encoding a gene product of interest is located between the two AAV ITR nucleotide sequences.

13. The insect cell according to claim 10, wherein the first nucleotide sequence, second nucleotide sequence, third nucleotide sequence and optionally fourth nucleotide sequence are stably integrated in the genome of the insect cell.

14. An AAV virion, comprising in its genome at least one nucleotide sequence encoding a gene product of interest, whereby the at least one nucleotide sequence is not a native AAV nucleotide sequence, and wherein the AAV VP1 capsid protein comprises, from N terminus to C terminus

(i) a first amino acid residue, which is encoded by a translation initiation codon, preferably by a suboptimal translation initiation codon selected from the group consisting of CTG, ACG, TTG and GTG;

(ii) a second amino acid residue selected from the group consisting of alanine, glycine, valine, aspartic acid and glutamic acid;

(iii) optionally, one or more additional amino acid residues following the second amino acid residue; and,

(iv) an amino acid sequence of the AAV VP1 capsid protein, whereby the sequence lacks only the amino acid residue that is encoded by the VP1 translation initiation codon.

15. A method for producing an AAV in an insect cell, comprising the steps of: (a) culturing an insect cell as defined in any one of claims 9 - 13 under conditions such that AAV is produced; and optionally (b) recovery of the AAV.

16. An AAV virion according to claim 14, wherein the gene product of interest encodes a Factor IX or a Factor VIII protein.

Fig. 1

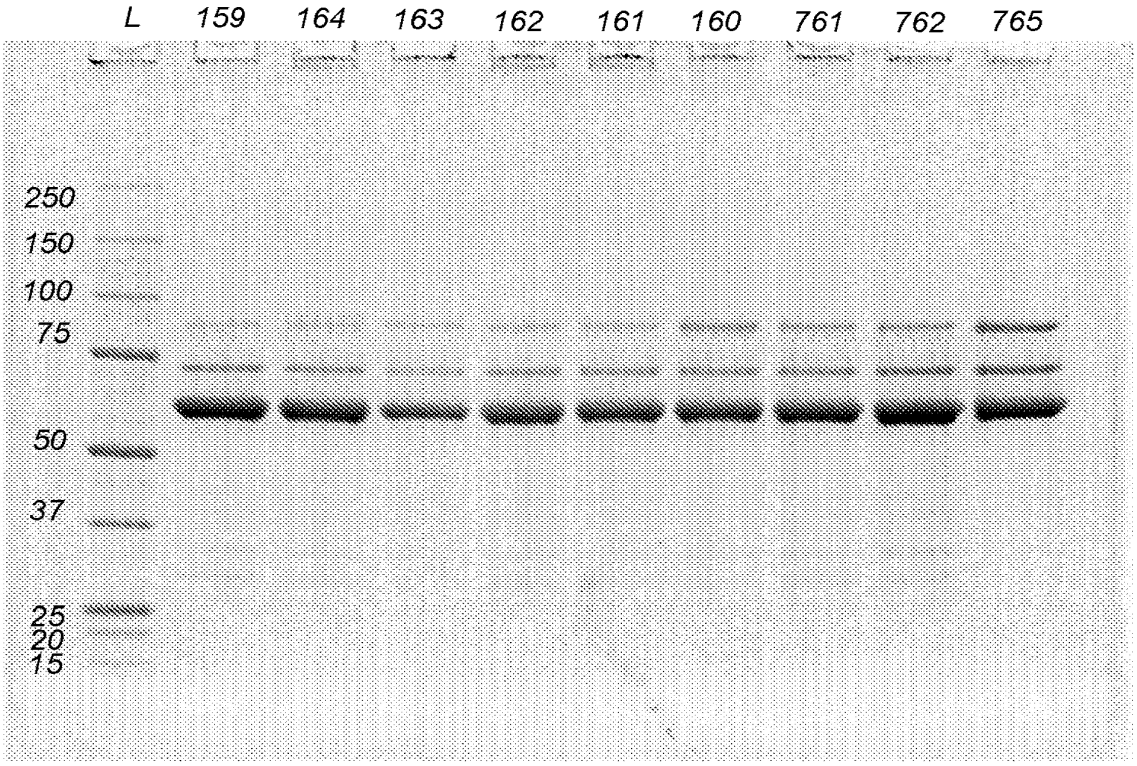


Fig. 2

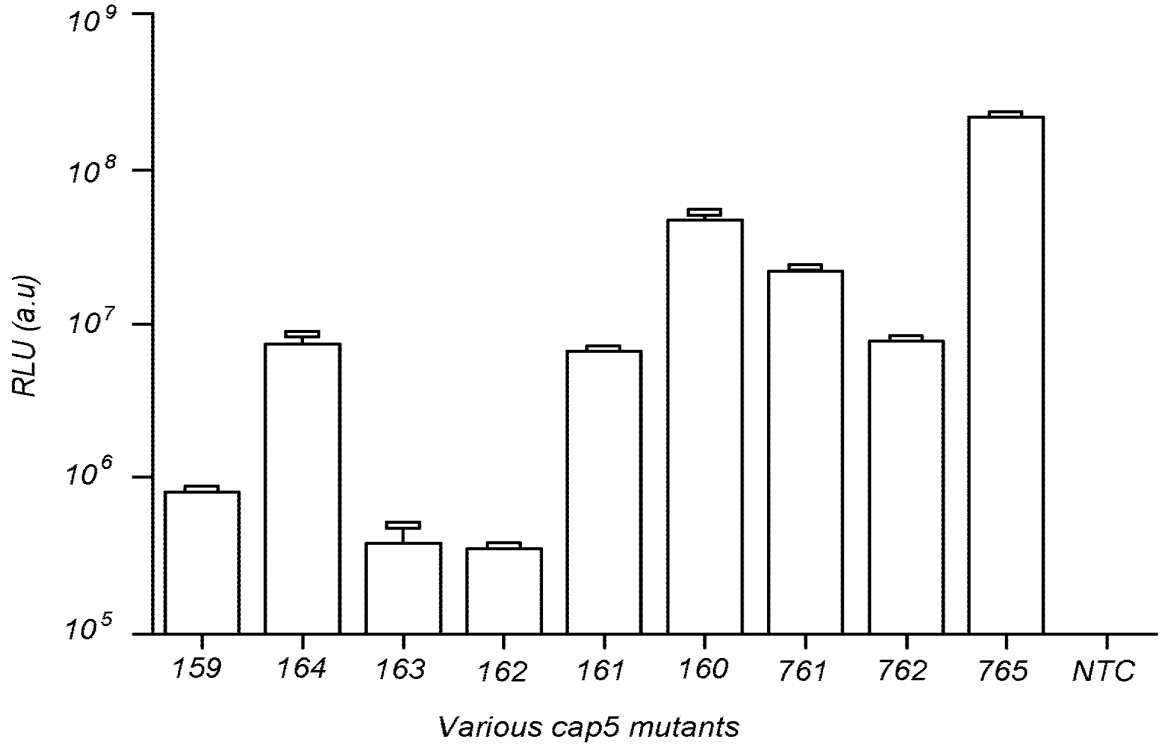

10^9
10^8
10^7
10^6
10^5
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159
164
163
162
161
160
761
762
765
NTC
Various cap5 mutants

Fig. 3

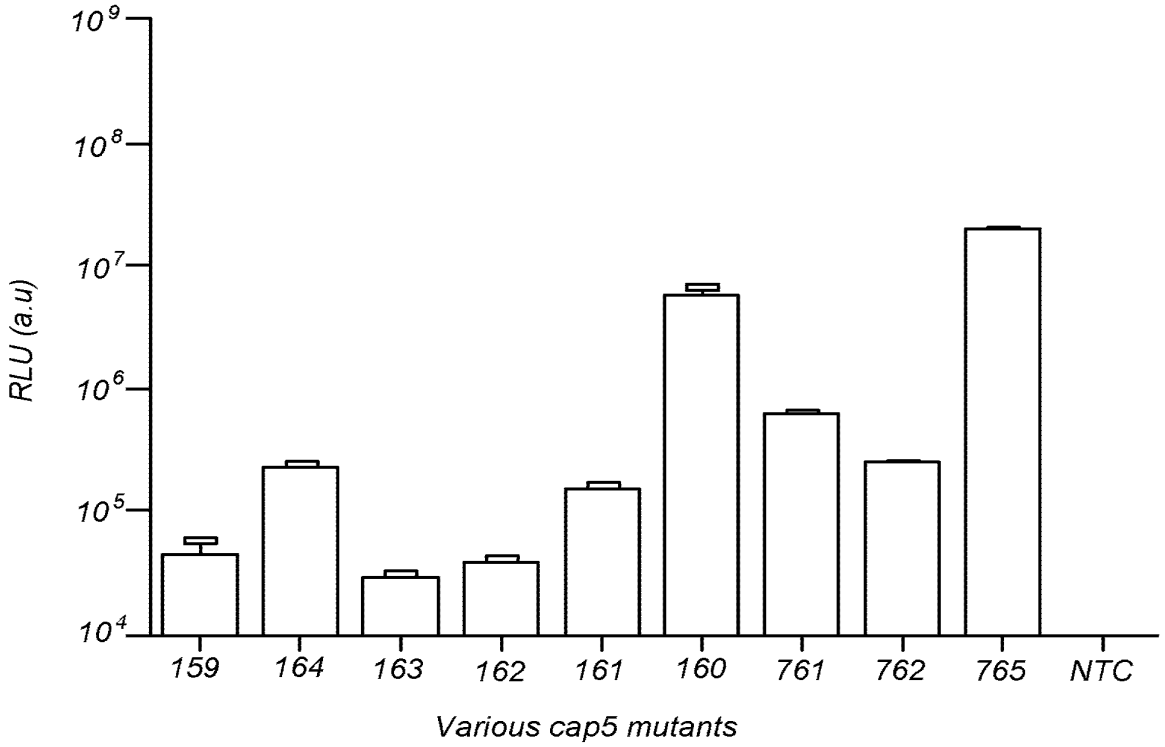

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10^6
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10^4
RLU (a.u)
159
164
163
162
161
160
761
762
765
NTC
Various cap5 mutants

Fig. 4

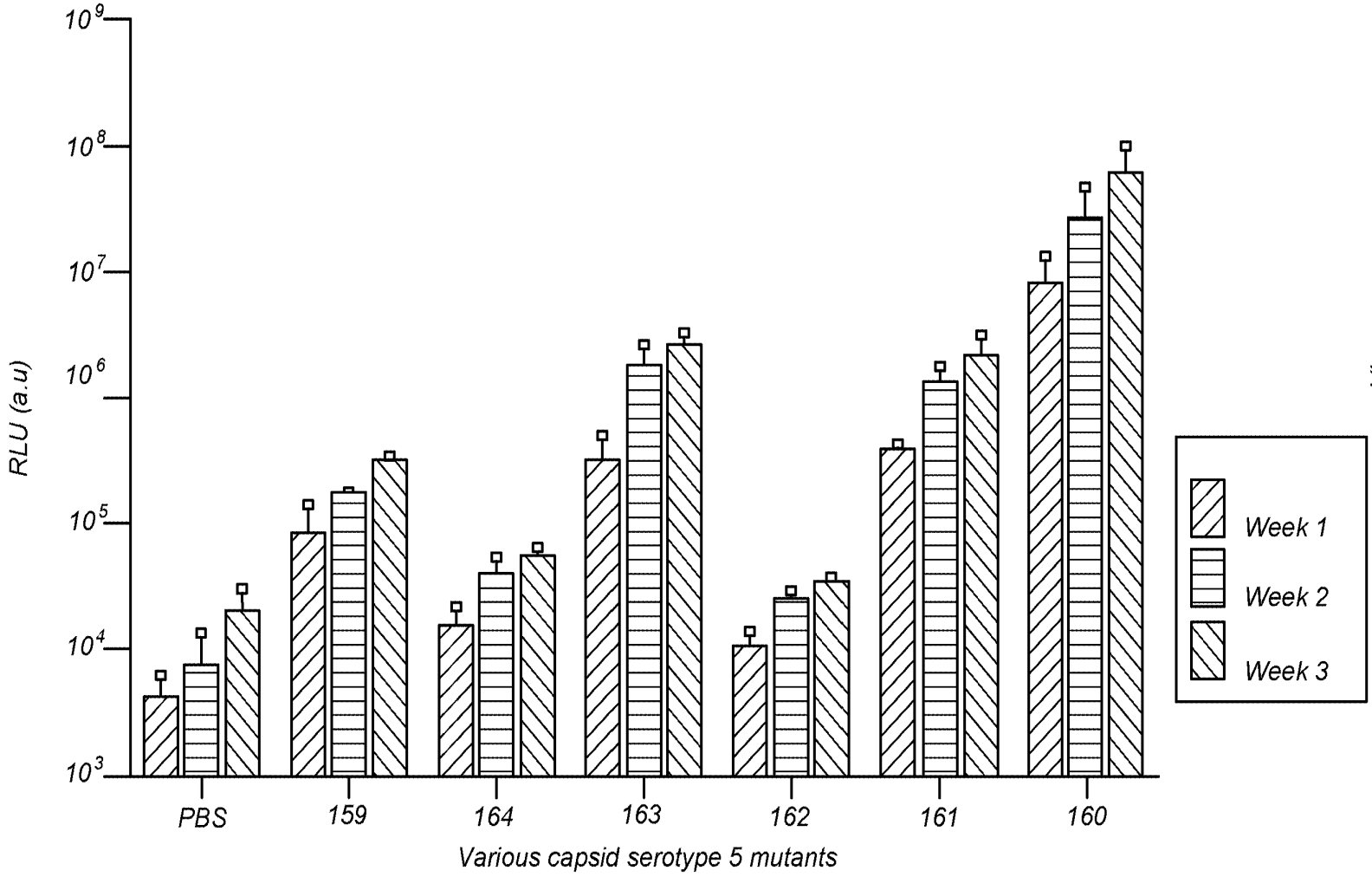

10^9
10^8
10^7
10^6
10^5
10^4
10^3
RLU (a.u)
PBS
159
164
163
162
161
160
Various capsid serotype 5 mutants
Week 1
Week 2
Week 3

Fig. 5

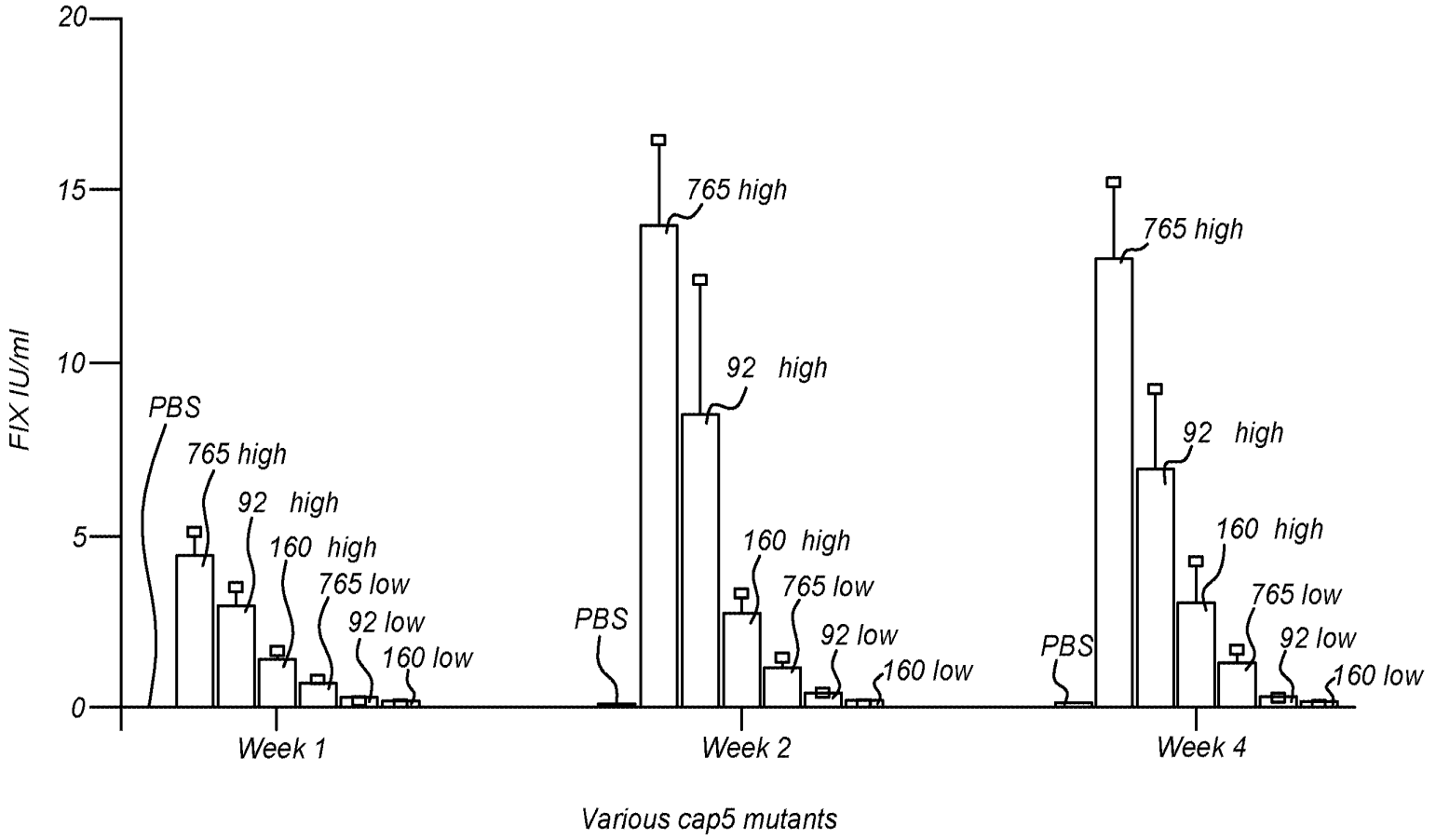

20
15
10
5
0
FIX IU/ml
PBS
765 high
92 high
160 high
765 low
92 low
160 low
Week 1
PBS
765 high
92 high
160 high
765 low
92 low
160 low
Week 2
PBS
765 high
92 high
160 high
765 low
92 low
160 low
Week 4
Various cap5 mutants

Fig. 6

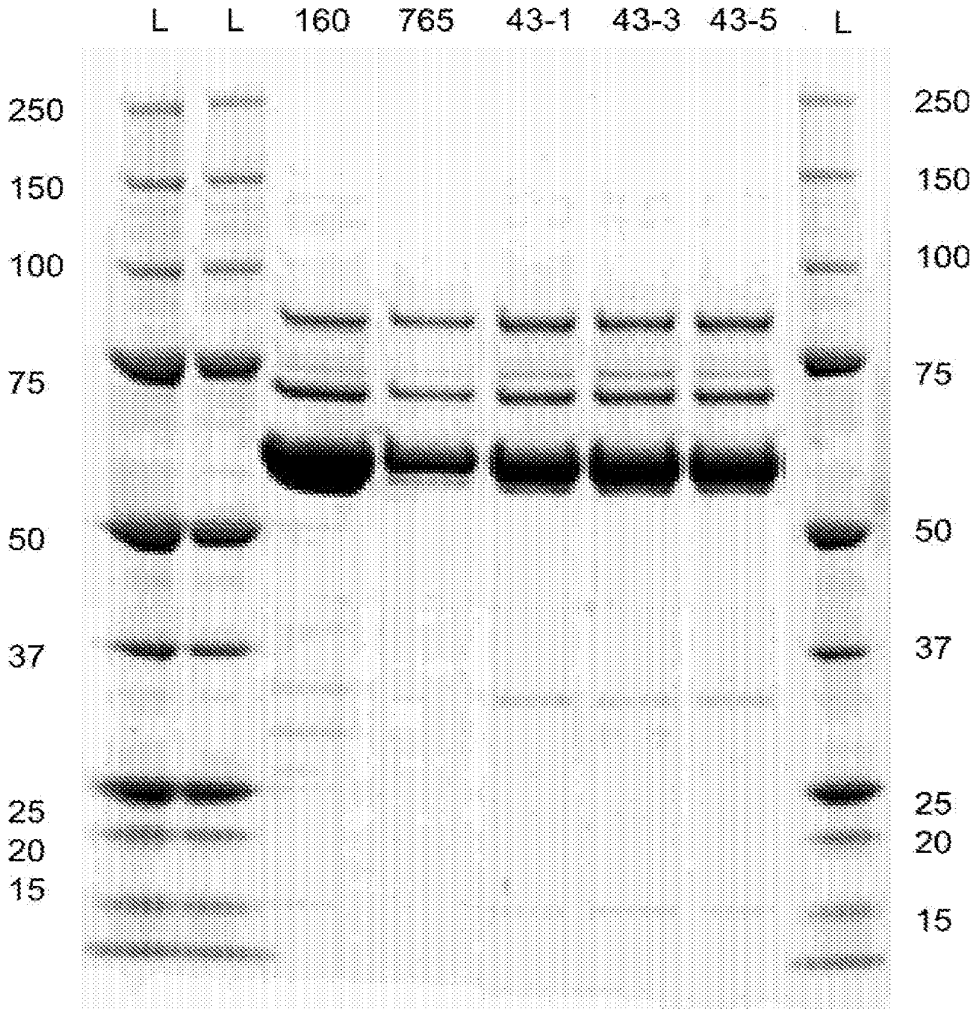

L
L
160
765
43-1
43-3
43-5
L
250
150
100
75
50
37
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20
15
250
150
100
75
50
37
25
20
15

