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(54) **FURTHER IMPROVED AAV VECTORS PRODUCED IN INSECT CELLS**

WEITER VERBESSERTE, IN INSEKTENZELLEN HERGESTELLTE AAV-VEKTOREN

VECTEURS DE VIRUS ADÉNO ASSOCIÉS ENCORE AMÉLIORÉS PRODUITS DANS DES CELLULES D'INSECTES

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(56) References cited:
**WO-A2-03/042361 WO-A2-2007/046703**

- **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**
- **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**

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## Description

### Field of the invention

**[0001]** 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

**[0002]** 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.

**[0003]** 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.

**[0004]** 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.

**[0005]** 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.

**[0006]** 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.

**[0007]** 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*).

**[0008]** 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.

**[0009]** 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.

**[0010]** 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

**[0011]** The invention is defined by the appended claims.

**[0012]** The first aspect of the invention relates to a nucleic acid molecule having a nucleotide sequence comprising a modified open reading frame encoding adeno-associated virus (AAV) capsid proteins, wherein the reading frame in 5' to 5 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 after the first codon encoding an amino acid residue selected from the group consisting of alanine, glycine, valine, aspartic acid and glutamic acid.
(iii) a sequence encoding adeno-associated virus (AAV) serotype 5 capsid proteins, whereby the sequence lacks only the ATG VP1 translation initiation codon, and whereby the sequence comprises the remainder of an open reading frame encoding AAV5 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. In a preferred embodiment, the AAV capsid proteins comprise the amino acid sequence of: SEQ ID NO: 22.

**[0013]** Alternatively or in combination with any previous embodiments, in a further preferred embodiment the second codon encodes alanine.

**[0014]** 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.

**[0015]** 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.

**[0016]** 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.

**[0017]** 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.

**[0018]** In a third aspect, the present invention relates to an insect cell comprising a nucleic acid construct according to the invention.

**[0019]** 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.

**[0020]** 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.

**[0021]** 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.

**[0022]** 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.

**[0023]** 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.

**[0024]** In a fourth aspect, the present invention relates to an AAV5 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.

**[0025]** Preferably, an AAV5 virion according to the invention comprises a gene product of interest that encodes a Factor IX or a Factor VIII protein.

**[0026]** In a fifth aspect, the present invention relates to a method for producing an AAV5 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

**[0027]** 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.

**[0028]** "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.

**[0029]** 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.

**[0030]** 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.

**[0031]** 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 × 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 × 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.

**[0032]** 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 × 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 × 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

**[0033]** The present description 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.

**[0034]** 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.

**[0035]** 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.

**[0036]** 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 5 (AAV5) capsid proteins. Preferably, the reading frame encoding the capsid proteins is modified, compared to a wild type open reading frame encoding AAV5 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.

**[0037]** 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.

**[0038]** 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.

**[0039]** 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.

**[0040]** 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

**[0041]** 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).

**[0042]** 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.

**[0043]** 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.

**[0044]** 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.

**[0045]** 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.

**[0046]** The sequence encoding AAV5 capsid proteins in step (iv) can be a capsid sequence as found in nature.

**[0047]** A preferred capsid protein is AAV5, preferably as provided in SEQ ID NO: 22.

**[0048]** 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.

**[0049]** 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.

**[0050]** 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.

**[0051]** A further preferred nucleotide sequence of the description 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.

**[0052]** 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.

**[0053]** 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.

**[0054]** 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.

**[0055]** 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.

**[0056]** 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.

**[0057]** 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.

**[0058]** 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.

**[0059]** 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.

**[0060]** 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.

**[0061]** 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.

**[0062]** 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.

**[0063]** 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.

**[0064]** 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.

**[0065]** 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).

**[0066]** 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.

**[0067]** 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 $\alpha$1-antitrypsin 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).