Fig. 7a

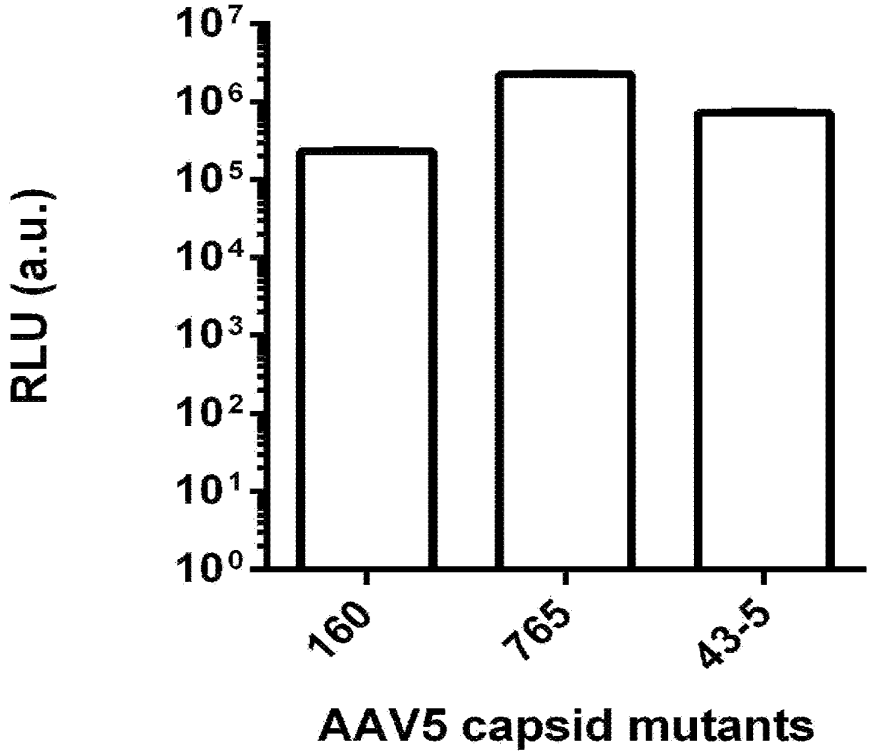
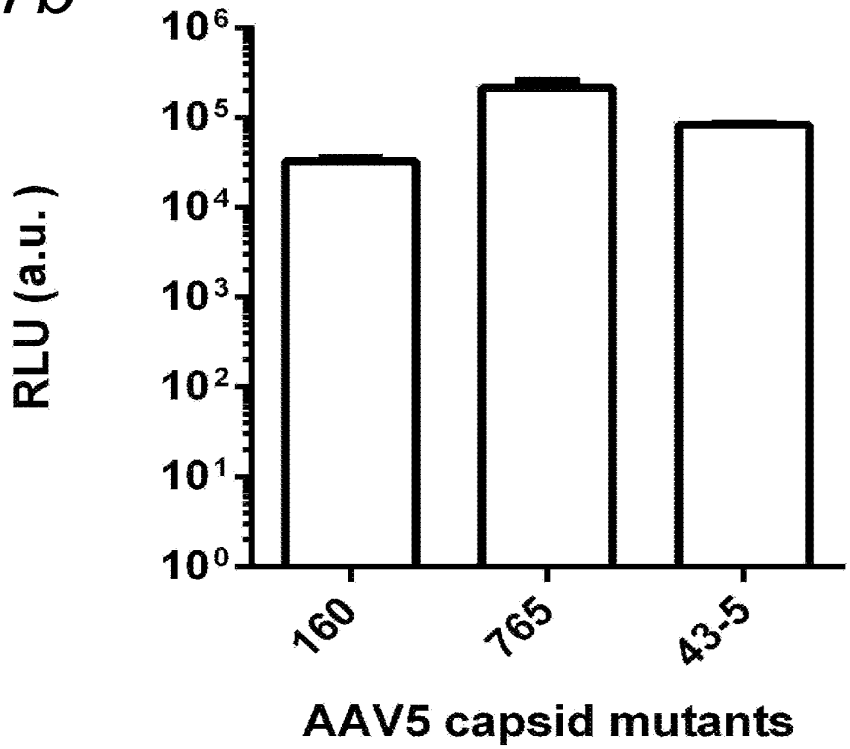

RLU (a.u.)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
160
765
43-5
AAV5 capsid mutants

Fig. 7b


RLU (a.u.)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
160
765
43-5
AAV5 capsid mutants

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2015/050149

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/86
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/046703 A2 (AMSTERDAM MOLECULAR THERAPEUTI [NL]; URABE MASASHI [JP]; OZAWA KEIYA []) 26 April 2007 (2007-04-26) cited in the application	1-7,9-16
Y	page 23 - page 24; claims 3, 4,9, 16,17	8
X	WO 03/042361 A2 (US GOV HEALTH & HUMAN SERV [US]; KOTIN ROBERT M [US]; URABE MASASHI [U) 22 May 2003 (2003-05-22)	1-7,9-16
Y	examples 10,11; tables 4,5	8

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 May 2015	15/06/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Renggli-Zulliger, N

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2015/050149

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	URABE MASASHI ET AL: "Scalable generation of high-titer recombinant adeno-associated virus type 5 in insect cells", JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 80, no. 4, 1 February 2006 (2006-02-01), pages 1874-1885, XP002429140, ISSN: 0022-538X, DOI: 10.1128/JVI.80.4.1874-1885.2006 cited in the application	1-7,9-16
Y	abstract; figure 2	8
A	URABE M ET AL: "Insect cells as a factory to produce adeno-associated virus type 2 vectors", HUMAN GENE THERAPY, MARY ANN LIEBERT, NEW YORK ,NY, US, vol. 13, no. 16, 1 November 2002 (2002-11-01), pages 1935-1943, XP002394454, ISSN: 1043-0342, DOI: 10.1089/10430340260355347 the whole document	1-16

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2015/050149

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007046703 A2	26-04-2007	AU 2006304997 A1	26-04-2007
		CA 2622233 A1	26-04-2007
		CN 101287837 A	15-10-2008
		DK 1945779 T3	03-06-2013
		EP 1945779 A2	23-07-2008
		EP 2311966 A2	20-04-2011
		EP 2311967 A2	20-04-2011
		ES 2410133 T3	01-07-2013
		HK 1115413 A1	01-11-2013
		JP 5305913 B2	02-10-2013
		JP 2009512436 A	26-03-2009
		SI 1945779 T1	31-07-2013
		US 2012028357 A1	02-02-2012
		WO 2007046703 A2	26-04-2007
WO 03042361 A2	22-05-2003	AU 2002360355 B2	07-07-2005
		CA 2467959 A1	22-05-2003
		EP 1572893 A2	14-09-2005
		US 2004197895 A1	07-10-2004
		WO 03042361 A2	22-05-2003

Electronic Patent Application Fee Transmittal

Application Number:	16325653
Filing Date:	14-Feb-2019
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Filer:	Brian Arthur Donahue/Aurelia Caparas
Attorney Docket Number:	15979-20141.00

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 1 month with $0 paid	1251	1	220	220
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	1806	1	260	260
		Total in USD ($)		**480**

Electronic Acknowledgement Receipt

EFS ID:	44242772
Application Number:	16325653
International Application Number:	
Confirmation Number:	1002
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Customer Number:	89300
Filer:	Brian Arthur Donahue/Aurelia Caparas
Filer Authorized By:	Brian Arthur Donahue
Attorney Docket Number:	15979-20141.00
Receipt Date:	09-NOV-2021
Filing Date:	14-FEB-2019
Time Stamp:	20:42:17
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$480
RAM confirmation Number	E2021A9K42410599
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		ROA.pdf	166405 e52e131a8ebbb41602394e655f8241a2c594163e	yes	16
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Amendment/Req. Reconsideration-After Non-Final Reject		1	1	
	Claims		2	9	
	Applicant Arguments/Remarks Made in an Amendment		10	16	
Warnings:					
Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	IDS.pdf	78353 4c10f025f2f2df39724d43b529f6f880908bd2e5	no	5
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Foreign Reference	WO2007046703A2.pdf	2545014 7699cf570d165579870d48bf246462c5eb0b5087	no	33
Warnings:					
Information:					
4	Foreign Reference	WO2010093784A2.pdf	10404839 f8c024293a76515445eee265d42759a994afcc3a	no	87
Warnings:					
Information:					

5	Foreign Reference	WO2015114365A1.pdf	8400800 c97ebd21ad3f61415a5a2c8b1cbfb3aa1e2a9cb0	no	82
Warnings:					
Information:					
6	Foreign Reference	WO2015137802A1.pdf	4263789 56c5a881fc03dd534c0cb7bdb56110147bbca863	no	60
Warnings:					
Information:					
7	Non Patent Literature	RIPA-BUFFER_brochure_9pgs.pdf	1289400 d76d543ed77aa87413526c0b586cc06642660fc1	no	9
Warnings:					
Information:					
8	Non Patent Literature	BENEVENTO_2014_11421-11430.pdf	2152452 da84dcb58f99a15a63646b6b77693c4555189f2a	no	10
Warnings:					
Information:					
9	Non Patent Literature	CALDERARO_2014_1-10.pdf	2765785 8dd0f912225eabff4cec5fc4de33c8f680c067d0	no	10
Warnings:					
Information:					
10	Non Patent Literature	CHENG_2013_e57339_12pgs.pdf	4871090 d891aabd2631ff76904a385beb85885b29e95489	no	12
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Information:					
11	Non Patent Literature	DONG_2014_e86453_7pgs.pdf	1723922 1c5415cdca35f0934d683217c553f410677206e9	no	7
Warnings:					
Information:					

12	Non Patent Literature	KONIETZNY_2012_1-9_10pgs.pdf	1239305 d7d9e373bfb85613efe9b73f1d970185c8a2a318	no	10
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Information:					
13	Non Patent Literature	MICROMASS_PDF-Quattro-Users-Guide_164pgs.pdf	25273339 20e0cc7a05df729410241b711249298cde9e4891	no	164
Warnings:					
Information:					
14	Non Patent Literature	Notice_Definition-IP-Limited_opponent_OCT062021_39pgs.pdf	261445 71c49c81aa6050a17c3eeacad7d0e9892e351866	no	39
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Information:					
15	Non Patent Literature	Notice_JA-Kemp_LLP_OCT062021_13pgs.pdf	79002 af26eb8011d0e406089cb8a94a4e0ce1310c4a31	no	13
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Information:					
16	Non Patent Literature	PIERSON_2016_6718-6725.pdf	944271 30127c04d394d022daa507e9208a92af3e4fd86d	no	21
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Information:					
17	Non Patent Literature	SHE_2001_20039-20047.pdf	1503149 9afe1c8b041d6bec82f35034bf5ea602fd8bf7d8	no	9
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Information:					
18	Non Patent Literature	STROBEL_2015_147-157.pdf	2914074 8762886c78803a420891c8d0e858ec60ec7d709f	no	11
Warnings:					
Information:					

19	Non Patent Literature	TAN_2000_93-99.pdf	571623 e6187eee7a2a9706fde936a08b169340dd2b5b38	no	7
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20	Non Patent Literature	Team-TFS_2016_WEB-URL_9pgs.pdf	1710091 2c50870c03a6413888e209a0131f90c1be42c2fa	no	9
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21	Non Patent Literature	WATERS_BeginnersGuide_WEB_2pgs_.pdf	272037 b93901f11d6adee86972f4b4639f4b27b23476ca	no	2
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Information:					
22	Fee Worksheet (SB06)	fee-info.pdf	40918 b2713442 0f8bc6aa706d3696606f0e49a1f3a62e	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	73471103		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

<u>New Applications Under 35 U.S.C. 111</u>
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
<u>National Stage of an International Application under 35 U.S.C. 371</u>
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
<u>New International Application Filed with the USPTO as a Receiving Office</u>
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/325,653	02/14/2019	Xiaoying JIN	15979-20141.00	1002

89300 7590 11/17/2021

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

EXAMINER
XU, XIAOYUN

ART UNIT	PAPER NUMBER
1797	

NOTIFICATION DATE	DELIVERY MODE
11/17/2021	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

EOfficePA@mofo.com
PatentDocket@mofo.com
pair_mofo@firsttofile.com

Office Action Summary	Application No. 16/325,653	Applicant(s) JIN et al.	
	Examiner XIAOYUN R XU, Ph. D.	Art Unit 1797	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 11/09/2021.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☑ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1,3,29,31,61-63,89-92,100,128,130,134,136,142 and 150-154 is/are pending in the application.

5a) Of the above claim(s) 3,31,62-63,89-92,100,130,136,142 and 150-154 is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1,29,61,128,134 and 181-218 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date 11/09/2021.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

1. The amendment filed on 11/09/2021 has been entered and fully considered. Claims 1, 3, 29, 31, 61-63, 89-92, 100, 128, 130, 134, 136, 142, 150-154 and 181-218 are pending. Claims 3, 31, 62-63, 89-92, 100, 130, 136, 142 and 150-154 have been withdrawn from consideration. Claims 1, 29, 61, 128, 134 and 181-218 are considered on merits, of which claim 1, 29, 61, 128 and 134 are amended, and Claims 181-218 are newly added.

Response to Amendment

2. In response to amendment, the examiner establish rejection under 35 U.S.C 112(b), and modifies rejection over the prior art established in the previous Office action.

Notice of Pre-AIA or AIA Status

3. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Claim Rejections - 35 USC § 112

4. The following is a quotation of 35 U.S.C. 112(b):

> (b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

> The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

5. **Claim 198-199** are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor (or for applications subject to pre-AIA 35 U.S.C. 112, the applicant), regards as the invention.

6. Claim 198 recites the limitation "the digestion" in line 1. There is insufficient antecedent basis for this limitation in the claim.

Claim Rejections - 35 USC § 103

7. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

8. **Claim 1, 29, 61, 128 and 134, 181, 183-186, 189-191, 193-206 and 209-218** is/are rejected under 35 U.S.C. 103 as being unpatentable over Vliet et al. (Journal of Virological Methods, 2009, IDS) (Vliet) in view of Bark et al. (J. Am. Chem. Soc. 2001, IDS) (Bark).

Regarding claim 1, Vliet teaches a method to determine the serotype of an adeno-associated virus (AAV) particle (abstract) comprising

a) denaturing the AAV particle (boiled for 3 min) (page 169, par 4),

b) subjecting the denatured and in-gel digested AAV particle to liquid chromatography/mass spectrometry (LC/MS) (page 169, par 6), and

c) determining the masses of VP1, VP2 and VP3 of the AAV particle (page 171, par 1);

wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype (Table 2, page 171, par 1).

Vliet does not specifically teach wherein the method is performed in the absence of a gel separation step. However, Bark teaches directly subjecting the denatured and digested virus particle (HK97) to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step (Fig. 1, page 1774, par 4). Bark teaches that “The primary techniques for proteomic analysis consist of 2-D gel electrophoresis followed by proteolytic digestion, mass spectrometry, and computer-facilitated data analysis.” (page 1774, par 1); “Two limitations of this method are the time required for adequate proteolysis and the resistance of some proteins to digestion.” (page 1774, par 2). “We have taken advantage of thermal denaturation to generate a facile proteolysis method for identifying proteins.” (page 1774, par 3). At time before the filing, it would have been obvious to one of ordinary skill in the art to directly subjecting the denatured and digested AAV particle to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step, in order to save time that is required for gel separation.

Regarding claim 29, Vliet teaches a method to determine the serotype of an adeno-associated virus (AAV) particle (abstract) comprising

a) denaturing the AAV particle (boiled for 3 min) (page 169, par 4),

b) subjecting the denatured AAV particle to reduction and/or alkylation (page 169, par 5),

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle (page 169, par 5),

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS) (page 170, par 2), and

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle (page 171, par 1);

wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype (Table 2, page 171, par 1).

Again, Vliet does not specifically teach wherein the method is performed in the absence of a gel separation step. However, Bark teaches directly subjecting the denatured and digested virus particle (HK97) to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step (Fig. 1, page 1774, par 4). Bark teaches that “The primary techniques for proteomic analysis consist of 2-D gel electrophoresis followed by proteolytic digestion, mass spectrometry, and computer-facilitated data analysis.” (page 1774, par 1); “Two limitations of this method are the time required for adequate proteolysis and the resistance of some proteins to digestion.” (page 1774, par 2). “We have taken advantage of thermal denaturation to generate a facile proteolysis method for identifying proteins.” (page 1774, par 3). At time before the filing, it would have been obvious to one of ordinary skill in the art to directly subjecting the denatured and digested AAV particle to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step, in order to save time that is required for gel separation.

Regarding claim 61, Vliet teaches a method of determining the serotype of an AAV particle comprising the method of claim 1 combined with a method to determine the serotype of an adeno-associated virus (AAV) particle (abstract) comprising

a) denaturing the AAV particle (boiled for 3 min) (page 169, par 4),

b) subjecting the denatured AAV particle to reduction and/or alkylation (page 169, par 5),

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle (page 169, par 5),

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS) (page 169, par 6, page 170, par 2), and

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle (page 169, par 6);

wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype (Table 2, page 171, par 1).

Again, Vliet does not specifically teach wherein the method is performed in the absence of a gel separation step. However, Bark teaches directly subjecting the denatured and digested virus particle (HK97) to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step (Fig. 1, page 1774, par 4). Bark teaches that "The primary techniques for proteomic analysis consist of 2-D gel electrophoresis followed by proteolytic digestion, mass spectrometry, and computer-facilitated data analysis." (page 1774, par 1); "Two limitations of this method are the time required for adequate proteolysis and the resistance of some proteins to digestion." (page 1774, par 2). "We have taken advantage of thermal denaturation to generate a facile proteolysis method for identifying proteins." (page 1774, par 3). At time before the filing, it would have been obvious to one of ordinary skill in the art to directly subjecting the denatured and digested AAV particle to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step, in order to save time that is required for gel separation.

Regarding claim 128, Vliet teaches a method to determine the serotype of a viral particle (abstract) comprising

a) denaturing the viral particle (boiled for 3 min) (page 169, par 4),

b) subjecting the denatured and in-gel digested viral particle to liquid chromatography/mass spectrometry (LC/MS) (page 169, par 6), and

c) determining the masses of one or more capsid proteins of the viral particle (page 171, par 1);

wherein the specific combination of masses of the one or more capsid proteins are indicative of the virus serotype (Table 2, page 171, par 1).

Again, Vliet does not specifically teach wherein the method is performed in the absence of a gel separation step. However, Bark teaches directly subjecting the denatured and digested virus particle (HK97) to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step (Fig. 1, page 1774, par 4). Bark teaches that "The primary techniques for proteomic analysis consist of 2-D gel electrophoresis followed by proteolytic digestion, mass spectrometry, and computer-facilitated data analysis." (page 1774, par 1); "Two limitations of this method are the time required for adequate proteolysis and the resistance of some proteins to digestion." (page 1774, par 2). "We have taken advantage of thermal denaturation to generate a facile proteolysis method for identifying proteins." (page 1774, par 3). At time before the filing, it would have been obvious to one of ordinary skill in the art to directly subjecting the denatured and digested AAV particle to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step, in order to save time that is required for gel separation.

Regarding claim 134, Vliet teaches a method to determine the serotype of a viral particle (abstract) comprising

a) denaturing the viral particle (boiled for 3 min) (page 169, par 4),

b) subjecting the denatured viral particle to reduction and/or alkylation (page 169, par 5),

c) subjecting the denatured viral particle to digestion to generate fragments of one or more capsid proteins of the viral particle (page 169, par 5),

d) subjecting the fragments of the one or more capsid proteins to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS) (page 170, par 2), and

e) determining the masses of fragments of the one or more capsid proteins of the viral particle (pae 169, par 6);

wherein the specific combination of masses of fragments of the one or more capsid proteins are indicative of the viral serotype (Table 2, page 171, par 1).

Again, Vliet does not specifically teach wherein the method is performed in the absence of a gel separation step. However, Bark teaches directly subjecting the denatured and digested virus particle (HK97) to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step (Fig. 1, page 1774, par 4). Bark teaches that "The primary techniques for proteomic analysis consist of 2-D gel electrophoresis followed by proteolytic digestion, mass spectrometry, and computer-facilitated data analysis." (page 1774, par 1); "Two limitations of this method are the time required for adequate proteolysis and the resistance of some proteins to digestion." (page 1774, par 2). "We have taken advantage of thermal denaturation to generate a facile proteolysis method for identifying proteins." (page 1774, par 3). At time before the filing, it would have been obvious to one of ordinary skill in the art to directly subjecting the denatured and digested AAV particle to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step, in order to save time that is required for gel separation.

Regarding claim 181, 191, 202 and 210, Vliet teaches that wherein the calculated masses of VP1, VP2 and VP3 are compared to the theoretical masses of VP 1, VP2 and VP3 of one or more AAV serotypes (Fig. 2, page 168, par 3).

Regarding claim 183, 193, 203 and 211, Vliet teaches that wherein the liquid chromatography is reverse phase liquid chromatography (C18 column), size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography (page 169, par 6).

Regarding claim 184, 194, 204 and 212, Vliet teaches that wherein the liquid chromatography is reverse phase chromatography (page 169, par 6).

Regarding claim 185 and 205, while Vliet teaches that wherein the reverse phase chromatography is a C18 reverse chromatography (page 169, par 6), Vliet does not teach that wherein the reverse phase chromatography is a C4 or C8 reverse chromatography. However, like C18, C4 and C8 are known reverse phase columns. C18 has 18 carbon atoms while C8 has only 8 carbon atoms. C18 has a longer carbon chain, but C8 has a shorter one. C18 has higher retention while C8 has shorter

retention. C18 has higher hydrophobicity, but C8 has a lower hydrophobicity. At time before the filing it would have been obvious to one of ordinary skill in the art to optimize the hydrophobicity of the reverse phase column, by routine experimentation.

Regarding claim 186 and 206, Vliet teaches that wherein the liquid chromatography is ultra- performance liquid chromatography (UPLC) (page 169, par 6).

Regarding claim 189 and 200, Vliet teaches that wherein the AAV particle is a recombinant AAV (rAAV) particle (abstract).

Regarding claim 190 and 201, Vliet teaches that wherein the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV I1 capsid, an AAV 12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, a mouse AAV capsid rAAV2/HBoVI (chimeric AAV / human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid (Fig. 1, page 167, par 2).

Regarding claim 195 and 213, Vliet teaches that wherein the reverse phase chromatography is a C18 reverse chromatography (page 169, par 6).

Regarding claim 196 and 214, Vliet teaches that wherein the reduction is by subjecting the AAV particle to dithiothreitol, beta-mercaptoethanol, or tris(2-arboxyethyl)phosphine (TCEP) (page 169, par 5).

Regarding claim 197 and 215, Vliet teaches that wherein the alkylation is by subjecting the AAV particle to iodoacetic acid, iodoacetamide, or 4-vinylpyridine (page 169, par 5).

Regarding claim 198 and 216, Vliet teaches that wherein the digestion is an endopeptidase digestion (page 169, par 5).

Regarding claim 199 and 217, Vliet teaches that wherein the endopeptidase digestion is a trypsin digestion (page 169, par 5).

Regarding claim 209 and 218, Vliet teaches that wherein the viral particle comprises a viral vector encoding a heterologous transgene (page 167, par 1).

9. **Claim 182, 192** is/are rejected under 35 U.S.C. 103 as being unpatentable over Vliet in view of Bark as applied to claims 1, 29, 61, 128 and 134, 181, 183-186, 189-191, 193-206 and 209-218 above, and further in view of Anderson (US 2014/0017716).

Regarding claim 182 and 192, Vliet does not teach that wherein the AAV particle is denatured with acetic acid, guanidine hydrochloride, and/or an organic solvent. However, Anderson teaches that protein sample is denatured with guanidine hydrochloride, and/or an organic solvent (par 0022]). At time before the filing, it would have been obvious to one of ordinary skill in the art to select guanidine hydrochloride, and/or an organic solvent for denaturizing the AAV particle, because the selection is based on its suitability for the intended use.

10. **Claim 187-188 and 207-208** is/are rejected under 35 U.S.C. 103 as being unpatentable over Vliet in view of Bark as applied to claims 1, 29, 61, 128 and 134, 181, 183-186, 189-191, 193-206 and 209-218 above, and further in view of Taylor et al. (US 2013/0217789)(Taylor).

Regarding claim 187-188 and 207-208, Vliet does not specifically teach that wherein the mass spectrometry comprises assisted calibration and sodium iodide is used as a calibrant. However, Taylor teaches that the mass spectrometry comprises assisted calibration and sodium iodide is used as a calibrant (par [0125]). At time before the filing it would have been obvious to one of ordinary skill in the art to incorporate assisted calibration in mass spectrometry and use sodium iodide as a calibrant, in order to obtain accurate mass measurement.

Response to Arguments

11. Applicant's arguments filed 11/09/2021 have been fully considered but they are not persuasive.

Applicant argues that "Vliet teaches away from performing the disclosed method in the absence of a gel separation step. Vliet explains that "The SDS-PAGE gel functions as a separation step for the three overlapping AAV capsid proteins that comprise the virion as well as impurities that may be present in the sample; therefore, samples of varying levels of purity can be analyzed." See 3. Results. Therefore, one of ordinary skill in the art reading Vliet would not have a reasonable expectation of

success in performing the disclosed method in the absence of a gel separation step, as required by the claims, since according to Vliet, this would not enable separation of the overlapping AAV capsid proteins and analysis of samples of varying levels of purity.." (remark, page 13, par 2).

This agreement is not persuasive. Vliet teaches the benefit of performing gel separation. However, the benefit of performing gel separation is not teach away for without gel separation, because Vliet teaches that gel separation is a good to have step, instead of a must have step.

Applicant argues that "The method disclosed in Bark is directed to thermolysin-mediated proteolysis of proteins, and does not teach performing the method without a protease. Bark attempts to overcome the challenges associated with proteolysis in proteomic techniques by taking advantage of "thermal denaturation to generate a facile proteolysis method for identifying proteins." Importantly, Bark states that "The key to this method is the use of a thermophilic enzyme, thermolysin, that exhibits optimal activity at elevated temperatures." See page 1774, paragraph 3. Thermolysin is well-known in the art to be a thermostable protease. Nowhere in Bark does it describe directly subjecting denatured viral capsid proteins to LC/MS without first subjecting them to proteolytic digestion with a protease." (remark, page 14,par 1).

This argument is not persuasive. First, performing the method without a protease is not recited in the instant claims. Second, Bark is cited for directly subjecting the denatured and digested virus particle (HK97) to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step, in order to save time that is required for the gel separation (Fig. 1, page 1774, par 4).

Conclusion

12. Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within

TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

13. Any inquiry concerning this communication or earlier communications from the examiner should be directed to XIAOYUN R XU, Ph. D. whose telephone number is (571)270-5560. The examiner can normally be reached on M-F 8am-5pm.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Lyle Alexander can be reached on 571-272-1254. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see https://ppair-my.uspto.gov/pair/PrivatePair. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/XIAOYUN R XU, Ph.D./
Primary Examiner, Art Unit 1797

Notice of References Cited	Application/Control No. 16/325,653	Applicant(s)/Patent Under Reexamination JIN et al.	
	Examiner XIAOYUN R XU, Ph. D.	Art Unit 1797	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-20140017716-A1	01-2014	ANDERSON; N. Leigh	G01N27/62	435/23
*	B	US-20130217789-A1	08-2013	Taylor; Darlene K.	C08L101/005	514/772.1
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
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	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

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	16/325,653	JIN et al.
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✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

CLAIMS

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	06/22/2021	11/12/2021							
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CLAIM		DATE								
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CPC - Searched*		
Symbol	Date	Examiner
C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848 (text limited)	06/22/2021	RX

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner
436	173 (text limited)	06/22/2021	RX

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
EAST (CPC C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848 ; USPC 436/173 with key words and inventor names) and Google	06/22/2021	RX
updated search in EAST and Google	11/12/2021	RX

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/XIAOYUN R XU, Ph.D./ Primary Examiner, Art Unit 1797	

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	4	wo-2007046703-$.DID.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/11/12 10:23
L2	4	wo-2010093784-$.DID.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/11/12 10:24
L3	2	wo-2015114365-$.DID.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/11/12 10:24
L4	2	wo-2015137802-$.DID.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/11/12 10:25
S1	20	(US-6989264-$ | US-7588772-$ | US-7629322-$ | US-8137948-$ | US-8283151-$ | US-9169299-$ | US-20110275529-$ | US-20120066783-$ | US-20120164106-$ | US-20130323226-$).DID.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 12:05
S2	1	(US-6989264-$ | US-7588772-$ | US-7629322-$ | US-8137948-$ | US-8283151-$ | US-9169299-$ | US-20110275529-$ | US-20120066783-$ | US-20120164106-$ | US-20130323226-$).DID. and acetylation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:19
S3	1	(US-20100129405-$).DID. and acetylation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:21

S4	1	(US-6989264-$ | US-7588772-$ | US-7629322-$ | US-8137948-$ | US-8283151-$ | US-9169299-$ | US-20110275529-$ | US-20120066783-$ | US-20120164106-$ | US-20130323226-$).DID. and deamidation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:25
S5	1	16/325653.app.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:25
S6	13,645	adeno-associated virus AND ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:26
S7	5,489	adeno-associated virus AND serotype and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:27
S8	836	adeno-associated virus AND serotype and denature$1 and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:27
S9	125	adeno-associated virus AND serotype and denature$1 and heterogeneity and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:28
S10	91	adeno-associated virus AND serotype and denature$1 and heterogeneity and mass$2 and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:28

S11	25	adeno-associated virus AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:29
S12	9	adeno-associated virus AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016" and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:29
S13	9	(adeno-associated virus or AAV)AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016" and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:30
S15	3	436/173.ccls. and (adeno-associated virus or AAV) AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:31
S16	10	(WO-2003042397-$ | WO-2004027019-$ | WO-2010148143-$ | WO-2013158879-$).DID.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:33
S17	0	(WO-2003042397-$ | WO-2004027019-$ | WO-2010148143-$ | WO-2013158879-$).DID. and deamidation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:34
S18	0	(WO-2003042397-$ | WO-2004027019-$ | WO-2010148143-$ | WO-2013158879-$).DID. and acetylation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO;	ADJ	ON	2021/03/08 13:34

			DERWENT; IBM_TDB			
S19	5	((("JIN") near3 ("Xiaoying"))).INV.	US-PGPUB; USPAT; USOCR	ADJ	ON	2021/03/08 13:35
S20	42	((("O'RIORDAN") near3 ("Catherine"))).INV.	US-PGPUB; USPAT; USOCR	ADJ	ON	2021/03/08 13:35
S21	1,105	((("LIU") near3 ("Lin"))).INV.	US-PGPUB; USPAT; USOCR	ADJ	ON	2021/03/08 13:36
S22	5	((("ZHANG") near3 ("Kate"))).INV.	US-PGPUB; USPAT; USOCR	ADJ	ON	2021/03/08 13:37
S23	9	(adeno-associated virus or AAV) AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016" and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/06/22 12:01

EAST Search History (Interference)

<This search history is empty>

11/12/2021 10:25:59 AM
C:\Users\xxu\Documents\EAST\Workspaces\16325653.wsp

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /R.X./
/Rosa Xu/ 11/09/2021

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (03-15)
Approved for use through 07/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16325653
Filing Date	2017-08-14
First Named Inventor	XIAOYING JIN
Art Unit	1797
Examiner Name	Xu, Xiaolin
Attorney Docket Number	15979-20141.00

U.S.PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

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U.S.PATENT APPLICATION PUBLICATIONS

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

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FOREIGN PATENT DOCUMENTS

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	2007046703	WO	A2	2007-04-26	AMSTERDAM MOLECULAR THERAPEUTICS B.V.		☐
	2	2010093784	WO	A2	2010-08-19	THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL		☐
	3	2015114365	WO	A1	2015-08-06	THE SECRETARY OF STATE FOR HEALTH		☐

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /R.X./
Examiner Signature Date Considered 11/09/2021

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16325653
	Filing Date	2017-08-14
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	Art Unit	1797
	Examiner Name	Xu, Xiaolin
	Attorney Docket Number	15979-20141.00

	4	2015137802	WO	A1	2015-09-17	UNIQURE IP B.V.		☐

If you wish to add additional Foreign Patent Document citation information please click the Add button

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1	"RIPA Buffer" (2021). Brochure for product data, located at https://www.sigmaaldrich.com/GB/en/product/sigma/r0278, last visited on November 9, 2021, 9 pages.	☐
	2	BENEVENTO, M. et al. (April 18, 2014). "Adenovirus Composition, Proteolysis, And Disassembly Studied By In-Depth Qualitative And Quantitative Proteomics," Journal of Biological Chemistry 289(16):11421-11430.	☐
	3	CALDERARO, A. et al (October 30, 2014). "Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) 5 Mass Spectrometry Applied To Virus Identification", Scientific Reports 4(6803):1-10.	☐
	4	CHENG, K. et al. (February 21, 2013). "MS-H: A Novel Proteomic Approach to Isolate and Type the E.coli H Antigen Using Membrane Filtration and Liquid Chromatography-Tandem Mass Spectrometry(LCMS/MS)", PLoS One 8(2): e57339, 12 pages.	☐
	5	DONG, B. et al (February 3, 2014). "Proteomics Analysis of Co-Purifying Cellular Proteins Associated with rAAV Vectors", PLoS One 9(2):e86453, 7 pages.	☐
	6	KONIETZNY, R. et al. (May 2012). "Detection of BK Virus In Urine From Renal Transplant Subjects By Mass Spectrometry", Clinical Proteomics 9(4):1-9, 10 pages.	☐
	7	MICROMASS UK Limited. (not dated). "Quattro LC User's Guide", PDF located at https://www.waters.com/webassets/cms/support/docs/quattro_lc_guide_issue2.pdf, last visited on November 9, 2021, 164 pages.	☐
	8	Notice of Opposition for European Patent No. 3497207, dated January 06, 2021, Proprietor Genzyme Corporation, Opponent Definition IP Limited, 13 pages.	☐

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /R.X./
Typed/Printed name of Applicant or Representative

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16325653
	Filing Date	2017-08-14
	First Named Inventor	XIAOYING JIN
	Art Unit	1797
	Examiner Name	Xu, Xiaolin
	Attorney Docket Number	15979-20141.00

	9	Notice of Opposition for European Patent No. 3497207, dated January 06, 2021, Proprietor Genzyme Corporation, Opponent Definition IP Limited, 39 pages.	☐
	10	PIERSON, E.E. et al. (July 5, 2016). "Resolving Adeno-Associated Viral Particle Diversity With Charge Detection Mass Spectrometry," Analytical Chemistry 88:6718-6725.	☐
	11	SHE, Y-M. et al. (June 8, 2001). "Determination Of The Complete Amino Acid Sequence For The CoatProtein Of Brome Mosaic Virus By Time-Of-Flight Mass Spectrometry", Journal of BiologicalChemistry 276(23):20039-20047.	☐
	12	STROBEL, B. et al. (July 28, 2015). "Comparative Analysis of Cesium Chloride- and Iodixanol-Based Purification of Recombinant Adeno-Associated Viral Vectors for Preclinical Applications", Human Gene Therapy Methods 26 (4):147-157.	☐
	13	TAN, S. et al (2000). "Rapid Simultaneous Detection Of Two Orchid Viruses Using LC- And/Or MALDI-mass Spectrometry", Journal of Virological Methods 85:93-99.	☐
	14	Team TFS (July 22, 2016). "Liquid Chromatography as the Future Replacement for Gel Electrophoresis", a website article available at URL: https://www.analyteguru.com/t5/Blog/Liquid-Chromatography-as-10 the-Future-Replacement-for-Gel/ba-p/3171, last visited on November 9, 2021, 9 pages.	☐
	15	WATERS (The Science of What's Possible) (Copyright Date 2021). "Beginner's Guide to UPLC", Introduction, a website article located at:https://www.waters.com/waters/en US/UPLC---Ultra-Performance-Liguid-Chromatography-Beginner's-Guide/nav.htm cid=134803622&1ocale=en US, last visited on November 9, 2021, 2 pages.	☐

If you wish to add additional non-patent literature document citation information please click the Add button

EXAMINER SIGNATURE

Examiner Signature	/XIAOYUN R XU, Ph.D./	Date Considered	11/12/2021

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /R.X./
/Rachel Bess/ 11/09/2021

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16325653
Filing Date	2017-08-14
First Named Inventor	XIAOYING JIN
Art Unit	1797
Examiner Name	Xu, Xiaolin
Attorney Docket Number	15979-20141.00

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☒ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☐ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Brian A. Donahue/	Date (YYYY-MM-DD)	2021-11-09
Name/Print	Brian Donahue	Registration Number	58,206

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /R.X./
/Rachel Bussard/ 11/09/2021

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The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

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4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.



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Document Code:WFEE

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Refund Accounting Date:02/09/2022

Effective Date	Sale Item Reference Number	Refund Total
07/23/2019	16325653	$5,260.00

Document Number	Fee Code	Fee Code Description	Amount Paid	Payment Method	Account Number
I202229919402881	1615	CLAIMS IN EXCESS OF 20	$200.00	DA	031952
I202229919402881	1614	INDEPENDENT CLAIMS IN EXCESS OF 3	$5,060.00	DA	031952

United States Patent and Trademark Office
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Document Code:WFEE

User :C47803

Sale Adjustment Accounting Date:02/09/2022

Effective Date	Sale Accounting Date	Sale Item Reference Number
07/23/2019	02/09/2022	16325653

Document Number	Fee Code	Fee Code Description	Amount Paid	Payment Method
I202229919402881	1615	CLAIMS IN EXCESS OF 20	$200.00	DA
I202229919402881	1614	INDEPENDENT CLAIMS IN EXCESS OF 3	$5,060.00	DA

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INTERNATIONAL PATENT LEGAL ADM

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto CA CALIFORNIA 94304

In re Application of :
GENZYME CORPORATION :
U.S. Application No.: 16/325,653 :
PCT Application No.: PCT/US2017/046814 : DECISION ON PETITION
Int. Filing Date: 14 August 2017 :
Priority Date: 15 August 2016 :
Attorney Docket No.: 15979-20141.00 :
For: METHODS FOR DETECTING AAV :

This decision is in response to applicant's request for refund filed 11 November 2020 in the United States Patent and Trademark Office (USPTO).

BACKGROUND

On 14 August 2017, applicant filed international application PCT/US2017/046814 which claimed priority to an earlier application filed 15 August 2016. Pursuant to 37 CFR 1.495, the thirty-month period for paying the basic national fee in the United States was set to expire at midnight on 15 February 2019.

On 14 February 2019 applicant electronically filed for entry into the national stage in the United States, among other items, $1980 in fees including payment of the full U.S. basic national fee; an Application Data Sheet (ADS); a PTO-1390 transmittal letter and a preliminary amendment.

On 23 April 2019, applicant was mailed a notification to filed corrected papers indicating that a substitute specification was required as pages 81 and 82 of the specification contained shading. Applicant was also advised that payment of additional claims fees in the amount of $26,120 was due. Applicant was afforded two months to file a response and advised that this period could be extended pursuant to 37 CFR 1.136(a).

On 23 July 2019, applicant filed a response including a substitute specification; a preliminary amendment and a fee payment of $5460 which included a one month extension of time. Applicant's response was timely filed.

On 19 February 2020, applicant was mailed a "Notification of Abandonment" (Form PCT/DO/EO/909) indicating that the application was abandoned noting that applicant had failed to respond to provide a proper reply to the notification mailed 23 April 2019 within the time period set therein. Specifically, applicant was informed that the status of each claim must be provided after the specific claim numbering by the proper status identifiers. The claim identifier for claims 32-60 was "original."

On 21 February 2020, applicant filed a petition discussed arguing that claims 32-60 "were properly indicated as abandoned in the Remarks section of the reply...and respectfully request that the Notice of Abandonment be withdrawn."

On 13 October 2020, applicant's petition to withdraw the holding of abandonment was granted. It was explained to applicant that while the preliminary amendment reducing the number of claims was non-complaint and could not be entered applicant provided deposit account authorization to charge all necessary fees. As such, the fees should have been taken and the abandonment was premature.

On 11 November 2020, applicant filed the request for refund considered herein.

DISCUSSION

As detailed in the previous decision, applicant contends that the application should not have been held abandoned as the Remarks section of the preliminary amendment indicated that claims 32-60 were cancelled. However, a review of the amended claims filed on 23 July 2019 finds that the amended claim listing does not comply with 37 CFR 1.121. 37 CFR 1.121 (c) states that, "In the claim listing, the status of every claim must be indicated after its claim number by using one of the following identifiers in a parenthetical expression: (Original), (Currently amended), (Canceled), (Withdrawn), (Previously presented), (New), and (Not entered)." (See also the Manual of Patent Examining Procedure (MPEP) section 1893.01(a)(4)). Therefore, the non-compliant amendment could be entered and the claims remain the original claims as filed.

The concluding paragraph of the Remarks stated that, "Applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account no. 03-1952." The only fee not authorized was payment of the issue fee. Applicant therefore timely provided authorization to charge all fees due in connection with the response filed on 23 July 2019 and rather than holding the application abandoned the Office should have charged the required fees as authorized.

Applicant seeks a refund of the amount charged based on the additional preliminary amendment concurrently with the petition. In order to avoid abandonment the fees charged must calculated based on the original set of claims. The amended claims filed 21 February 2020 were filed after the expiration of all extendable time. Further, MPEP section 1893.01 (c) provides

that, "A subsequently filed amendment canceling claims and/or eliminating multiple dependent claims will not entitle applicant to a refund of fees previously paid. See MPEP §§ 607 and 608."

However, applicant is correct that excess claims fees were paid by applicant on 23 July 2019. These fees were not considered by the Office when the excess claim fees were charged on 14 October 2020. Applicant is entitled to a refund of $5060.00 for the fee for each independent claim over three and $200.00 for the fee for each claim over 20 paid on 23 July 2019. Applicant is not entitled to refund of the $200.00 paid for the one-month extension of time as this payment made the filing of 23 July 2019 a timely response to the notice mailed on 23 April 2019.