**[0068]** 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).

**[0069]** 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).

**[0070]** 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.

**[0071]** 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.

**[0072]** 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.

**[0073]** 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.

**[0074]** 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.

**[0075]** 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.

**[0076]** In a further aspect the invention relates to an AAV5 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.

**[0077]** 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.

**[0078]** 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.

**[0079]** 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).

**[0080]** 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.

**[0081]** 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.

**[0082]** 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.

**[0083]** In another aspect the invention thus relates to a method for producing an AAV5 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.

**[0084]** 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.

**[0085]** 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

**[0086]**

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

**[0087]** 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.

**[0088]** 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.

**[0089]** 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 stoi-

chiometry, and similar or superior potency as compared to the chimeric AAV2/5.

## 2. Methods

### *2.1. Generation of rAAV5 vectors*

**[0090]** 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 10× 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*

**[0091]** 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.

**[0092]** 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 | 50 |

*2.3. VP protein composition of rAAV5 variants*

**[0093]** 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*

**[0094]** To investigate *in vitro* potency of the different serotype 5 capsid variants, two continuous cell lines were used. Here, $1\times10^5$ 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*

**[0095]** 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 $5\times10^{12}$ 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.

**[0096]** 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 $2\times10^{12}$ gc/kg and $2\times10^{13}$ 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*

**[0097]** 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.

**[0098]** 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.

**[0099]** 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.....<br>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.....<br>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.....<br>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.....<br>**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.....<br>**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.....<br>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.....<br>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.....<br>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.....<br>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.....<br>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.....<br>S F V D H P P D W | 50 |

(continued)

| 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.....<br>S F V D H P P D W | 39 |
| **Changes surrounding the VP1 translation initiation start** | | | | | |
| 765 | - | CTG | **GCT<br>A** | TCT TTT GTT GAT CAC CC**A** CC**C** GAT TGG T.....<br>S F V D H P P D W | 51 |
| 766 | - | GTG | **GCT<br>A** | TCT TTT GTT GAT CAC CC**A** CC**C** GAT TGG T.....<br>S F V D H P P D W | 52 |
| 43 | CCTGTTAAG | CTG | **GCT<br>A** | TCT TTT GTT GAT CAC CC**A** CC**C** GAT TGG T.....<br>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)<br>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*

**[0100]** 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.

**[0101]** 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 VP 3 is responsible for a low potency of true type 5 AAV mutants in BEVS.*

**[0102]** 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).

**[0103]** 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 5 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 AA15 (765) performs superior to the chimeric type 2/5 mutant in vivo*

**[0104]** 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

**[0105]** 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*).

**[0106]** 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).

**[0107]** 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.*

**[0108]** 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.

**[0109]** 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 a modified open reading frame encoding adeno-associated virus (AAV) capsid proteins, 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 after the first codon encoding an amino acid residue selected from the group consisting of alanine, glycine, valine, aspartic acid and glutamic acid.
   (iii) a sequence encoding adeno-associated virus (AAV) serotype 5 capsid proteins, whereby the sequence lacks only the ATG VP1 translation initiation codon, and whereby the sequence comprises the remainder of an open reading frame encoding AAV5 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.

2. A nucleic acid molecule according to claim 1, wherein the AAV capsid proteins comprise the amino acid sequence of: SEQ ID NO: 22.

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 5 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 selected from the group consisting of CTG, ACG, TTG and GTG;
(ii) 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;

wherein a second amino acid residue is inserted after the first amino acid residue encoding an amino acid residue selected from the group consisting of alanine, glycine, valine, aspartic acid and glutamic acid, and, optionally, one or more additional amino acid residues following the second amino acid residue.

**15.** A method for producing an AAV5 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 AAV5 virion according to claim 14, wherein the gene product of interest encodes a Factor IX or a Factor VIII protein.