CONCLUSION

For the reasons detailed above, applicant's request for refund is **GRANTED**.

$5260.00 will be refunded to applicant's deposit account in due course.

This application is being returned to Group Art Unit 1797 for continued examination.

/ Derek A. Putonen /

Derek A. Putonen
Attorney Advisor
International Patent Legal Administration
571-272-3294

Docket No.: 15979-20141.00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Xiaoying JIN et al.

Application No.: 16/325,653

Filed: August 14, 2017 (Int'l)

For: METHODS FOR DETECTING AAV

Confirmation No.: 1002

Art Unit: 1797

Examiner: X. Xu

AMENDMENT AFTER FINAL ACTION UNDER 37 C.F.R. 1.116

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

INTRODUCTORY COMMENTS

This is in response to the final Office Action dated November 17, 2021, for which a response is due on February 17, 2022. Accordingly, this response is timely filed. This response accompanies a Certification and Request for Consideration under the After Final Consideration Pilot Program 2. 0. Reconsideration and allowance of the pending claims, as amended, in light of the remarks presented herein are respectfully requested.

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 10 of this paper.

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INTRODUCTORY COMMENTS

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Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 10 of this paper.

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions, and listings, of claims in the application:

In the claims:

Claim 1 (currently amended): A method to determine the serotype of an adeno-associated virus (AAV) particle comprising

a) denaturing the AAV particle,

b) directly subjecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis, and

c) determining the masses of VP1, VP2 and VP3 of the AAV particle;

wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype,

and wherein the method is performed in the absence of a gel separation step.

Claims 2-28 (canceled)

Claim 29 (previously presented): A method to determine the serotype of an adeno-associated virus (AAV) particle comprising

a) denaturing the AAV particle,

b) subjecting the denatured AAV particle to reduction and/or alkylation,

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle,

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle;

wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype,

and wherein the method is performed in the absence of a gel separation step.

Claims 30-60 (canceled)

Claim 61 (previously presented): A method of determining the serotype of an AAV particle comprising the method of claim 1 combined with a method to determine the serotype of an adeno-associated virus (AAV) particle comprising

a) denaturing the AAV particle,

b) subjecting the denatured AAV particle to reduction and/or alkylation,

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle,

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle;

wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype,

and wherein the method is performed in the absence of a gel separation step.

Claims 62-127 (canceled)

Claim 128 (currently amended): A method to determine the serotype of a viral particle comprising

a) denaturing the viral particle,

b) directly subjecting the denatured viral particle to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis, and

c) determining the masses of one or more capsid proteins of the viral particle;

wherein the specific combination of masses of the one or more capsid proteins are indicative of the virus serotype,

and wherein the method is performed in the absence of a gel separation step.

Claims 129-133 (canceled)

Claim 134 (previously presented): A method to determine the serotype of a viral particle comprising

a) denaturing the viral particle,

b) subjecting the denatured viral particle to reduction and/or alkylation,

c) subjecting the denatured viral particle to digestion to generate fragments of one or more capsid proteins of the viral particle,

d) subjecting the fragments of the one or more capsid proteins to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and

e) determining the masses of fragments of the one or more capsid proteins of the viral particle;

wherein the specific combination of masses of fragments of the one or more capsid proteins are indicative of the viral serotype,

and wherein the method is performed in the absence of a gel separation step.

Claims 135-180 (canceled)

Claim 181 (previously presented): The method of claim 1, wherein the calculated masses of VP1, VP2 and VP3 are compared to the theoretical masses of VP1, VP2 and VP3 of one or more AAV serotypes.

Claim 182 (previously presented): The method of claim 1, wherein the AAV particle is denatured with acetic acid, guanidine hydrochloride, and/or an organic solvent.

Claim 183 (previously presented): The method of claim 1, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

Claim 184 (previously presented): The method of claim 1, wherein the liquid chromatography is reverse phase chromatography.

Claim 185 (previously presented): The method of claim 184, wherein the reverse phase chromatography is a C4 or C8 reverse chromatography.

Claim 186 (previously presented): The method of claim 1, wherein the liquid chromatography is ultra-performance liquid chromatography (UPLC).

Claim 187 (previously presented): The method of claim 1, wherein the mass spectrometry comprises assisted calibration.

Claim 188 (previously presented): The method of claim 187, wherein sodium iodide is used as a calibrant.

Claim 189 (previously presented): The method of claim 1, wherein the AAV particle is a recombinant AAV (rAAV) particle.

Claim 190 (previously presented): The method of claim 1, wherein the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid.

Claim 191 (previously presented): The method of claim 29, wherein the calculated masses of the fragments of VP1, VP2 and/or VP3 are compared to the theoretical masses of fragments of VP1, VP2 and/or VP3 of one or more AAV serotypes.

Claim 192 (previously presented): The method of claim 29, wherein the AAV particle is denatured with acetic acid, guanidine hydrochloride and/or an organic solvent.

Claim 193 (previously presented): The method of claim 29, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

Claim 194 (currently amended): The method of claim [[1]]29, wherein the liquid chromatography is reverse phase liquid chromatography.

Claim 195 (previously presented): The method of claim 194, wherein the reverse phase chromatography is a C18 reverse chromatography.

Claim 196 (previously presented): The method of claim 29, wherein the reduction is by subjecting the AAV particle to dithiothreitol, beta-mercaptoethanol, or tris(2-carboxyethyl)phosphine (TCEP).

Claim 197 (previously presented): The method of claim 29, wherein the alkylation is by subjecting the AAV particle to iodoacetic acid, iodoacetamide, or 4-vinylpyridine.

Claim 198 (currently amended): The method of claim [[1]]29, wherein the digestion is an endopeptidase digestion.

Claim 199 (previously presented): The method of claim 198, wherein the endopeptidase digestion is a trypsin digestion, a LysC digestion, an Asp-N digestion or a Glu-C digestion.

Claim 200 (previously presented): The method of claim 29, wherein the AAV particle is a recombinant AAV (rAAV) particle.

Claim 201 (previously presented): The method of claim 29, wherein the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid.

Claim 202 (previously presented): The method of claim 128, wherein the calculated masses of the one or more capsid proteins are compared to the theoretical masses of the one or more capsid proteins of one or more virus serotypes.

Claim 203 (previously presented): The method of claim 128, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

Claim 204 (previously presented): The method of claim 128, wherein the liquid chromatography is reverse phase chromatography.

Claim 205 (previously presented): The method of claim 204, wherein the reverse phase chromatography is a C4 or C8 reverse chromatography.

Claim 206 (previously presented): The method of claim 128, wherein the liquid chromatography is ultra-performance liquid chromatography (UPLC).

Claim 207 (previously presented): The method of claim 128, wherein the mass spectrometry comprises assisted calibration.

Claim 208 (previously presented): The method of claim 207, wherein sodium iodide is used as a calibrant.

Claim 209 (previously presented): The method of claim 128, wherein the viral particle comprises a viral vector encoding a heterologous transgene.

Claim 210 (previously presented): The method of claim 134, wherein the calculated masses of the fragments of the one or more capsid proteins are compared to the theoretical masses of fragments of the one or more capsid proteins of one or more viral serotypes.

Claim 211 (previously presented): The method of claim 134, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

Claim 212 (previously presented): The method of claim 134, wherein the liquid chromatography is reverse phase liquid chromatography.

Claim 213 (previously presented): The method of claim 212, wherein the reverse phase chromatography is a C18 reverse chromatography.

Claim 214 (previously presented): The method of claim 134, wherein the reduction is by subjecting the viral particle to dithiothreitol, beta-mercaptoethanol, or tris(2-carboxyethyl)phosphine (TCEP).

Claim 215 (previously presented): The method of claim 134, wherein the alkylation is by subjecting the viral particle to iodoacetic acid, iodoacetamide, or 4-vinylpyridine.

Claim 216 (previously presented): The method of claim 134, wherein the digestion is an endopeptidase digestion.

Claim 217 (previously presented): The method of claim 216, wherein the endopeptidase digestion is a trypsin digestion, a LysC digestion, an Asp-N digestion or a Glu-C digestion.

Claim 218 (previously presented): The method of claim 134, wherein the viral particle comprises a viral vector encoding a heterologous transgene.

REMARKS

Claims 1, 29, 61, 128, 134, and 181-218 were pending in the present application. By virtue of this response, claims 1, 128, 194, and 198 have been amended. Accordingly, claims 1, 29, 61, 128, 134, and 181-218 are currently under consideration. Support for the amendments to claims 1 and 128 can be found at least in paragraphs [0189], [0192], and [0194] of the specification as filed. Claims 194 and 198 were amended to depend from claim 29. No new matter has been added. Entry of the claim amendments is respectfully requested.

With respect to all claim amendments, Applicant has not dedicated or abandoned any unclaimed subject matter and moreover has not acquiesced to any rejections and/or objections made by the patent office. Applicant reserves the right to pursue prosecution of any presently excluded claim embodiments in a future continuation and/or divisional application.

Rejections under 35 U.S.C. § 112

Claims 198-199 are rejected under 35 U.S.C. § 112(b) or 35 U.S.C. § 112 (pre-AIA), second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor (or for applications subject to pre-AIA 35 U.S.C. 112, the applicant), regards as the invention. Specifically, the Examiner alleges that there is insufficient antecedent basis for the limitation "the digestion" in line 1 of claim 198.

Without acquiescing to the Examiners rejection, and solely for purposes of expediting prosecution, claim 198 has been amended to depend from claim 29. Claim 29 recites "subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle". Therefore, claims 198 and 199 as amended have sufficient antecedent basis for the limitation "the digestion".

In view of the foregoing, Applicant respectfully requests withdrawal of the rejection.

Rejections under 35 U.S.C. § 103

Claims 1, 29, 61,128, 134,181, 183-186, 189-191, 193-206 and 209-218 are rejected under 35 U.S.C. § 103 as allegedly being unpatentable over Vliet *et al.* (*Journal of Virological Methods*, 2009,"Vliet") in view of Bark *et al.* (*J. Am. Chem. Soc.* 2001, Bark).

Applicant respectfully traverses.

Without acquiescing to the Examiner's rejection, and solely for purposes of expediting prosecution, claims 1 and 128 have been amended to recite directly subjecting the denatured AAV or viral particle to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis.

Applicant respectfully submits that it would not have been obvious to a person of ordinary skill in the art to combine the teachings of Vliet and Bark with a reasonable expectation of success to arrive at the presently claimed invention. Further, there is no teaching, suggestion, or motivation in Vliet and Bark that would have led one of ordinary skill to modify or combine the references to arrive at the presently claimed invention.

By virtue of the amendment filed on November 9, 2021, the claims require that the method is carried out in the absence of a gel separation step. The Examiner acknowledges that Vliet does not teach performing the method in the absence of a gel separation, but alleges that Bark teaches performing the method in the absence of a gel separation step in order to save time required for gel separation. *See* the Office Action dated November 17, 2021, hereafter "Office Action", *e.g.*, page 3. Applicant respectfully disagrees.

Applicant respectfully submits that Bark does not cure the defects of Vliet, because it does not teach performing the method in the absence of a gel separation step. Bark is directed to the development of a method for high-temperature proteolytic digestion using a thermotolerant protease (thermolysin) to generate protein fragments for analysis via mass spectrometry. The Examiner highlights specific passages of Bark: "The ***primary techniques for proteomic analysis consist of 2-D gel electrophoresis followed by proteolytic digestion***, mass spectrometry, and computer-facilitated data analysis" (page 1774, par. 1; emphasis added); "Two ***limitations of this***

method are the time required for adequate proteolysis and the resistance of some proteins to digestion" (page 1774, par. 2; *emphasis added*); and "We have taken advantage of thermal denaturation to generate a facile proteolysis method for identifying proteins" (page 1774, par. 2) *See* the Office Action, *e.g.*, page 3. These passages do not teach that the time required for gel separation is a limitation of proteomic methods; rather, these passages indicate that 2-D gel electrophoresis is a primary technique that is carried out prior to proteolytic digestion. Except for the passage above, Bark is completely silent about gel separation. Instead, Bark describes high-temperature proteolysis methods to reduce the time required for ***proteolysis***, which is the limitation identified in the passages cited by the Examiner. In fact, Bark provides no description of how the samples were prepared prior to proteolysis, including whether or not a gel separation step was performed. Since, as Bark points out in paragraph 1 of page 1774, gel separation is carried out prior to proteolysis in proteomics techniques, it is not clear that the various proteins enumerated in paragraph 4 of page 1774 were not separated in a gel separation step prior to digestion. Given that Bark teaches that gel separation is a primary technique carried out prior to proteolytic digestion, one skilled in the art would assume that gel separation was used prior to the analysis. Therefore, Bark neither teaches performing the method in the absence of a gel separation step, nor suggests that proteomics methods could be performed in the absence of a gel separation step in order to save the time required for gel separation.

Further, as discussed in Applicant's remarks filed on November 9, 2021, Vliet teaches away from performing the disclosed method in the absence of a gel separation step. Vliet explains that "The SDS-PAGE gel functions as a separation step for the three overlapping AAV capsid proteins that comprise the virion as well as impurities that may be present in the sample; therefore, samples of varying levels of purity can be analyzed." *See* 3. Results. One of ordinary skill in the art reading Vliet would not have a reasonable expectation of success in performing the disclosed method in the absence of a gel separation step, as required by the claims, since according to Vliet, this would not enable separation of the overlapping AAV capsid proteins and analysis of samples of varying levels of purity.

The Examiner alleges that Vliet does not teach away from performing the method in the absence of a gel separation step, because it allegedly teaches that "gel separation is a good to

have step, instead of a must have step." *See* Office Action, page 10. Applicant respectfully disagrees. Nowhere in Vliet does it teach or suggest that a gel separation step prior to digestion is optional, or that the method would be successful if carried out in the absence of a gel separation step. Rather, the references cited by the Examiner identify gel separation as a standard step in proteomic methods. *See* 3. Results of Vliet and page 1774, paragraph 1 of Bark. The fact that gel separation is a standard step is also emphasized in paragraph [0174] of the specification as filed, which states that "Conventionally, a Gel-LC/MS method (SDS-PAGE, in-gel tryptic digestion and LC/MS/MS) was used in characterization of VPs". Applicant respectfully submits that the Examiner has not identified a cited reference that teaches or suggests that gel separation prior to digestion is optional in existing proteomic methods, or that such methods could be successfully executed without a gel separation step, prior to the priority date of the present application.

For at least the above reasons, claims 1, 29, 128, and 134, and all claims depending therefrom, are not obvious over Vliet and Bark.

With respect to claims 1 and 29, one of skill in the art would not look to the teachings of Bark to overcome the deficiencies of Vliet to arrive at method of determining the serotype of an AAV particle. HK97 is a phage with only one major capsid protein which interlinks to form the viral capsid. *See* page 1774, paragraphs 5 and 6 of Bark. HK97 stands in stark contrast to AAVs, which have three capsid proteins (VP1, VP2, and VP3) with serotype-dependent sequences and masses. As explained above, Vliet discusses the need for an SDS-PAGE gel for analyzing AAV, which "functions as a separation step for the three overlapping AAV capsid proteins." *See* 3. Results of Vliet. Therefore, one of ordinary skill in the art reading Vliet and Bark would not have a reasonable expectation of success in combining the teachings thereof to determine the serotype of an AAV particle, since Bark discloses only a simple phage particle and do provide a means to analyze VP1, VP2, and VP3 in the absence of a gel separation step. For at least these reasons, claims 1 and 129, and all claims depending therefrom, are not obvious over Vliet and Bark.

With respect to claims 1 and 128, by virtue of the amendment filed herewith, claims 1 and 128 require that the method comprises directly subjecting the denatured AAV or viral particle to liquid chromatography/mass spectrometry (LC/MS) ***intact protein analysis***. As discussed in Applicant's remarks filed on November 9, 2021, neither Vliet nor Bark teaches or suggests directly subjecting a denatured AAV or viral particle to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis, *i.e.*, without using a protease. For at least this reason, claims 1 and 128 as amended, and all claims depending therefrom, are not obvious over Vliet and Bark.

In view of the foregoing, Applicant respectfully requests withdrawal of the rejection of the claims over Vliet and Bark.

Claims 182, 192 are rejected under 35 U.S.C. § 103 as allegedly being unpatentable over Vliet in view of Bark as applied to claims 1, 29, 61, 128 and 134, 181, 183-186, 189-191, 193-206 and 209-218 above, and further in view of Anderson *et al.* (US 2014/0017716, "Anderson").

As discussed above, claims 1 and 29 and their dependents, including claims 182 and 192, are not obvious over Vliet and Bark. Anderson does not cure the defects of Vliet and Bark. Anderson is completely silent regarding determining the serotype of an viral particle in the absence of a gel separation step, and does not teach or suggest directly subjecting a denatured viral particle to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis. For at least these reasons, claims 182 and 192 are not obvious over Vliet, Bark, and Anderson.

In view of the foregoing, Applicant respectfully requests withdrawal of the rejection of the claims over Vliet, Bark and Anderson.

Claims 187-188 and 207-208 are rejected under 35 U.S.C. § 103 as allegedly being unpatentable over Vliet in view of Bark as applied to claims 1, 29, 61, 128 and 134, 181, 183-186, 189-191, 193-206 and 209-218 above, and further in view of Taylor *et al.* (US 2013/0217789, "Taylor").

As discussed above, claim 1 and its dependents, including claims 187 and 188, are not obvious over Vliet and Bark. Taylor is completely silent regarding determining the serotype of a viral particle in the absence of a gel separation step, and does not teach or suggest directly subjecting a denatured viral particle to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis. For at least these reasons, claims 187 and 188 are not obvious over Vliet, Bark, and Taylor.

In view of the foregoing, Applicant respectfully requests withdrawal of the rejection of the claims over Vliet, Bark and Taylor.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark Office determines that an extension and/or other relief is required, Applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. **03-1952** referencing docket no. **15979-20141.00**. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: February 17, 2022

Respectfully submitted,

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Doc Code: A.NE.AFCP
Document Description: After Final Consideration Pilot Program Request

PTO/SB/434 (12-20)

CERTIFICATION AND REQUEST FOR CONSIDERATION UNDER THE AFTER FINAL CONSIDERATION PILOT PROGRAM 2.0

Practitioner Docket No.:	Application No.:	Filing Date:
15979-20141.00	16/325,653	August 14, 2017 (Int'l)
First Named Inventor:	**Title:**	
Xiaoying JIN	METHODS FOR DETECTING AAV	

APPLICANT HEREBY CERTIFIES THE FOLLOWING AND REQUESTS CONSIDERATION UNDER THE AFTER FINAL CONSIDERATION PILOT PROGRAM 2.0 (AFCP 2.0) OF THE ACCOMPANYING RESPONSE UNDER 37 CFR 1.116.

1. The above-identified application is (i) an original utility, plant, or design nonprovisional application filed under 35 U.S.C. 111(a) [a continuing application (*e.g.*, a continuation or divisional application) is filed under 35 U.S.C. 111(a) and is eligible under (i)], or (ii) an international application that has entered the national stage in compliance with 35 U.S.C. 371(c).
2. The above-identified application contains an outstanding final rejection.
3. Submitted herewith is a response under 37 CFR 1.116 to the outstanding final rejection. The response includes an amendment to at least one independent claim, and the amendment does not broaden the scope of the independent claim in any aspect.
4. This certification and request for consideration under AFCP 2.0 is the only AFCP 2.0 certification and request filed in response to the outstanding final rejection.
5. Applicant is willing and available to participate in any interview requested by the examiner concerning the present response.
6. This certification and request is being filed electronically using the Office's electronic filing system (EFS-Web).
7. Any fees that would be necessary consistent with current practice concerning responses after final rejection under 37 CFR 1.116, *e.g.*, extension of time fees, are being concurrently filed herewith. [There is no additional fee required to request consideration under AFCP 2.0.]
8. By filing this certification and request, applicant acknowledges the following:
    - Reissue applications and reexamination proceedings are not eligible to participate in AFCP 2.0.
    - The examiner will verify that the AFCP 2.0 submission is compliant, *i.e.*, that the requirements of the program have been met (see items 1 to 7 above). For compliant submissions:
        - The examiner will review the response under 37 CFR 1.116 to determine if additional search and/or consideration (i) is necessitated by the amendment and (ii) could be completed within the time allotted under AFCP 2.0. If additional search and/or consideration is required but cannot be completed within the allotted time, the examiner will process the submission consistent with current practice concerning responses after final rejection under 37 CFR 1.116, *e.g.*, by mailing an advisory action.
        - If the examiner determines that the amendment does not necessitate additional search and/or consideration, or if the examiner determines that additional search and/or consideration is required and could be completed within the allotted time, then the examiner will consider whether the amendment places the application in condition for allowance (after completing the additional search and/or consideration, if required). If the examiner determines that the amendment does not place the application in condition for allowance, then the examiner will contact the applicant and request an interview.
            - The interview will be conducted by the examiner, and if the examiner does not have negotiation authority, a primary examiner and/or supervisory patent examiner will also participate.
            - If the applicant declines the interview, or if the interview cannot be scheduled within ten (10) calendar days from the date that the examiner first contacts the applicant, then the examiner will proceed consistent with current practice concerning responses after final rejection under 37 CFR 1.116.

Signature	Date	Practitioner Registration No.
/Brian A. Donahue/	February 17, 2022	58,206
Name (Print/Typed)	**Telephone Number**	
Brian A. Donahue	+1 (650) 813-5632	

Note: *This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.*

☐ * Total of _____ forms are submitted.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	45023269
Application Number:	16325653
International Application Number:	
Confirmation Number:	1002
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Customer Number:	89300
Filer:	Brian Arthur Donahue/Aurelia Caparas
Filer Authorized By:	Brian Arthur Donahue
Attorney Docket Number:	15979-20141.00
Receipt Date:	17-FEB-2022
Filing Date:	14-FEB-2019
Time Stamp:	17:17:09
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		Resp_to_FOA_.pdf	171320 aa92c6407f1197dae6339d94d522cba3e4b43577	yes	16

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Response After Final Action	1	1
	Claims	2	9
	Applicant Arguments/Remarks Made in an Amendment	10	16

Warnings:					
Information:					
2	After Final Consideration Program Request	AFCPilot_program.pdf	202515 d5021d65b1fa324304224491ba4e18d40f4ce141	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	373835		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 16/325,653	Filing Date 02/14/2019	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $100 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $460 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	02/17/2022	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 43	Minus	** 204	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 4	Minus	*** 18	= 0		x $480 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/VERONICA EVERETTE/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/325,653	02/14/2019	Xiaoying JIN	15979-20141.00	1002

89300 7590 02/28/2022

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

EXAMINER	
XU, XIAOYUN	

ART UNIT	PAPER NUMBER
1797	

NOTIFICATION DATE	DELIVERY MODE
02/28/2022	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

EOfficePA@mofo.com
PatentDocket@mofo.com
pair_mofo@firsttofile.com

Advisory Action Before the Filing of an Appeal Brief	Application No. 16/325,653	Applicant(s) JIN et al.	
	Examiner XIAOYUN R XU, Ph. D.	Art Unit 1797	AIA (FITF) Status Yes

--The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

THE REPLY FILED 17 February 2022 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE.

NO NOTICE OF APPEAL FILED

1. ☑ The reply was filed after a final rejection. No Notice of Appeal has been filed. To avoid abandonment of this application, applicant must timely file one of the following replies: (1) an amendment, affidavit, or other evidence, which places the application in condition for allowance; (2) a Notice of Appeal (with appeal fee) in compliance with 37 CFR 41.31; or (3) a Request for Continued Examination (RCE) in compliance with 37 CFR 1.114 if this is a utility or plant application. Note that RCEs are not permitted in design applications. The reply must be filed within one of the following time periods:
   a) ☑ The period for reply expires 3 months from the mailing date of the final rejection.
   b) ☐ The period for reply expires on: (1) the mailing date of this Advisory Action; or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection.
   c) ☐ A prior Advisory Action was mailed more than 3 months after the mailing date of the final rejection in response to a first after-final reply filed within 2 months of the mailing date of the final rejection.The current period for reply expires ____ months from the mailing date of *the prior Advisory Action* or SIX MONTHS from the mailing date of the final rejection, whichever is earlier.
   Examiner Note: If box 1 is checked, check either box (a), (b) or (c). ONLY CHECK BOX (b) WHEN THIS ADVISORY ACTION IS THE FIRST RESPONSE TO APPLICANTS FIRST AFTER-FINAL REPLY WHICH WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. ONLY CHECK BOX (c) IN THE LIMITED SITUATION SET FORTH UNDER BOX (c). See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) or (c) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

NOTICE OF APPEAL

2. ☐ The Notice of Appeal was filed on _____. A brief in compliance with 37 CFR 41.37 must be filed within two months of the date of filing the Notice of Appeal (37 CFR 41.37(a)), or any extension thereof (37 CFR 41.37(e)), to avoid dismissal of the appeal. Since a Notice of Appeal has been filed, any reply must be filed within the time period set forth in 37CFR 41.37(a).

AMENDMENTS

3. ☑ The proposed amendments filed after a final rejection, but prior to the date of filing a brief, will not be entered because
   a) ☑ They raise new issues that would require further consideration and/or search (see NOTE below);
   b) ☐ They raise the issue of new matter (see NOTE below);
   c) ☐ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or
   d) ☐ They present additional claims without canceling a corresponding number of finally rejected claims.
   NOTE: See Continuation Sheet (See 37 CFR 1.116 and 41.33(a)).
4. ☐ The amendments are not in compliance with 37 CFR 1.121. See attached Notice of Non-Compliant Amendment (PTOL-324).
5. ☐ Applicant's reply has overcome the following rejection(s): _____
6. ☐ Newly proposed or amended claim(s) _____ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).
7. ☐ For purposes of appeal, the proposed amendment(s):(a)☐ will not be entered, or (b)☐ will be entered, and an explanation of how the new or amended claims would be rejected is provided below or appended.

AFFIDAVIT OR OTHER EVIDENCE

8. ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____
9. ☐ The affidavit or other evidence filed after final action, but before or on the date of filing a Notice of Appeal will not be entered because applicant failed to provide a showing of good and sufficient reasons why the affidavit or other evidence is necessary and was not earlier presented. See 37 CFR 1.116(e).
10. ☐ The affidavit or other evidence filed after the date of filing the Notice of Appeal, but prior to the date of filing a brief, will not be entered because the affidavit or other evidence failed to overcome all rejections under appeal and/or appellant fails to provide a showing of good and sufficient reasons why it is necessary and was not earlier presented. See 37 CFR 41.33(d)(1).
11. ☐ The affidavit or other evidence is entered. An explanation of the status of the claims after entry is below or attached.

REQUEST FOR RECONSIDERATION/OTHER

12. ☐ The request for reconsideration has been considered but does NOT place the application in condition for allowance because: _____.
13. ☐ Note the attached Information *Disclosure Statement*(s). (PTO/SB/08) Paper No(s). _____
14. ☑ Other: Interview summary and AFCP form are attached.

STATUS OF CLAIMS

15. The status of the claim(s) is (or will be) as follows:
    Claim(s) allowed:_____.
    Claim(s) objected to:_____.
    Claim(s) rejected:1,29,61,128,134 and 181-218.
    Claim(s) withdrawn from consideration:_____.

/XIAOYUN R XU, Ph.D./
Primary Examiner, Art Unit 1797

Continuation of 3. NOTE: The amendment in claim 1 and 128 raises new issue that would require further consideration and search.

Examiner-Initiated Interview Summary	**Application No.** 16/325,653	**Applicant(s)** JIN et al.		
	Examiner XIAOYUN R XU, Ph. D.	**Art Unit** 1797	**AIA (First Inventor to File) Status** Yes	**Page** 1 of 1

All Participants (applicant, applicants representative, PTO personnel)	**Title**	**Type**
XIAOYUN R XU, Ph. D.	Primary Examiner	Telephonic
Brian Donahue	Attorney of Record	

Date of Interview: 23 February 2022

Issues Discussed:

35 U.S.C. 103

Applicant and examiner discussed the claimed subject matter in relation to the prior art. The amendment in claim 1 and 128 raises new issue that would require further consideration and search.

/XIAOYUN R XU, Ph.D./ Primary Examiner, Art Unit 1797	

Applicant is reminded that a complete written statement as to the substance of the interview must be made of record in the application file. It is the applicants responsibility to provide the written statement, unless the interview was initiated by the Examiner and the Examiner has indicated that a written summary will be provided. See MPEP 713.04
Please further see:
MPEP 713.04
Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews, paragraph (b)
37 CFR § 1.2 Business to be transacted in writing

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

AFCP 2.0 Decision

Application No.	Applicant(s)	
16/325,653	JIN et al.	
Examiner	**Art Unit**	**AIA (FITF) Status**
XIAOYUN R XU, Ph. D.	1797	Yes

This is in response to the After Final Consideration Pilot request filed 17 February 2022.

1. **Improper Request** – The AFCP 2.0 request is improper for the following reason(s) and the after final amendment submitted with the request will be treated under pre-pilot procedure.

   ☐ An AFCP 2.0 request form PTO/SB/434 (or equivalent document) was not submitted.

   ☐ A non-broadening amendment to at least one independent claim was not submitted.

   ☐ The request is not the first proper AFCP 2.0 request submitted in response to the most recent final rejection.

   ☐ Other: _____

2. **Proper Request**

   A. After final amendment submitted with the request will not be treated under AFCP 2.0.
   The after final amendment cannot be reviewed and a search conducted within the guidelines of the pilot program.

   ☐ The after final amendment will be treated under pre-pilot procedure.

   B. Updated search and/or completed additional consideration.
   The examiner performed an updated search and/or completed additional consideration of the after final amendment within the time authorized for the pilot program. The result(s) of the updated search and/or completed additional consideration are:

   ☐ 1. All of the rejections in the most recent final Office action are overcome and a Notice of Allowance is issued herewith.

   ☐ 2. The after final amendment would not overcome all of the rejections in the most recent final Office action . See attached interview summary for further details.

   ☑ 3. The after final amendment was reviewed, and it raises a new issue(s). See attached interview summary for further details.

   ☐ 4. The after final amendment raises new issues, but would overcome all of the rejections in the most recent final Office action. A decision on determining allowability could not be made within the guidelines of the pilot. See attached interview summary for further details, including any newly discovered prior art.

   ☐ 5. Other: _____

   Examiner Note: Please attach an interview summary when necessary as described above.

DO NOT ENTER: /R.X./

Docket No.: 15979-20141.00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Xiaoying JIN et al.

Application No.: 16/325,653

Filed: August 14, 2017 (Int'l)

For: METHODS FOR DETECTING AAV

Confirmation No.: 1002

Art Unit: 1797

Examiner: X. Xu

AMENDMENT AFTER FINAL ACTION UNDER 37 C.F.R. 1.116

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

INTRODUCTORY COMMENTS

This is in response to the final Office Action dated November 17, 2021, for which a response is due on February 17, 2022. Accordingly, this response is timely filed. This response accompanies a Certification and Request for Consideration under the After Final Consideration Pilot Program 2. 0. Reconsideration and allowance of the pending claims, as amended, in light of the remarks presented herein are respectfully requested.

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 10 of this paper.

Doc code: RCEX
Doc description: Request for Continued Examination (RCE)

PTO/SB/30EFS (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
(Submitted Only via EFS-Web)

Application Number	16/325,653	Filing Date	2017-08-14	Docket Number (if applicable)	15979-20141.00	Art Unit	1797
First Named Inventor	Xiaoying JIN			Examiner Name	X. Xu		

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. The Instruction Sheet for this form is located at WWW.USPTO.GOV

SUBMISSION REQUIRED UNDER 37 CFR 1.114

Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

☒ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____

☒ Other Consider the Response to Final Office Action filed on February 17, 2022

☐ Enclosed

☐ Amendment/Reply

☐ Information Disclosure Statement (IDS)

☐ Affidavit(s)/ Declaration(s)

☐ Other _____

MISCELLANEOUS

☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____ (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

☐ Other _____

FEES

The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.

☒ The Director is hereby authorized to charge any underpayment of fees, or credit any overpayments, to Deposit Account No 03-1952

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

X Patent Practitioner Signature

Applicant Signature

PTO/SB/30EFS (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Signature of Registered U.S. Patent Practitioner			
Signature	/Brian A. Donahue/	Date (YYYY-MM-DD)	2022-03-10
Name	Brian A. Donahue	Registration Number	58206

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Patent Application Fee Transmittal

Application Number:	16325653
Filing Date:	14-Feb-2019
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Filer:	Brian Arthur Donahue/Aurelia Caparas
Attorney Docket Number:	15979-20141.00

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 1 month with $0 paid	1251	1	220	220
Miscellaneous:				
RCE- 1ST REQUEST	1801	1	1360	1360
		Total in USD ($)		**1580**

Electronic Acknowledgement Receipt

EFS ID:	45195933
Application Number:	16325653
International Application Number:	
Confirmation Number:	1002
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Customer Number:	89300
Filer:	Brian Arthur Donahue/Aurelia Caparas
Filer Authorized By:	Brian Arthur Donahue
Attorney Docket Number:	15979-20141.00
Receipt Date:	10-MAR-2022
Filing Date:	14-FEB-2019
Time Stamp:	18:07:16
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$1580
RAM confirmation Number	E202230I07372120
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Continued Examination (RCE)	RCE.PDF	1349832 e681da58d2bb59fd3c88b24a65cff986d7f2be67	no	3
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	40942 0e879a2834e85a6fd3876f98791eea7534122288	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	1390774		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/325,653	02/14/2019	Xiaoying JIN	15979-20141.00	1002

89300 7590 04/28/2022

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

EXAMINER
XU, XIAOYUN

ART UNIT	PAPER NUMBER
1797	

NOTIFICATION DATE	DELIVERY MODE
04/28/2022	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

EOfficePA@mofo.com
PatentDocket@mofo.com
pair_mofo@firsttofile.com

Office Action Summary	Application No. 16/325,653	Applicant(s) JIN et al.	
	Examiner XIAOYUN R XU, Ph. D.	Art Unit 1797	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 03/10/2022.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1,29,61,128,134 and 181-218 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☑ Claim(s) 1,128,181-190,194-195,198-199 and 202-209 is/are allowed.

7) ☑ Claim(s) 29,61,134,191-193,196-197,200-201 and 210-218 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

1. The amendment and RCE filed on 03/10/2022 has been entered and fully considered. Claims 1, 29, 61, 128, 134, and 181-218 are pending, of which claims 1 and 128 are amended.

Response to Amendment

2. In response to amendment, the examiner withdraws rejection to claim 1 and 128 as well as dependent claims. and maintains rejection to claim 29, 61 and 134 as well as dependent claims over the prior art established in the previous Office action.

Notice of Pre-AIA or AIA Status

3. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Claim Rejections - 35 USC § 103

4. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

5. **Claim 29, 61, 134, 191, 193, 196-197, 200-201 and 210-218** is/are rejected under 35 U.S.C. 103 as being unpatentable over Vliet et al. (Journal of Virological Methods, 2009, IDS) (Vliet) in view of Bark et al. (J. Am. Chem. Soc. 2001, IDS) (Bark).

Regarding claim 29, Vliet teaches a method to determine the serotype of an adeno-associated virus (AAV) particle (abstract) comprising

a) denaturing the AAV particle (boiled for 3 min) (page 169, par 4),

b) subjecting the denatured AAV particle to reduction and/or alkylation (page 169, par 5),

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle (page 169, par 5),

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS) (page 170, par 2), and

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle (page 171, par 1);

wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype (Table 2, page 171, par 1).

Again, Vliet does not specifically teach wherein the method is performed in the absence of a gel separation step. However, Bark teaches directly subjecting the denatured and digested virus particle (HK97) to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step (Fig. 1, page 1774, par 4). Bark teaches that "The primary techniques for proteomic analysis consist of 2-D gel electrophoresis followed by proteolytic digestion, mass spectrometry, and computer-facilitated data analysis. Masses for proteolytic fragments of the target protein generated by enzymatic degradation can be measured directly with high accuracy (±0.005 Da for a 1000 Da peptide). Alternatively, liquid chromatography tandem mass spectrometry can be used to analyze the protein digest fragments" (page 1774, par 1). Here Bark teaches that alternatively, one can directly subjects the denatured and digested virus particle (HK97) to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step. At time before the filing, it would have been obvious to one of ordinary skill in the art to directly subjecting the denatured and digested AAV particle to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step, in order to save time that is required for gel separation.

Regarding claim 61, Vliet teaches a method of determining the serotype of an AAV particle comprising the method of claim 1 combined with a method to determine the serotype of an adeno-associated virus (AAV) particle (abstract) comprising

a) denaturing the AAV particle (boiled for 3 min) (page 169, par 4),

b) subjecting the denatured AAV particle to reduction and/or alkylation (page 169, par 5),

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle (page 169, par 5),

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS) (page 169, par 6, page 170, par 2), and

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle (page 169, par 6);

wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype (Table 2, page 171, par 1).

Again, Vliet does not specifically teach wherein the method is performed in the absence of a gel separation step. However, Bark teaches directly subjecting the denatured and digested virus particle (HK97) to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step (Fig. 1, page 1774, par 4). Bark teaches that "The primary techniques for proteomic analysis consist of 2-D gel electrophoresis followed by proteolytic digestion, mass spectrometry, and computer-facilitated data analysis. Masses for proteolytic fragments of the target protein generated by enzymatic degradation can be measured directly with high accuracy (±0.005 Da for a 1000 Da peptide). Alternatively, liquid chromatography tandem mass spectrometry can be used to analyze the protein digest fragments" (page 1774, par 1). Here Bark teaches that alternatively, one can directly subjects the denatured and digested virus particle (HK97) to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step. At time before the filing, it would have been obvious to one of ordinary skill in the art to directly subjecting the denatured and digested AAV particle to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step, in order to save time that is required for gel separation.

Regarding claim 134, Vliet teaches a method to determine the serotype of a viral particle (abstract) comprising

a) denaturing the viral particle (boiled for 3 min) (page 169, par 4),

b) subjecting the denatured viral particle to reduction and/or alkylation (page 169, par 5),

c) subjecting the denatured viral particle to digestion to generate fragments of one or more capsid proteins of the viral particle (page 169, par 5),

d) subjecting the fragments of the one or more capsid proteins to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS) (page 170, par 2), and

e) determining the masses of fragments of the one or more capsid proteins of the viral particle (page 169, par 6);

wherein the specific combination of masses of fragments of the one or more capsid proteins are indicative of the viral serotype (Table 2, page 171, par 1).

Again, Vliet does not specifically teach wherein the method is performed in the absence of a gel separation step. However, Bark teaches directly subjecting the denatured and digested virus particle (HK97) to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step (Fig. 1, page 1774, par 4). Bark teaches that "The primary techniques for proteomic analysis consist of 2-D gel electrophoresis followed by proteolytic digestion, mass spectrometry, and computer-facilitated data analysis. Masses for proteolytic fragments of the target protein generated by enzymatic degradation can be measured directly with high accuracy (±0.005 Da for a 1000 Da peptide). Alternatively, liquid chromatography tandem mass spectrometry can be used to analyze the protein digest fragments" (page 1774, par 1). Here Bark teaches that alternatively, one can directly subjects the denatured and digested virus particle (HK97) to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step. At time before the filing, it would have been obvious to one of ordinary skill in the art to directly subjecting the denatured and digested AAV particle to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step, in order to save time that is required for gel separation.

Regarding claim 191 and 210, Vliet teaches that wherein the calculated masses of VP1, VP2 and VP3 are compared to the theoretical masses of VP 1, VP2 and VP3 of one or more AAV serotypes (Fig. 2, page 168, par 3).

Regarding claim 193 and 211, Vliet teaches that wherein the liquid chromatography is reverse phase liquid chromatography (C18 column), size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography (page 169, par 6).

Regarding claim 200, Vliet teaches that wherein the AAV particle is a recombinant AAV (rAAV) particle (abstract).

Regarding claim 201, Vliet teaches that wherein the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an

AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV I1 capsid, an AAV 12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, a mouse AAV capsid rAAV2/HBoVI (chimeric AAV / human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid (Fig. 1, page 167, par 2).

Regarding claim 212, Vliet teaches that wherein the liquid chromatography is reverse phase chromatography (page 169, par 6).

Regarding claim 213, Vliet teaches that wherein the reverse phase chromatography is a C18 reverse chromatography (page 169, par 6).

Regarding claim 196 and 214, Vliet teaches that wherein the reduction is by subjecting the AAV particle to dithiothreitol, beta-mercaptoethanol, or tris(2-arboxyethyl)phosphine (TCEP) (page 169, par 5).

Regarding claim 197 and 215, Vliet teaches that wherein the alkylation is by subjecting the AAV particle to iodoacetic acid, iodoacetamide, or 4-vinylpyridine (page 169, par 5).

Regarding claim 216, Vliet teaches that wherein the digestion is an endopeptidase digestion (page 169, par 5).

Regarding claim 217, Vliet teaches that wherein the endopeptidase digestion is a trypsin digestion (page 169, par 5).

Regarding claim 218, Vliet teaches that wherein the viral particle comprises a viral vector encoding a heterologous transgene (page 167, par 1).

6. **Claim 192** is/are rejected under 35 U.S.C. 103 as being unpatentable over Vliet in view of Bark as applied to claims 29, 61, 134, 191, 193, 196-197, 200-201 and 210-218 above, and further in view of Anderson (US 2014/0017716).

Regarding claim 192, Vliet does not teach that wherein the AAV particle is denatured with acetic acid, guanidine hydrochloride, and/or an organic solvent. However, Anderson teaches that protein sample is denatured with guanidine hydrochloride, and/or an organic solvent (par 0022]). At time before the filing, it would

have been obvious to one of ordinary skill in the art to select guanidine hydrochloride, and/or an organic solvent for denaturizing the AAV particle, because the selection is based on its suitability for the intended use.

Allowable Subject Matter

7. **Claim 1, 128, 181-190, 194-195, 198-199 and 202-209** are allowed.