## Patentansprüche

1. Nukleinsäuremolekül mit einer Nukleotidsequenz, die einen modifizierten offenen Leserahmen umfasst, der für Adeno-assoziierte Virus (AAV)-Kapsidproteine codiert, wobei der Leserahmen in der Reihenfolge 5' zu 3' Folgendes umfasst:

   (i) ein erstes Codon, das ein suboptimales Translationsinitiationscodon ist, ausgewählt aus der Gruppe bestehend aus CTG, ACG, TTG und GTG;
   (ii) ein zweites Codon nach dem ersten Codon, das für einen Aminosäurerest codiert, der aus der Gruppe ausgewählt ist, die aus Alanin, Glycin, Valin, Asparaginsäure und Glutaminsäure besteht.
   (iii) eine Sequenz, die für Adeno-assoziierte Virus (AAV)-Serotyp-5-Kapsidproteine codiert, wobei der Sequenz nur das ATG-VP1-Translationsinitiationscodon fehlt und wobei die Sequenz den Rest eines offenen Leserahmens umfasst, der für AAV5-Kapsidproteine codiert, wobei der Rest an der Position beginnt, die der zweiten Aminosäureposition in einem offenen Leserahmen vom Wildtyp entspricht, der für die Kapsidproteine codiert.

2. Nukleinsäuremolekül nach Anspruch 1, wobei die AAV-Kapsidproteine die Aminosäuresequenz von: SEQ ID NO: 22 umfassen.

3. Nukleinsäuremolekül nach Anspruch 1 oder Anspruch 2, wobei das zweite Codon für Alanin codiert.

4. Nukleinsäuremolekül nach Anspruch 1 oder Anspruch 2, wobei das zweite Codon aus der Gruppe ausgewählt ist, die aus GCT, GCC, GCA, GCG und GGU besteht, wobei das Codon vorzugsweise GCT ist.

5. Nukleinsäurekonstrukt, umfassend ein Nukleinsäuremolekül nach einem der Ansprüche 1-4, wobei die Nukleotidsequenz des Leserahmens, der für die Adenoassoziierten Virus (AAV)-Kapsidproteine codiert, funktionsfähig mit Expressionskontrollsequenzen zur Expression in einer Insektenzelle verbunden ist.

6. Nukleinsäurekonstrukt nach Anspruch 5, wobei die Nukleotidsequenz des Leserahmens funktionsfähig mit einem Promotor verknüpft ist, der ausgewählt ist aus der Gruppe bestehend aus: Polyeder-Promotor, p10-Promotor, 4xHsp27 EcRE+minimal Hsp70-Promotor, deltaE1-Promotor, E1-Promotor.

7. Nukleinsäurekonstrukt nach Anspruch 5 oder Anspruch 6, wobei das Konstrukt ein insektenkompatibler Vektor ist, vorzugsweise ein baculoviraler Vektor.

8. Nukleinsäurekonstrukt nach einem der Ansprüche 5-7, wobei das Nukleinsäuremolekül einen offenen Leserahmen umfasst, der ausgewählt ist aus der Gruppe bestehend aus: SEQ ID NO: 51, 69, 42, 47, 48 und 50, vorzugsweise SEQ ID NO: 51.

9. Insektenzelle, umfassend ein Nukleinsäurekonstrukt nach einem der Ansprüche 5-8.

10. Insektenzelle nach Anspruch 9, wobei die Insektenzelle weiter Folgendes umfasst:

    (a) eine zweite Nukleotidsequenz, die mindestens eine AAV - Inverted Terminal Repeat (ITR)-Nukleotidsequenz umfasst;
    (b) eine dritte Nukleotidsequenz, die eine Rep78- oder eine Rep68-Codiersequenz umfasst, die funktionsfähig mit Expressionskontrollsequenzen zur Expression in einer Insektenzelle verbunden ist;
    (c) optional eine vierte Nukleotidsequenz, die eine Rep52- oder eine Rep40-Codiersequenz umfasst, die funktionsfähig mit Expressionskontrollsequenzen zur Expression in einer Insektenzelle verbunden ist.

11. Insektenzelle nach Anspruch 10, wobei die Insektenzelle Folgendes umfasst:

    (a) ein erstes Nukleinsäurekonstrukt nach einem der Ansprüche 5-8, wobei das erste Nukleinsäurekonstrukt weiter die dritte und vierte Nukleotidsequenz umfasst, wie sie in (b) und (c) von Anspruch 10 definiert sind; und,
    (b) ein zweites Nukleinsäurekonstrukt, das die zweite Nukleotidsequenz gemäß (a) von Anspruch 10 umfasst, wobei das zweite Nukleinsäurekonstrukt vorzugsweise ein mit Insektenzellen kompatibler Vektor ist, besonders bevorzugt ein baculoviraler Vektor.