8. The following is an examiner's statement of reasons for allowance: neither Vliet nor Bark teaches or fairly suggests directly subjecting a denatured AAV or viral particle to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis, i.e., without using a protease.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

Response to Arguments

9. Applicant's arguments filed 02/17/2022 have been fully considered but they are not persuasive to some rejections.

Applicant argues that "Applicant respectfully submits that Bark does not cure the defects of Vliet, because it does not teach performing the method in the absence of a gel separation step. Bark is directed to the development of a method for high-temperature proteolytic digestion using a thermotolerant protease (thermolysin) to generate protein fragments for analysis via mass spectrometry. The Examiner highlights specific passages of Bark: "The primary techniques for proteomic analysis consist of 2-D gel electrophoresis followed by proteolytic digestion, mass spectrometry, and computer-facilitated data analysis" (page 1774, par. 1; emphasis added); "Two limitations of this method are the time required for adequate proteolysis and the resistance of some proteins to digestion" (page 1774, par. 2; emphasis added), and "We have taken advantage of thermal denaturation to generate a facile proteolysis method for identifying proteins" (page 1774, par. 2) See the Office Action, e.g., page 3. These passages do not teach that the time required for gel separation is a limitation of

proteomic methods; rather, these passages indicate that 2-D gel electrophoresis is a primary technique that is carried out prior to proteolytic digestion. Except for the passage above, Bark is completely silent about gel separation. Instead, Bark describes high-temperature proteolysis methods to reduce the time required for proteolysis, which is the limitation identified in the passages cited by the Examiner. In fact, Bark provides no description of how the samples were prepared prior to proteolysis, including whether or not a gel separation step was performed. Since, as Bark points out in paragraph 1 of page 1774, gel separation is carried out prior to proteolysis in proteomics techniques, it is not clear that the various proteins enumerated in paragraph 4 of page 1774 were not separated in a gel separation step prior to digestion. Given that Bark teaches that gel separation is a primary technique carried out prior to proteolytic digestion, one skilled in the art would assume that gel separation was used prior to the analysis. Therefore, Bark neither teaches performing the method in the absence of a gel separation step, nor suggests that proteomics methods could be performed in the absence of a gel separation step in order to save the time required for gel separation." (remark, page 11-12).

Examiner respectfully disagrees. Bark teaches directly subjecting the denatured and digested virus particle (HK97) to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step (Fig. 1, page 1774, par 4). Bark teaches that "The primary techniques for proteomic analysis consist of 2-D gel electrophoresis followed by proteolytic digestion, mass spectrometry, and computer-facilitated data analysis. Masses for proteolytic fragments of the target protein generated by enzymatic degradation can be measured directly with high accuracy (±0.005 Da for a 1000 Da peptide). Alternatively, liquid chromatography tandem mass spectrometry can be used to analyze the protein digest fragments" (page 1774, par 1). Here Bark teaches that alternatively, one can directly subjects the denatured and digested virus particle (HK97) to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step. At time before the filing, it would have been obvious to one of ordinary skill in the art to directly subjecting the denatured and digested AAV particle to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step, in order to save time that is required for gel separation.

Applicant argues that "Vliet teaches away from performing the disclosed method in the absence of a gel separation step. Vliet explains that "The SDS-PAGE gel functions as a separation step for the three overlapping AAV capsid proteins that comprise the virion as well as impurities that may be present in the sample; therefore, samples of varying levels of purity can be analyzed." See 3. Results. Therefore, one of ordinary skill in the art reading Vliet would not have a reasonable expectation of success in performing the disclosed method in the absence of a gel separation step, as required by the claims, since according to Vliet, this would not enable separation of the overlapping AAV capsid proteins and analysis of samples of varying levels of purity." (remark, page 12, par 2).

This agreement is not persuasive. Vliet teaches the benefit of performing gel separation. However, the benefit of performing gel separation is not teach away for without gel separation, because Vliet teaches that gel separation is a good to have step, instead of a must have step.

Conclusion

10. Any inquiry concerning this communication or earlier communications from the examiner should be directed to XIAOYUN R XU, Ph. D. whose telephone number is (571)270-5560. The examiner can normally be reached on M-F 8am-5pm.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Lyle Alexander can be reached on 571-272-1254. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see https://ppair-

my.uspto.gov/pair/PrivatePair. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/XIAOYUN R XU, Ph.D./
Primary Examiner, Art Unit 1797

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
	Examiner	Art Unit
	XIAOYUN R XU, Ph. D.	1797

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

CLAIMS

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	06/22/2021	11/12/2021	03/14/2022						
	1	✓	✓	=						
	2	-	-	-						
	3	N	N	-						
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	29	✓	✓	✓						
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	38	-	-	-						
	39	-	-	-						
	40	-	-	-						
	41	-	-	-						
	42	-	-	-						

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
	Examiner	Art Unit
	XIAOYUN R XU, Ph. D.	1797

CLAIM		DATE								
Final	Original	06/22/2021	11/12/2021	03/14/2022						
	43	-	-	-						
	44	-	-	-						
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Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
	Examiner	Art Unit
	XIAOYUN R XU, Ph. D.	1797

CLAIM		DATE								
Final	Original	06/22/2021	11/12/2021	03/14/2022						
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Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
	Examiner	**Art Unit**
	XIAOYUN R XU, Ph. D.	1797

CLAIM		DATE								
Final	Original	06/22/2021	11/12/2021	03/14/2022						
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	196		✓	✓						
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Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
	Examiner	**Art Unit**
	XIAOYUN R XU, Ph. D.	1797

CLAIM		DATE								
Final	Original	06/22/2021	11/12/2021	03/14/2022						
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	215		✓	✓						
	216		✓	✓						
	217		✓	✓						
	218		✓	✓						

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
	Examiner	Art Unit
	XIAOYUN R XU, Ph. D.	1797

CPC - Searched*		
Symbol	Date	Examiner
C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848 (text limited)	06/22/2021	RX

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner
436	173 (text limited)	06/22/2021	RX

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
EAST (CPC C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848 ; USPC 436/173 with key words and inventor names) and Google	06/22/2021	RX
updated search in EAST and Google	11/12/2021	RX
updated search in EAST and Google	03/14/2022	RX

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/XIAOYUN R XU, Ph.D./ Primary Examiner, Art Unit 1797	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
	Examiner	**Art Unit**
	XIAOYUN R XU, Ph. D.	1797

/XIAOYUN R XU, Ph.D./ Primary Examiner, Art Unit 1797	

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	20	(US-6989264-$ | US-7588772-$ | US-7629322-$ | US-8137948-$ | US-8283151-$ | US-9169299-$ | US-20110275529-$ | US-20120066783-$ | US-20120164106-$ | US-20130323226-$).DID.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 12:05
S2	1	(US-6989264-$ | US-7588772-$ | US-7629322-$ | US-8137948-$ | US-8283151-$ | US-9169299-$ | US-20110275529-$ | US-20120066783-$ | US-20120164106-$ | US-20130323226-$).DID. and acetylation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:19
S3	1	(US-20100129405-$).DID. and acetylation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:21
S4	1	(US-6989264-$ | US-7588772-$ | US-7629322-$ | US-8137948-$ | US-8283151-$ | US-9169299-$ | US-20110275529-$ | US-20120066783-$ | US-20120164106-$ | US-20130323226-$).DID. and deamidation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:25
S5	1	16/325653.app.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:25
S6	13,645	adeno-associated virus AND ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:26
S7	5,489	adeno-associated virus AND serotype and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:27

		G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)				
S8	836	adeno-associated virus AND serotype and denature$1 and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:27
S9	125	adeno-associated virus AND serotype and denature$1 and heterogeneity and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:28
S10	91	adeno-associated virus AND serotype and denature$1 and heterogeneity and mass$2 and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:28
S11	25	adeno-associated virus AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:29
S12	9	adeno-associated virus AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016" and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:29
S13	9	(adeno-associated virus or AAV)AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016" and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:30

		G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)				
S15	3	436/173.ccls. and (adeno-associated virus or AAV) AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:31
S16	10	(WO-2003042397-$ | WO-2004027019-$ | WO-2010148143-$ | WO-2013158879-$).DID.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:33
S17	0	(WO-2003042397-$ | WO-2004027019-$ | WO-2010148143-$ | WO-2013158879-$).DID. and deamidation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:34
S18	0	(WO-2003042397-$ | WO-2004027019-$ | WO-2010148143-$ | WO-2013158879-$).DID. and acetylation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:34
S19	5	((("JIN") near3 ("Xiaoying"))).INV.	US-PGPUB; USPAT; USOCR	ADJ	ON	2021/03/08 13:35
S20	42	((("O'RIORDAN") near3 ("Catherine"))).INV.	US-PGPUB; USPAT; USOCR	ADJ	ON	2021/03/08 13:35
S21	1,105	((("LIU") near3 ("Lin"))).INV.	US-PGPUB; USPAT; USOCR	ADJ	ON	2021/03/08 13:36
S22	5	((("ZHANG") near3 ("Kate"))).INV.	US-PGPUB; USPAT; USOCR	ADJ	ON	2021/03/08 13:37
S23	9	(adeno-associated virus or AAV) AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016" and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/06/22 12:01

S24	4	wo-2007046703-$.DID.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/11/12 10:23
S25	4	wo-2010093784-$.DID.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/11/12 10:24
S26	2	wo-2015114365-$.DID.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/11/12 10:24
S27	2	wo-2015137802-$.DID.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/11/12 10:25
S28	2,591	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent)and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/11/12 16:09
S29	585	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent) and AAV and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/11/12 16:09
S30	585	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent)and AAV and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/11/12 16:10
S31	175	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic	US-PGPUB; USPAT;	ADJ	ON	2021/11/12 16:11

		solvent)and AAV and mass spectrometry and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			
S32	172	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent)and AAV and mass spectrometry and digest$3 and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/11/12 16:11
S33	129	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent)and AAV and mass spectrometry and digest$3 and @ay< "2016" and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/11/12 16:12
S34	99	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent)and AAV and mass spectrometry and digest$3 and @ay< "2016" and trypsin and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/11/12 16:17
S35	3	ep-1879912-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/11/12 16:33
S36	2	wo-2012031245-$.DID.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/11/12 16:54

EAST Search History (Interference)

<This search history is empty>

3/14/2022 12:51:08 PM
C:\Users\xxu\Documents\EAST\Workspaces\16325653.wsp

PTO/SB/06 (09-11)
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 16/325,653	Filing Date 02/14/2019	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $100 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $460 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	10/27/2022	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 21	Minus	** 204	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 2	Minus	*** 3	= 0		x $480 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/ANNIE C SINGLETON/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Docket No.: 15979-20141.00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Xiaoying JIN et al.

Application No.: 16/325,653

Filed: August 14, 2017 (Int'l)

For: METHODS FOR DETECTING AAV

Confirmation No.: 1002

Art Unit: 1797

Examiner: X. Xu

AMENDMENT IN RESPONSE TO NON-FINAL OFFICE ACTION UNDER 37 C.F.R. 1.111

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

INTRODUCTORY COMMENTS

This is in response to the non-final Office Action dated April 28, 2022, for which a response was due on July 28, 2022. We hereby petition and submit the fee for a three-month extension of time, thereby extending the deadline for response to October 28, 2022. Accordingly, this response is timely filed. Reconsideration and allowance of the pending claims, as amended, in light of the remarks presented herein are respectfully requested.

Amendments to the Claims are reflected in the listing of claims, which begins on page 2 of this paper.

Remarks/Arguments begin on page 6 of this paper.

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions, and listings, of claims in the application:

In the claims:

Claim 1 (previously presented): A method to determine the serotype of an adeno-associated virus (AAV) particle comprising

a) denaturing the AAV particle,

b) directly subjecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis, and

c) determining the masses of VP1, VP2 and VP3 of the AAV particle;

wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype,

and wherein the method is performed in the absence of a gel separation step.

Claims 2-60 (canceled)

Claim 61 (previously presented): A method of determining the serotype of an AAV particle comprising the method of claim 1 combined with a method to determine the serotype of an adeno-associated virus (AAV) particle comprising

a) denaturing the AAV particle,

b) subjecting the denatured AAV particle to reduction and/or alkylation,

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle,

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle;

wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype,

and wherein the method is performed in the absence of a gel separation step.

Claims 62-127 (canceled)

Claim 128 (previously presented): A method to determine the serotype of a viral particle comprising

a) denaturing the viral particle,

b) directly subjecting the denatured viral particle to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis, and

c) determining the masses of one or more capsid proteins of the viral particle;

wherein the specific combination of masses of the one or more capsid proteins are indicative of the virus serotype,

and wherein the method is performed in the absence of a gel separation step.

Claims 129-180 (canceled)

Claim 181 (previously presented): The method of claim 1, wherein the calculated masses of VP1, VP2 and VP3 are compared to the theoretical masses of VP1, VP2 and VP3 of one or more AAV serotypes.

Claim 182 (previously presented): The method of claim 1, wherein the AAV particle is denatured with acetic acid, guanidine hydrochloride, and/or an organic solvent.

Claim 183 (previously presented): The method of claim 1, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

Claim 184 (previously presented): The method of claim 1, wherein the liquid chromatography is reverse phase chromatography.

Claim 185 (previously presented): The method of claim 184, wherein the reverse phase chromatography is a C4 or C8 reverse chromatography.

Claim 186 (previously presented): The method of claim 1, wherein the liquid chromatography is ultra-performance liquid chromatography (UPLC).

Claim 187 (previously presented): The method of claim 1, wherein the mass spectrometry comprises assisted calibration.

Claim 188 (previously presented): The method of claim 187, wherein sodium iodide is used as a calibrant.

Claim 189 (previously presented): The method of claim 1, wherein the AAV particle is a recombinant AAV (rAAV) particle.

Claim 190 (previously presented): The method of claim 1, wherein the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid.

Claim 191-201 (canceled)

Claim 202 (previously presented): The method of claim 128, wherein the calculated masses of the one or more capsid proteins are compared to the theoretical masses of the one or more capsid proteins of one or more virus serotypes.

Claim 203 (previously presented): The method of claim 128, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

Claim 204 (previously presented): The method of claim 128, wherein the liquid chromatography is reverse phase chromatography.

Claim 205 (previously presented): The method of claim 204, wherein the reverse phase chromatography is a C4 or C8 reverse chromatography.

Claim 206 (previously presented): The method of claim 128, wherein the liquid chromatography is ultra-performance liquid chromatography (UPLC).

Claim 207 (previously presented): The method of claim 128, wherein the mass spectrometry comprises assisted calibration.

Claim 208 (previously presented): The method of claim 207, wherein sodium iodide is used as a calibrant.

Claim 209 (previously presented): The method of claim 128, wherein the viral particle comprises a viral vector encoding a heterologous transgene.

Claim 210-218 (canceled)

REMARKS

Claims 1, 29, 61, 128, 134, and 181-217 were pending in the present application. By virtue of this response, claims 29, 134, 191-201, and 210-218 have been canceled. Accordingly, following entry of this amendment, claims 1, 61, 128, 181-190, and 202-209 will be under consideration. No new matter has been added. Entry of the claim amendments is respectfully requested.

With respect to all claim amendments, Applicant has not dedicated or abandoned any unclaimed subject matter and moreover has not acquiesced to any rejections and/or objections made by the patent office. Applicant reserves the right to pursue prosecution of any presently excluded claim embodiments in a future continuation and/or divisional application.

Rejections under 35 U.S.C. §103

Claims 29, 61, 134, 191, 193, 196-197, 200-201 and 210-218 are rejected under 35 U.S.C. 103 as allegedly being unpatentable over Vliet et al. (Journal of Virological Methods, 2009, IDS) (Vliet) in view of Bark et al. (J. Am. Chem. Soc. 2001, IDS) (Bark).

Applicant traverses the rejection and its supporting remarks. However, without acquiescing to the Examiner's rejection, and solely to expedite prosecution, claims 29, 134, 191, 193, 196-197, 200-201 and 210-218 have been canceled without prejudice, thus rendering the rejection of said claims moot.

Applicant respectfully submits that claim 61 depends from claim 1, and therefore incorporates all of the limitations of, claim 1, which was not rejected as allegedly obvious over Vliet and Bark. Therefore, claim 61 is not obvious over Vliet and Bark. Applicant respectfully requests withdrawal of claim 61 as allegedly obvious over Vliet and Bark.

Claim 192 is rejected under 35 U.S.C. 103 as allegedly being unpatentable over Vliet in view of Bark as applied to claims 29, 61, 134, 191, 193, 196-197, 200-201 and 210-218 above, and further in view of Anderson (US 2014/0017716).

Applicant traverses the rejection and its supporting remarks. However, without acquiescing to the Examiner's rejection, and solely to expedite prosecution, claim 192 has been canceled without prejudice, thus rendering the rejection moot.

Allowed Claims

Applicant thanks the Examiner for acknowledging that 1, 128, 181-190, and 202-209 are patentable.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark Office determines that an extension and/or other relief is required, Applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. **03-1952** referencing docket no. **15979-20141.00**. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: October 27, 2022

Respectfully submitted,

Electronic signature: /Sarah E. Bloch/
Sarah Bloch
Registration No.: 79,763
MORRISON & FOERSTER LLP
755 Page Mill Road
Palo Alto, CA 94304-1018
+1 (650) 813-4046

PTO/SB/08a (03-15)
Approved for use through 07/31/2016. OMB 0651-0031
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16325653
Filing Date	2017-08-14
First Named Inventor	XIAOYING JIN
Art Unit	1797
Examiner Name	X. Xu
Attorney Docket Number	15979-20141.00

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16325653
	Filing Date	2017-08-14
	First Named Inventor	XIAOYING JIN
	Art Unit	1797
	Examiner Name	X. Xu
	Attorney Docket Number	15979-20141.00

	1	SNIJDER, J. et al. (May 1, 2014). "Defining the Stoichiometry and Cargo Load of Viral and Bacterial Nanoparticles by Orbitrap Mass Spectrometry," J. Am. Chem. Soc. 136: 7295-7299.
	2	Submission in Opposition Proceedings for European Patent No. 3497207, dated February 25, 2022, Proprietor Genzyme Corporation, Opponent Definition IP Limited, 2 pages.

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

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[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16325653
Filing Date	2017-08-14
First Named Inventor	XIAOYING JIN
Art Unit	1797
Examiner Name	X. Xu
Attorney Docket Number	15979-20141.00

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

☒ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

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A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Sarah E. Bloch/	Date (YYYY-MM-DD)	2022-10-27
Name/Print	Sarah Bloch	Registration Number	79,763

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2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Patent Application Fee Transmittal

Application Number:	16325653
Filing Date:	14-Feb-2019
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Filer:	Sarah Elizabeth Bloch/Aurelia Caparas
Attorney Docket Number:	15979-20141.00

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 3 months with $0 paid	1253	1	1480	1480
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	1806	1	260	260
		Total in USD ($)		**1740**

Electronic Acknowledgement Receipt

EFS ID:	46904577
Application Number:	16325653
International Application Number:	
Confirmation Number:	1002
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Customer Number:	89300
Filer:	Sarah Elizabeth Bloch/Aurelia Caparas
Filer Authorized By:	Sarah Elizabeth Bloch
Attorney Docket Number:	15979-20141.00
Receipt Date:	27-OCT-2022
Filing Date:	14-FEB-2019
Time Stamp:	13:09:00
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$1740
RAM confirmation Number	E20220QD09272146
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The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		ROA.pdf	96045 96943f8cd7d5812deb910bfa21cf1b227437c530	yes	8
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Amendment/Request for Reconsideration-After Non-Final Rejection		1	1	
	Claims		2	5	
	Applicant Arguments/Remarks Made in an Amendment		6	8	
Warnings:					
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2	Information Disclosure Statement (IDS) Form (SB08)	SIDS_SB08.PDF	1060531 a63c309480e16f5e02a8db199e1bbd6aabe52493	no	4
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A U.S. Patent Number Citation or a U.S. Publication Number Citation is required in the Information Disclosure Statement (IDS) form for autoloading of data into USPTO systems. You may remove the form to add the required data in order to correct the Informational Message if you are citing U.S. References. If you chose not to include U.S. References, the image of the form will be processed and be made available within the Image File Wrapper (IFW) system. However, no data will be extracted from this form. Any additional data such as Foreign Patent Documents or Non Patent Literature will be manually reviewed and keyed into USPTO systems.					
3	Non Patent Literature	4_NPL_1_Snijder_2014_7295-7299.pdf	1598150 d939b58aff160ad06b17ff94084878fa12239bac	no	5
Warnings:					
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4	Non Patent Literature	4_NPL_2_Sub_in_Opp_Proc_EP3497207_02252022_2pgs.pdf	184391 26633891237b661cdfd6b13876bfdb066dc134fe	no	2
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5	Fee Worksheet (SB06)	fee-info.pdf	40911 a17247313e8401ab3c5496818443b54ed58f33ef	no	2

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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/325,653	02/14/2019	Xiaoying JIN	15979-20141.00	1002

89300 7590 11/08/2022

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

EXAMINER
XU, XIAOYUN

ART UNIT	PAPER NUMBER
1797	

NOTIFICATION DATE	DELIVERY MODE
11/08/2022	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

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PatentDocket@mofo.com
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Office Action Summary	Application No. 16/325,653	Applicant(s) JIN et al.	
	Examiner XIAOYUN R XU, Ph. D.	Art Unit 1797	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 10/27/2022.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☑ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1,61,128,181-190 and 202-209 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☑ Claim(s) 1,128,181-190 and 202-209 is/are allowed.

7) ☑ Claim(s) 61 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date 10/27/2022.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

1. The amendment filed on 10/27/2022 has been entered and fully considered. Claim 29, 134, 191-201 and 210-218 are canceled. Claims 1, 61, 128, 181-190 and 202-209 are pending.

Response to Amendment

2. In response to amendment, the examiner maintains rejection to claim 61 over the prior art established in the previous Office action.

Notice of Pre-AIA or AIA Status

3. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Claim Rejections - 35 USC § 103

4. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

5. **Claim 61** is/are rejected under 35 U.S.C. 103 as being unpatentable over Vliet et al. (Journal of Virological Methods, 2009, IDS) (Vliet) in view of Bark et al. (J. Am. Chem. Soc. 2001, IDS) (Bark).

Regarding claim 61, Vliet teaches a method of determining the serotype of an AAV particle comprising the method of claim 1 combined with a method to determine the serotype of an adeno-associated virus (AAV) particle (abstract) comprising

a) denaturing the AAV particle (boiled for 3 min) (page 169, par 4),

b) subjecting the denatured AAV particle to reduction and/or alkylation (page 169, par 5),

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle (page 169, par 5),

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS) (page 169, par 6, page 170, par 2), and

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle (page 169, par 6);

wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype (Table 2, page 171, par 1).

Vliet does not specifically teach wherein the method is performed in the absence of a gel separation step. However, Bark teaches directly subjecting the denatured and digested virus particle (HK97) to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step (Fig. 1, page 1774, par 4). Bark teaches that "The primary techniques for proteomic analysis consist of 2-D gel electrophoresis followed by proteolytic digestion, mass spectrometry, and computer-facilitated data analysis. Masses for proteolytic fragments of the target protein generated by enzymatic degradation can be measured directly with high accuracy (±0.005 Da for a 1000 Da peptide). Alternatively, liquid chromatography tandem mass spectrometry can be used to analyze the protein digest fragments" (page 1774, par 1). Here Bark teaches that alternatively, one can directly subjects the denatured and digested virus particle (HK97) to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step. At time before the filing, it would have been obvious to one of ordinary skill in the art to directly subjecting the denatured and digested AAV particle to liquid chromatography/mass spectrometry (LC/MS) without a gel separation step, in order to save time that is required for gel separation.

While claim 61 comprises limitations of claim 1, claim 61 overwrites claim 1's limitation of "directly subjecting the denatured AA V particle to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis" by adding limitation "subjecting the denatured AAV particle to digestion generate fragments of VP1, VP2 and/or VP3 of the AAV particle". Thus, claim 61 is not allowable.

Allowable Subject Matter

6. **Claim 1, 128, 181-190 and 202-209** are allowed.

7. The following is an examiner's statement of reasons for allowance: neither Vliet nor Bark teaches or fairly suggests directly subjecting a denatured AAV or viral particle

to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis, i.e., without using a protease.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

Response to Arguments

8. Applicant's arguments filed 10/27/2022 have been fully considered but they are not persuasive to claim 61 rejections.

Applicant submits that "claim 61 depends from claim 1, and therefore incorporates all of the limitations of, claim 1, which was not rejected as allegedly obvious over Vliet and Bark. Therefore, claim 61 is not obvious over Vliet and Bark. Applicant respectfully requests withdrawal of claim 61 as allegedly obvious over Vliet and Bark." (remark, page 6, par 5).

This argument is not persuasive. As has been discussed in Allowable Subject Matter above, claim 1 is allowable because neither Vliet nor Bark teaches or fairly suggests "directly subjecting a denatured AAV or viral particle to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis", i.e., without using a protease. While claim 61 comprises limitations of claim 1, claim 61 overwrites claim 1's limitation of "directly subjecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis" by adding a limitation "subjecting the denatured AAV particle to digestion generate fragments of VP1, VP2 and/or VP3 of the AAV particle". Thus, claim 61 is not allowable.

Conclusion

9. **THIS ACTION IS MADE FINAL.** Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not

mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

10. Any inquiry concerning this communication or earlier communications from the examiner should be directed to XIAOYUN R XU, Ph. D. whose telephone number is (571)270-5560. The examiner can normally be reached on M-F 8am-5pm.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Lyle Alexander can be reached on 571-272-1254. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see https://ppair-my.uspto.gov/pair/PrivatePair. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/XIAOYUN R XU, Ph.D./
Primary Examiner, Art Unit 1797

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
	Examiner	Art Unit
	XIAOYUN R XU, Ph. D.	1797

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

CLAIMS

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	06/22/2021	11/12/2021	03/14/2022	11/03/2022					
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	Examiner	**Art Unit**
	XIAOYUN R XU, Ph. D.	1797

CLAIM		DATE								
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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
	Examiner	Art Unit
	XIAOYUN R XU, Ph. D.	1797

CPC - Searched*		
Symbol	Date	Examiner
C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848 (text limited)	06/22/2021	RX

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner
436	173 (text limited)	06/22/2021	RX

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
EAST (CPC C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848 ; USPC 436/173 with key words and inventor names) and Google	06/22/2021	RX
updated search in EAST and Google	11/12/2021	RX
updated search in EAST and Google	03/14/2022	RX
updated search in EAST and Google	11/03/2022	RX

/XIAOYUN R XU, Ph.D./ Primary Examiner, Art Unit 1797	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
	Examiner	Art Unit
	XIAOYUN R XU, Ph. D.	1797

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/XIAOYUN R XU, Ph.D./ Primary Examiner, Art Unit 1797	

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (03-15)
Approved for use through 07/31/2016. OMB 0651-0031
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16325653
Filing Date	2017-08-14
First Named Inventor	XIAOYING JIN
Art Unit	1797
Examiner Name	X. Xu
Attorney Docket Number	15979-20141.00

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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /R.X./

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16325653
Filing Date	2017-08-14
First Named Inventor	XIAOYING JIN
Art Unit	1797
Examiner Name	X. Xu
Attorney Docket Number	15979-20141.00

	1	SNIJDER, J. et al. (May 1, 2014). "Defining the Stoichiometry and Cargo Load of Viral and Bacterial Nanoparticles by Orbitrap Mass Spectrometry," J. Am. Chem. Soc. 136: 7295-7299.
	2	Submission in Opposition Proceedings for European Patent No. 3497207, dated February 25, 2022, Proprietor Genzyme Corporation, Opponent Definition IP Limited, 2 pages.

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature	/XIAOYUN R XU, Ph.D./	Date Considered	11/03/2022

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /R.X./

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16325653
Filing Date	2017-08-14
First Named Inventor	XIAOYING JIN
Art Unit	1797
Examiner Name	X. Xu
Attorney Docket Number	15979-20141.00

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

☒ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Sarah E. Bloch/	Date (YYYY-MM-DD)	2022-10-27
Name/Print	Sarah Bloch	Registration Number	79,763

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /R.X./

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

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5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

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7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /R.X./

PE2E SEARCH - Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	British Equivalents	Time Stamp
L1	20	(US-6989264-$ | US-7588772-$ | US-7629322-$ | US-8137948-$ | US-8283151-$ | US-9169299-$ | US-20110275529-$ | US-20120066783-$ | US-20120164106-$ | US-20130323226-$).DID.	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 12:05 PM
L2	1	(US-6989264-$ | US-7588772-$ | US-7629322-$ | US-8137948-$ | US-8283151-$ | US-9169299-$ | US-20110275529-$ | US-20120066783-$ | US-20120164106-$ | US-20130323226-$).DID. and acetylation	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:19 PM
L3	1	(US-20100129405-$).DID. and acetylation	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:21 PM
L4	1	(US-6989264-$ | US-7588772-$ | US-7629322-$ | US-8137948-$ | US-8283151-$ | US-9169299-$ | US-20110275529-$ | US-20120066783-$ | US-20120164106-$ | US-20130323226-$).DID. and deamidation	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:25 PM
L5	1	16/325653.app.	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:25 PM
L6	13645	adeno-associated virus AND ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:26 PM

L7	5489	adeno-associated virus AND serotype and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:27 PM
L8	836	adeno-associated virus AND serotype and denature$1 and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:27 PM
L9	125	adeno-associated virus AND serotype and denature$1 and heterogeneity and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:28 PM
L10	91	adeno-associated virus AND serotype and denature$1 and heterogeneity and mass$2 and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:28 PM
L11	25	adeno-associated virus AND serotype and denature$1 and	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT;	ADJ	ON	ON	2021/03/08 01:29 PM

		heterogeneity and mass$2 and spectromet$2 and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	IBM_TDB)				
L12	9	adeno-associated virus AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016" and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:29 PM
L13	9	(adeno-associated virus or AAV)AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016" and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:30 PM
L15	3	436/173.ccls. and (adeno-associated virus or AAV) AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016"	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:31 PM
L16	10	(WO-2003042397-$ | WO-2004027019-$ |	(US-PGPUB; USPAT; USOCR; FPRS; EPO;	ADJ	ON	ON	2021/03/08 01:33 PM

		WO-2010148143-$ | WO-2013158879-$).DID.	JPO; DERWENT; IBM_TDB)				
L17	0	(WO-2003042397-$ | WO-2004027019-$ | WO-2010148143-$ | WO-2013158879-$).DID. and deamidation	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:34 PM
L18	0	(WO-2003042397-$ | WO-2004027019-$ | WO-2010148143-$ | WO-2013158879-$).DID. and acetylation	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:34 PM
L19	5	((("JIN") near3 ("Xiaoying"))).INV.	(US-PGPUB; USPAT; USOCR)	ADJ	ON	ON	2021/03/08 01:35 PM
L20	42	((("O'RIORDAN") near3 ("Catherine"))).INV.	(US-PGPUB; USPAT; USOCR)	ADJ	ON	ON	2021/03/08 01:35 PM
L21	1105	((("LIU") near3 ("Lin"))).INV.	(US-PGPUB; USPAT; USOCR)	ADJ	ON	ON	2021/03/08 01:36 PM
L22	5	((("ZHANG") near3 ("Kate"))).INV.	(US-PGPUB; USPAT; USOCR)	ADJ	ON	ON	2021/03/08 01:37 PM
L23	9	(adeno-associated virus or AAV) AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016" and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/06/22 12:01 PM
L24	4	wo-2007046703-$.DID.	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 10:23 AM
L25	4	wo-2010093784-$.DID.	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 10:24 AM
L26	2	wo-2015114365-$.DID.	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 10:24 AM
L27	2	wo-2015137802-$.DID.	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT;	ADJ	ON	ON	2021/11/12 10:25 AM

			IBM_TDB)				
L28	2591	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent)and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 04:09 PM
L29	585	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent) and AAV and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 04:09 PM
L30	585	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent)and AAV and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 04:10 PM
L31	175	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent)and AAV and mass spectrometry and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 04:11 PM

		G01N30/7233 OR G01N33/6848).CPC.)					
L32	172	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent)and AAV and mass spectrometry and digest$3 and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 04:11 PM
L33	129	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent)and AAV and mass spectrometry and digest$3 and @ay< "2016" and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 04:12 PM
L34	99	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent)and AAV and mass spectrometry and digest$3 and @ay< "2016" and trypsin and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 04:17 PM
L35	3	ep-1879912-$.did.	(US-PGPUB; USPAT; USOCR; FPRS; EPO;	ADJ	ON	ON	2021/11/12 04:33 PM

L36	2	wo-2012031245-$.DID.	JPO; DERWENT; IBM_TDB) (US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 04:54 PM

PE2E SEARCH - Search History (Interference)

There are no Interference searches to show.

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 16/325,653	Filing Date 02/14/2019	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $100 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $460 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	02/03/2023	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 20	Minus	** 204	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 2	Minus	*** 4	= 0		x $480 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/DIANE FLOYD/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Docket No.: 15979-20141.00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Xiaoying JIN et al.

Application No.: 16/325,653

Filed: August 14, 2017 (Int'l)

For: METHODS FOR DETECTING AAV

Confirmation No.: 1002

Art Unit: 1797

Examiner: X. Xu

AMENDMENT AFTER FINAL ACTION UNDER 37 C.F.R. 1.116

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

INTRODUCTORY COMMENTS

This is in response to the final Office Action dated November 8, 2022, for which a response is due on February 8, 2023. Accordingly, this response is timely filed. Reconsideration and allowance of the pending claims, as amended, in light of the remarks presented herein are respectfully requested.

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 6 of this paper.

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions, and listings, of claims in the application:

In the claims:

Claim 1 (previously presented): A method to determine the serotype of an adeno-associated virus (AAV) particle comprising

a) denaturing the AAV particle,

b) directly subjecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis, and

c) determining the masses of VP1, VP2 and VP3 of the AAV particle;

wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype,

and wherein the method is performed in the absence of a gel separation step.

Claims 2-127 (canceled)

Claim 128 (previously presented): A method to determine the serotype of a viral particle comprising

a) denaturing the viral particle,

b) directly subjecting the denatured viral particle to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis, and

c) determining the masses of one or more capsid proteins of the viral particle;

wherein the specific combination of masses of the one or more capsid proteins are indicative of the virus serotype,

and wherein the method is performed in the absence of a gel separation step.

Claims 129-180 (canceled)

Claim 181 (previously presented): The method of claim 1, wherein the calculated masses of VP1, VP2 and VP3 are compared to the theoretical masses of VP1, VP2 and VP3 of one or more AAV serotypes.

Claim 182 (previously presented): The method of claim 1, wherein the AAV particle is denatured with acetic acid, guanidine hydrochloride, and/or an organic solvent.

Claim 183 (previously presented): The method of claim 1, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

Claim 184 (previously presented): The method of claim 1, wherein the liquid chromatography is reverse phase chromatography.

Claim 185 (previously presented): The method of claim 184, wherein the reverse phase chromatography is a C4 or C8 reverse chromatography.

Claim 186 (previously presented): The method of claim 1, wherein the liquid chromatography is ultra-performance liquid chromatography (UPLC).

Claim 187 (previously presented): The method of claim 1, wherein the mass spectrometry comprises assisted calibration.

Claim 188 (previously presented): The method of claim 187, wherein sodium iodide is used as a calibrant.

Claim 189 (previously presented): The method of claim 1, wherein the AAV particle is a recombinant AAV (rAAV) particle.

Claim 190 (previously presented): The method of claim 1, wherein the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid.

Claims 191-201 (canceled)

Claim 202 (previously presented): The method of claim 128, wherein the calculated masses of the one or more capsid proteins are compared to the theoretical masses of the one or more capsid proteins of one or more virus serotypes.

Claim 203 (previously presented): The method of claim 128, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

Claim 204 (previously presented): The method of claim 128, wherein the liquid chromatography is reverse phase chromatography.

Claim 205 (previously presented): The method of claim 204, wherein the reverse phase chromatography is a C4 or C8 reverse chromatography.

Claim 206 (previously presented): The method of claim 128, wherein the liquid chromatography is ultra-performance liquid chromatography (UPLC).

Claim 207 (previously presented): The method of claim 128, wherein the mass spectrometry comprises assisted calibration.

Claim 208 (previously presented): The method of claim 207, wherein sodium iodide is used as a calibrant.

Claim 209 (previously presented): The method of claim 128, wherein the viral particle comprises a viral vector encoding a heterologous transgene.

Claims 210-218 (canceled)

REMARKS

Claims 1, 61, 128, 181-190 and 202-209 were pending in the present application. By virtue of this response, claim 61 has been cancelled. Accordingly, following entry of this amendment, claims 1, 128, 181-190 and 202-209 will be under consideration. No new matter has been added. Applicant respectfully requests entry of the claim amendments as the number of claims is not increased and the amendments place the claims in condition for allowance.

With respect to all claim amendments, Applicant has not dedicated or abandoned any unclaimed subject matter and moreover has not acquiesced to any rejections and/or objections made by the patent office. Applicant reserves the right to pursue prosecution of any presently excluded claim embodiments in a future continuation and/or divisional application.

Rejections under 35 U.S.C. § 103

Claim 61 is rejected under 35 U.S.C. 103 as allegedly being unpatentable over Vliet et al. (Journal of Virological Methods, 2009, IDS; hereafter "Vliet") in view of Bark et al. (J. Am. Chem. Soc. 2001, IDS; hereafter "Bark"). Claim 61 incorporates the limitations of claim 1, which the Examiner acknowledges is not obvious over Vliet and Bark. However, the Examiner alleges that claim 61 "overwrites" claim 1's limitation of directly subjecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis by adding the limitation "subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/of VP3 of the AAV particle."

Applicant respectfully disagrees. However, without acquiescing to the Examiner's rejection, and solely to expedite prosecution, claim 61 has been canceled without prejudice, thus rendering the rejection moot.

Allowed Claims

Applicant thanks the Examiner for acknowledging that claims 1, 128, 181-190, and 202-209 are patentable.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark Office determines that an extension and/or other relief is required, Applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. **03-1952** referencing docket no. **15979-20141.00**. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: February 3, 2023

Respectfully submitted,

Electronic signature: /Seth Snyder/
Seth Snyder
Registration No.: 60,243
MORRISON & FOERSTER LLP
755 Page Mill Road
Palo Alto, CA 94304-1018
+1 (650) 813-4046

Electronic Acknowledgement Receipt

EFS ID:	47478271
Application Number:	16325653
International Application Number:	
Confirmation Number:	1002
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Customer Number:	89300
Filer:	Seth Elliot Snyder/Aurelia Caparas
Filer Authorized By:	Seth Elliot Snyder
Attorney Docket Number:	15979-20141.00
Receipt Date:	03-FEB-2023
Filing Date:	14-FEB-2019
Time Stamp:	18:51:09
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		Resp_to_FOA.pdf	95122 42afeda29bc510214eaf99764b3d666c0f1c95ae	yes	7

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Response After Final Action	1	1
	Claims	2	5
	Applicant Arguments/Remarks Made in an Amendment	6	7
Warnings:			
Information:			
	Total Files Size (in bytes):	95122	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

89300 7590 02/21/2023

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

EXAMINER
XU, XIAOYUN

ART UNIT	PAPER NUMBER
1797	

DATE MAILED: 02/21/2023

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/325,653	02/14/2019	Xiaoying JIN	15979-20141.00	1002

TITLE OF INVENTION: METHODS FOR DETECTING AAV

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1200	$0.00	$0.00	$1200	05/22/2023

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 40% the amount of undiscounted fees, and micro entity fees are 20% the amount of undiscounted fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

89300 7590 02/21/2023

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/325,653	02/14/2019	Xiaoying JIN	15979-20141.00	1002

TITLE OF INVENTION: METHODS FOR DETECTING AAV

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1200	$0.00	$0.00	$1200	05/22/2023

EXAMINER	ART UNIT	CLASS-SUBCLASS
XU, XIAOYUN	1797	436-173000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/AIA/122 or PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/AIA/47 or PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. Fees submitted: ❑ Issue Fee ❑ Publication Fee (if required) ❑ Advance Order - # of Copies _____

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

❑ Electronic Payment via EFS-Web ❑ Enclosed check ❑ Non-electronic payment by credit card (Attach form PTO-2038)

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

❑ Applicant asserting small entity status. See 37 CFR 1.27

❑ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/325,653	02/14/2019	Xiaoying JIN	15979-20141.00	1002

89300 7590 02/21/2023

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

EXAMINER	
XU, XIAOYUN	
ART UNIT	PAPER NUMBER
1797	

DATE MAILED: 02/21/2023

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 16/325,653	Applicant(s) JIN et al.	
	Examiner XIAOYUN R XU, Ph. D.	Art Unit 1797	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to 02/03/2023.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☑ The allowed claim(s) is/are See Continuation Sheet . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☐ All b) ☐ Some* c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☐ Examiner's Amendment/Comment
6. ☑ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/XIAOYUN R XU, Ph.D./
Primary Examiner, Art Unit 1797

Continuation of 3. The allowed claim(s) is/are: 1,128,181-190 and 202-209

DETAILED ACTION

Notice of Pre-AIA or AIA Status

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Allowable Subject Matter

2. **Claim 1,128181-190 and 202-209** are allowed.

3. The following is an examiner's statement of reasons for allowance: Neither Vliet nor Bark teaches or fairly suggests directly subjecting a denatured AAV or particle to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis, i.e. without using a protease.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

4. Any inquiry concerning this communication or earlier communications from the examiner should be directed to XIAOYUN R XU, Ph. D. whose telephone number is (571)270-5560. The examiner can normally be reached M-F 8am-5pm.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Lyle Alexander can be reached on 571-272-1254. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of published or unpublished applications may be obtained from Patent Center. Unpublished application information in Patent Center is available to registered users. To file and manage patent submissions in Patent Center, visit: https://patentcenter.uspto.gov. Visit https://www.uspto.gov/patents/apply/patent-

center for more information about Patent Center and https://www.uspto.gov/patents/docx for information about filing in DOCX format. For additional questions, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/XIAOYUN R XU, Ph.D./
Primary Examiner, Art Unit 1797

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
	Examiner	Art Unit
	XIAOYUN R XU, Ph. D.	1797

CPC - Searched*		
Symbol	Date	Examiner
C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848 (text limited)	06/22/2021	RX

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner
436	173 (text limited)	06/22/2021	RX

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
EAST (CPC C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848 ; USPC 436/173 with key words and inventor names) and Google	06/22/2021	RX
updated search in EAST and Google	11/12/2021	RX
updated search in EAST and Google	03/14/2022	RX
updated search in EAST and Google	11/03/2022	RX

/XIAOYUN R XU, Ph.D./ Primary Examiner, Art Unit 1797	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
	Examiner	Art Unit
	XIAOYUN R XU, Ph. D.	1797

Interference Search			
US Class/CPC Symbol	**US Subclass/CPC Group**	**Date**	**Examiner**
C07K, C12N, G01N	C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848	02/08/2023	RX
436	173	02/08/2023	RX

/XIAOYUN R XU, Ph.D./ Primary Examiner, Art Unit 1797	

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
	Examiner	**Art Unit**
	XIAOYUN R XU, Ph. D.	1797

CPC						
Symbol					**Type**	**Version**
G01N	/	33	/	6848	F	2013-01-01
C12N	/	15	/	86	I	2013-01-01
G01N	/	30	/	72	I	2013-01-01
C12N	/	2750	/	14123	A	2013-01-01
C12N	/	2750	/	14143	A	2013-01-01
G01N	/	2030	/	027	A	2013-01-01

CPC Combination Sets							
Symbol					**Type**	**Set**	**Ranking**	**Version**
	/		/					

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	20	
/XIAOYUN R XU, Ph.D./ Primary Examiner, Art Unit 1797	08 February 2023	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	1

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
	Examiner	**Art Unit**
	XIAOYUN R XU, Ph. D.	1797

INTERNATIONAL CLASSIFICATION				
CLAIMED				
G01N	/	33	/	68

NON-CLAIMED				
	/		/	

US ORIGINAL CLASSIFICATION	
CLASS	**SUBCLASS**
436	173

CROSS REFERENCES(S)						
CLASS	**SUBCLASS (ONE SUBCLASS PER BLOCK)**					
436	173					

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	20	
/XIAOYUN R XU, Ph.D./ Primary Examiner, Art Unit 1797	08 February 2023	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	1

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
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	XIAOYUN R XU, Ph. D.	1797

☑ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIMS

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	20	
/XIAOYUN R XU, Ph.D./ Primary Examiner, Art Unit 1797	08 February 2023	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	1

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
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✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

CLAIMS

☑ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	06/22/2021	11/12/2021	03/14/2022	11/03/2022	02/08/2023				
1	1	✓	✓	=	=	=				
	2	-	-	-	-	-				
	3	N	N	-	-	-				
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	28	-	-	-	-	-				
	29	✓	✓	✓	-	-				
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	39	-	-	-	-	-				
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	42	-	-	-	-	-				

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	16/325,653	JIN et al.
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CLAIM		DATE								
Final	Original	06/22/2021	11/12/2021	03/14/2022	11/03/2022	02/08/2023				
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Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
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CLAIM		DATE								
Final	Original	06/22/2021	11/12/2021	03/14/2022	11/03/2022	02/08/2023				
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CLAIM		DATE								
Final	Original	06/22/2021	11/12/2021	03/14/2022	11/03/2022	02/08/2023				
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	192		✓	✓	-	-				
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	195		✓	=	-	-				
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	197		✓	✓	-	-				
	198		✓	=	-	-				

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
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	Examiner	**Art Unit**
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CLAIM		DATE								
Final	Original	06/22/2021	11/12/2021	03/14/2022	11/03/2022	02/08/2023				
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15	204		✓	=	=	=				
16	205		✓	=	=	=				
17	206		✓	=	=	=				
18	207		✓	=	=	=				
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	216		✓	✓	-	-				
	217		✓	✓	-	-				
	218		✓	✓	-	-				

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BIB DATA SHEET

CONFIRMATION NO. 1002

SERIAL NUMBER	FILING or 371(c) DATE RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
16/325,653	02/14/2019	436	1797	15979-20141.00

APPLICANTS

Genzyme Corporation, Cambridge, MA;

INVENTORS

Xiaoying JIN, Bridgewater, NJ;
Catherine O'RIORDAN, Bridgewater, NJ;
Lin LIU, Bridgewater, NJ;
Kate ZHANG, Bridgewater, NJ;

**** CONTINUING DATA ***************************

This application is a 371 of PCT/US2017/046814 08/14/2017
which claims benefit of 62/375,314 08/15/2016

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****

04/20/2019

Foreign Priority claimed	35 USC 119(a-d) conditions met	Verified and Acknowledged	Met after Allowance	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
☐ Yes ☑ No	☐ Yes ☐ No	/ROBERT XU/ Examiner's Signature	☐ Initials	NJ	18	22	14

ADDRESS

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304
UNITED STATES

TITLE

METHODS FOR DETECTING AAV

FILING FEE RECEIVED		
33360	FEES: Authority has been given in Paper No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit

OK TO ENTER: /R.X./

Docket No.: 15979-20141.00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Xiaoying JIN et al.

Application No.: 16/325,653

Filed: August 14, 2017 (Int'l)

For: METHODS FOR DETECTING AAV

Confirmation No.: 1002

Art Unit: 1797

Examiner: X. Xu

AMENDMENT AFTER FINAL ACTION UNDER 37 C.F.R. 1.116

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

INTRODUCTORY COMMENTS

This is in response to the final Office Action dated November 8, 2022, for which a response is due on February 8, 2023. Accordingly, this response is timely filed. Reconsideration and allowance of the pending claims, as amended, in light of the remarks presented herein are respectfully requested.

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 6 of this paper.

PE2E SEARCH - Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	British Equivalents	Time Stamp
L1	20	(US-6989264-$ | US-7588772-$ | US-7629322-$ | US-8137948-$ | US-8283151-$ | US-9169299-$ | US-20110275529-$ | US-20120066783-$ | US-20120164106-$ | US-20130323226-$).DID.	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 12:05 PM
L2	1	(US-6989264-$ | US-7588772-$ | US-7629322-$ | US-8137948-$ | US-8283151-$ | US-9169299-$ | US-20110275529-$ | US-20120066783-$ | US-20120164106-$ | US-20130323226-$).DID. and acetylation	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:19 PM
L3	1	(US-20100129405-$).DID. and acetylation	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:21 PM
L4	1	(US-6989264-$ | US-7588772-$ | US-7629322-$ | US-8137948-$ | US-8283151-$ | US-9169299-$ | US-20110275529-$ | US-20120066783-$ | US-20120164106-$ | US-20130323226-$).DID. and deamidation	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:25 PM
L5	1	16/325653.app.	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:25 PM
L6	13645	adeno-associated virus AND ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:26 PM

L7	5489	adeno-associated virus AND serotype and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:27 PM
L8	836	adeno-associated virus AND serotype and denature$1 and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:27 PM
L9	125	adeno-associated virus AND serotype and denature$1 and heterogeneity and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:28 PM
L10	91	adeno-associated virus AND serotype and denature$1 and heterogeneity and mass$2 and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:28 PM
L11	25	adeno-associated virus AND serotype and denature$1 and	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT;	ADJ	ON	ON	2021/03/08 01:29 PM

		heterogeneity and mass$2 and spectromet$2 and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	IBM_TDB)				
L12	9	adeno-associated virus AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016" and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:29 PM
L13	9	(adeno-associated virus or AAV)AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016" and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:30 PM
L15	3	436/173.ccls. and (adeno-associated virus or AAV) AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016"	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:31 PM
L16	10	(WO-2003042397-$ | WO-2004027019-$ |	(US-PGPUB; USPAT; USOCR; FPRS; EPO;	ADJ	ON	ON	2021/03/08 01:33 PM

		WO-2010148143-$ | WO-2013158879-$).DID.	JPO; DERWENT; IBM_TDB)				
L17	0	(WO-2003042397-$ | WO-2004027019-$ | WO-2010148143-$ | WO-2013158879-$).DID. and deamidation	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:34 PM
L18	0	(WO-2003042397-$ | WO-2004027019-$ | WO-2010148143-$ | WO-2013158879-$).DID. and acetylation	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/03/08 01:34 PM
L19	5	((("JIN") near3 ("Xiaoying"))).INV.	(US-PGPUB; USPAT; USOCR)	ADJ	ON	ON	2021/03/08 01:35 PM
L20	42	((("O'RIORDAN") near3 ("Catherine"))).INV.	(US-PGPUB; USPAT; USOCR)	ADJ	ON	ON	2021/03/08 01:35 PM
L21	1105	((("LIU") near3 ("Lin"))).INV.	(US-PGPUB; USPAT; USOCR)	ADJ	ON	ON	2021/03/08 01:36 PM
L22	5	((("ZHANG") near3 ("Kate"))).INV.	(US-PGPUB; USPAT; USOCR)	ADJ	ON	ON	2021/03/08 01:37 PM
L23	9	(adeno-associated virus or AAV) AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016" and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/06/22 12:01 PM
L24	4	wo-2007046703-$.DID.	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 10:23 AM
L25	4	wo-2010093784-$.DID.	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 10:24 AM
L26	2	wo-2015114365-$.DID.	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 10:24 AM
L27	2	wo-2015137802-$.DID.	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT;	ADJ	ON	ON	2021/11/12 10:25 AM

			IBM_TDB)				
L28	2591	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent)and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 04:09 PM
L29	585	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent) and AAV and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 04:09 PM
L30	585	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent)and AAV and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 04:10 PM
L31	175	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent)and AAV and mass spectrometry and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 04:11 PM

		G01N30/7233 OR G01N33/6848).CPC.)					
L32	172	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent)and AAV and mass spectrometry and digest$3 and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 04:11 PM
L33	129	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent)and AAV and mass spectrometry and digest$3 and @ay< "2016" and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 04:12 PM
L34	99	virus AND denatur$3 and (acetic acid or guanidine hydrochloride or organic solvent)and AAV and mass spectrometry and digest$3 and @ay< "2016" and trypsin and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 04:17 PM
L35	3	ep-1879912-$.did.	(US-PGPUB; USPAT; USOCR; FPRS; EPO;	ADJ	ON	ON	2021/11/12 04:33 PM

			JPO; DERWENT; IBM_TDB)				
L36	2	wo-2012031245-$.DID.	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2021/11/12 04:54 PM

PE2E SEARCH - Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	British Equivalents	Time Stamp
N1	100	virus AND denatur$3 AND (acetic acid OR guanidine hydrochloride OR organic solvent) AND AAV AND mass spectrometry AND digest$3 AND @ay< "2016" AND trypsin AND ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	(US-PGPUB; USPAT)	ADJ	ON	ON	2023/02/08 04:11 PM
N2	3	436/173.ccls. AND (adeno-associated virus OR AAV) AND serotype AND denature$1 AND heterogeneity AND mass$2 AND spectromet$2 AND @ay< "2016"	(US-PGPUB; USPAT)	ADJ	ON	ON	2023/02/08 04:11 PM

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Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

Courtesy Reminder for
Application Serial No: 16/325,653

Attorney Docket No: 15979-20141.00
Customer Number: 89300
Date of Electronic Notification: 02/22/2023

This is a courtesy reminder that new correspondence is available for this application. If you have not done so already, please review the correspondence. The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

An email notification regarding the correspondence was sent to the following email address(es) associated with your customer number:

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PatentDocket@mofo.com
pair_mofo@firsttofile.com

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If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov or call 1-866-217-9197.

Document Description: Issue Fee Payment (PTO-85B)

Issue Fee Transmittal Form

Application Number	Filing Date	First Named Inventor	Atty. Docket No.	Confirmation No.
16325653	14-Feb-2019	Xiaoying JIN	15979-20141.00	1002

TITLE OF INVENTION :

METHODS FOR DETECTING AAV

Entity Status	Application Type	Art Unit	Class - Subclass	EXAMINER
Regular Undiscounted	U.S. National Stage under 35 USC 371	1797	173000	XIAOYUN XU

Issue Fee Due	Publication Due	Total Fee(s) Due	Date Due	Prev. Paid Fee
$1200	$0	$1200	22-May-2023	$0

1.Change of Correspondence Address and/or Indication Of Fee Address (37 CFR 1.33 & 1.363)

Current Correspondence Address:	Current Indicated Fee Address :
89300 Sanofi/Genzyme c/o Morrison & Foerster LLP 755 Page Mill Road Palo Alto CA 94304 UNITED STATES 650-813-5600 EOfficePA@mofo.com	
☐ Change of correspondence address requested, system generated AIA/122-EFS form attached	☐ Fee Address indication requested, system generated SB/47-EFS form attached

2.Entity Status

Change in Entity Status

○ Applicant certifying micro entity status; system generated Micro Entity certification form attached. See 37 CFR 1.29.
Note: Absent a valid certification of micro entity status, issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment. If this box is checked, you will be prompted to choose a micro entity status on the gross income basis (37 CFR 1.29(a)) or the institution of higher education basis (37 CFR 1.29(d)), and make the applicable certification online.

○ Applicant asserting small entity status. See 37 CFR 1.27.
Note: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

◉ Applicant changing to regular undiscounted fee status.
Note: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

Document Description: Issue Fee Payment (PTO-85B)

3.The Following Fee(s) Are Submitted:

☒ Issue Fee

☐ I authorize USPTO to apply my previously paid issue fee to the current fees due

☐ Publication Fee

☐ The Director is hereby authorized to apply my previously paid issue fee to the current fee due and to charge deficient fees to Deposit Account Number _____

☒ If **in addition to** the payment of the issue fee amount submitted with this form, there are any discrepancies in any amount(s) due, the Director is authorized to charge any deficiency or credit any overpayment, to Deposit Account Number 03-1952 . The **issue fee must be submitted** with this form. **If payment of the issue fee does not accompany this form, checking this box and providing a deposit account number will NOT be effective to satisfy full payment of the fee(s) due.**

4.Firm and/or Attorney Names To Be Printed

NOTE: If no name is listed, no name will be printed
For printing on the patent front page, list to be displayed as entered

1. MORRISON & FOERSTER LLP
2.
3.

5.Assignee Name(s) and Residence Data To Be Printed

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

Name	City	State	Country	Category
Genzyme Corporation	Cambridge	MASSACHUSETTS	UNITED STATES	corporation

6.Signature

I certify, in accordance with 37 CFR 1.4(d)(4) that I am an attorney or agent registered to practice before the Patent and Trademark Office who has filed and has been granted power of attorney in this application. I also certify that this Fee(s) Transmittal form is being transmitted to the USPTO via EFS-WEB on the date indicated below.

Signature	/Sarah E. Bloch/	**Date**	05-22-2023
Name	Sarah Elizabeth Bloch	**Registration Number**	79763

Electronic Patent Application Fee Transmittal

Application Number:	16325653
Filing Date:	14-Feb-2019
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Filer:	Sarah Elizabeth Bloch/Aurelia Caparas
Attorney Docket Number:	15979-20141.00

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
UTILITY APPL ISSUE FEE	1501	1	1200	1200
PUBL. FEE- EARLY, VOLUNTARY, OR NORMAL	1504	1	0	0
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**1200**

Electronic Acknowledgement Receipt

EFS ID:	48035699
Application Number:	16325653
International Application Number:	
Confirmation Number:	1002
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Customer Number:	89300
Filer:	Sarah Elizabeth Bloch/Aurelia Caparas
Filer Authorized By:	Sarah Elizabeth Bloch
Attorney Docket Number:	15979-20141.00
Receipt Date:	22-MAY-2023
Filing Date:	14-FEB-2019
Time Stamp:	15:16:53
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$1200
RAM confirmation Number	E20235LF16526784
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Issue Fee Payment (PTO-85B)	Web85b.pdf	60957 d9f546c5bf95df039b135e13891441fb4328262b	no	2
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	40447 53b863327048284467c74feda7818f6cfa512e5f	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	101404		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Field

PTO/SB/08a (03-15)
Approved for use through 07/31/2016. OMB 0651-0031
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Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT
(Not for submission under 37 CFR 1.99)

Application Number	16/325,653
Filing Date	August 14, 2017 (Int'l)
First Named Inventor	Xiaoying JIN
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	15979-20141.00

U.S. PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1.	US-6,989,264-B2		01-24-2006	Atkinson et al.	
	2.	US-7,588,772-B2		09-15-2009	Kay et al.	
	3.	US-7,629,322-B2		12-08-2009	Kleinschmidt et al.	
	4.	US-8,137,948-B2		03-20-2012	Qu et al.	
	5.	US-8,283,151-B2		10-09-2012	Schmidt et al.	
	6.	US-9,169,299-B2		10-27-2015	Lisowski et al.	

U.S. PATENT APPLICATION PUBLICATIONS

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	7.	US-2011/0275529-A1		11-10-2011	Heilbronn, R.	
	8.	US-2012/0164106-A1		03-15-2012	Kay et al.	
Change(s) applied to document, /J.E./ 2/27/2023	9.	US-2013/0323226-A1		~~08-28-2012~~	~~Schaffer et al.~~ Wilson et al.	12/2013
	10.	US-2012/0066783-A1		~~12-05-2013~~	~~Wilson et al.~~ Kay et al.	03/2012

FOREIGN PATENT DOCUMENTS

Examiner Initial*	Cite No	Country Code[2]	Foreign Document Number[3]	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear	T[5]
	11.	WO	2003/042397	A2	05-22-2003	The Trustees of the University of Pennsylvania		
	12.	WO	2004/027019	A2	04-01-2004	University of Florida Research Foundation		
	13.	WO	2010/148143	A1	12-23-2010	Genzyme Corporation		
	14.	WO	2013/158879	A1	10-24-2013	The Children's Hospital of Philadelphia		

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	15.	AHMADIANKIA, A. et al. (July 1, 2013). "Generation of Helper Plasmids Encoding Mutant Adeno-Associated Virus Type 2 Capsid Proteins with Increased Resistance against Proteasomal Degradation", *Iran J Basic Med Sci* 16:813-821.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /R.X./

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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/325,653	07/11/2023	11698377	15979-20141.00	1002

89300 7590 06/21/2023

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

ISSUE NOTIFICATION

The projected patent number and issue date are specified above. The patent will issue electronically. The electronically issued patent is the official patent grant pursuant to 35 U.S.C. § 153. The patent may be accessed on or after the issue date through Patent Center at https://patentcenter.uspto.gov/. The patent will be available in both the public and the private sides of Patent Center. Further assistance in electronically accessing the patent, or about Patent Center, is available by calling the Patent Electronic Business Center at 1-888-217-9197.

The USPTO is implementing electronic patent issuance with a transition period, during which period the USPTO will mail a ceremonial paper copy of the electronic patent grant to the correspondence address of record. Additional copies of the patent (i.e., certified and presentation copies) may be ordered for a fee from the USPTO's Certified Copy Center at https://certifiedcopycenter.uspto.gov/index.html. The Certified Copy Center may be reached at (800)972-6382.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 359 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Patents Stakeholder Experience (OPSE), Stakeholder Support Division (SSD) at (571)-272-4200.

INVENTOR(s) (Please see PAIR WEB site http://pair.uspto.gov for additional inventors):

Xiaoying JIN, Bridgewater, NJ;
Catherine O'RIORDAN, Bridgewater, NJ;
Lin LIU, Bridgewater, NJ;
Kate ZHANG, Bridgewater, NJ;

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Genzyme Corporation, Cambridge, MA;

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/325,653	02/14/2019	Xiaoying JIN	15979-20141.00	1002

89300 7590 07/11/2023

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

EXAMINER
XU, XIAOYUN

ART UNIT	PAPER NUMBER
1797	

NOTIFICATION DATE	DELIVERY MODE
07/11/2023	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

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APPLICATION NO.	ISSUE DATE	PATENT NO.
16/325,653	11-Jul-2023	11698377

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

EGRANT NOTIFICATION

Your electronic patent grant (eGrant) is now available, which can be accessed via Patent Center at https://patentcenter.uspto.gov

The electronic patent grant is the official patent grant under 35 U.S.C. 153. For more information, please visit https://www.uspto.gov/electronicgrants

PTO/AIA/123 (08-12)
Approved for use through 03/31/2021. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Patent

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Alexandria, VA 22313-1450

Patent Number	11,698,377
Issue Date	July 11, 2023
Application Number	16/325,653
Filing Date	August 14, 2017 (Int'l)
First Named Inventor	Xiaoying JIN
Attorney Docket Number	15979-20141.00

Please change the Correspondence Address for the above-identified patent to:

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I am the:

☐ Patentee.

☐ If the Patentee was not the applicant for patent (37 CFR 1.42), then a Statement under 37 CFR 3.73(c) (Form PTO/AIA/96 or equivalent) is enclosed or was filed on _____. See 37 CFR 3.71.

☑ Attorney or agent of record. Registration Number 60243.

☐ Patent practitioner acting in a representative capacity whose correspondence address is the correspondence address of record. Notice has been given to the patentee or owner. Registration Number _____.

Signature /Seth Snyder/

Typed or Printed Name Seth Snyder

Date September 18, 2023 | Telephone +1 (650) 813-4046

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☐ *Total of _____ forms are submitted.

This collection of information is required by 37 CFR 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop Post Issue, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Electronic Acknowledgement Receipt

EFS ID:	48600876
Application Number:	16325653
International Application Number:	
Confirmation Number:	1002
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Customer Number:	89300
Filer:	Seth Elliot Snyder/Aurelia Caparas
Filer Authorized By:	Seth Elliot Snyder
Attorney Docket Number:	15979-20141.00
Receipt Date:	18-SEP-2023
Filing Date:	14-FEB-2019
Time Stamp:	14:46:37
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Change of Address	Change_of_Corr_Address.pdf	168238 34a45a509b3f17d5bf33772e3bce2f771370219c	no	1

Warnings:

Information:	
Total Files Size (in bytes):	168238

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.