12. Insektenzelle nach einem der Ansprüche 10-11, wobei die zweite Nukleotidsequenz weiter mindestens eine Nuk-

leotidsequenz umfasst, die für ein interessierendes Genprodukt (zur Expression in einer Säugetierzelle) codiert, und wobei die mindestens eine Nukleotidsequenz, die für ein interessierendes Genprodukt codiert, in das Genom eines in der Insektenzelle produzierten AAV-Serotyps 5 eingebaut wird, wobei die zweite Nukleotidsequenz vorzugsweise zwei AAV ITR-Nukleotidsequenzen umfasst und wobei die mindestens eine Nukleotidsequenz, die für ein interessierendes Genprodukt codiert, zwischen den beiden AAV ITR-Nukleotidsequenzen liegt.

**13.** Insektenzelle nach Anspruch 10, wobei die erste Nukleotidsequenz, die zweite Nukleotidsequenz, die dritte Nukleotidsequenz und optional die vierte Nukleotidsequenz stabil in das Genom der Insektenzelle integriert sind.

**14.** AAV5-Virion, das in seinem Genom mindestens eine Nukleotidsequenz umfasst, die für ein interessierendes Genprodukt codiert, wobei die mindestens eine Nukleotidsequenz keine native AAV-Nukleotidsequenz ist und wobei das AAV-VP1-Kapsidprotein vom N-Terminus bis zum C-Terminus Folgendes umfasst

(i) einen ersten Aminosäurerest, der durch ein Translationsinitiationscodon codiert wird, das vorzugsweise aus der Gruppe ausgewählt ist, die aus CTG, ACG, TTG und GTG besteht;
(ii) eine Aminosäuresequenz des AAV VP1-Kapsidproteins, wobei der Sequenz nur der Aminosäurerest fehlt, der durch das VP1-Translationsinitiationscodon codiert wird;

wobei ein zweiter Aminosäurerest nach dem ersten Aminosäurerest insertiert ist, der für einen Aminosäurerest codiert, der aus der Gruppe ausgewählt ist, die aus Alanin, Glycin, Valin, Asparaginsäure und Glutaminsäure besteht, und wobei dem zweiten Aminosäurerest optional ein oder mehrere zusätzliche Aminosäurereste folgen.

**15.** Verfahren zur Herstellung eines AAV5 in einer Insektenzelle, das die folgenden Schritte umfasst: (a) Kultivieren einer Insektenzelle, wie in einem der Ansprüche 9-13 definiert, unter solchen Bedingungen, dass AAV produziert wird; und optional (b) Gewinnen des AAV.

**16.** AAV5-Virion nach Anspruch 14, wobei das interessierende Genprodukt für ein Faktor IX- oder ein Faktor VIII-Protein codiert.

**Revendications**

**1.** Molécule d'acide nucléique présentant une séquence nucléotidique comprenant un cadre de lecture ouvert modifié codant pour des protéines de capside de virus adéno-associé (AAV), dans laquelle le cadre de lecture dans l'ordre 5' à 3' comprend :

(i) un premier codon, qui est un codon d'initiation de traduction sous-optimal choisi dans le groupe consistant en CTG, ACG, TTG et GTG;
(ii) un second codon après le premier codon codant pour un résidu d'acide aminé choisi dans le groupe consistant en alanine, glycine, valine, acide aspartique et acide glutamique.
(iii) une séquence codant pour des protéines de capside de sérotype 5 de virus adéno-associé (AAV), la séquence étant dépourvue uniquement du codon d'initiation de traduction ATG VP1, et la séquence comprenant le reste d'un cadre de lecture ouvert codant pour des protéines de capside d'AAV5, les parties restantes commençant à la position correspondant à la seconde position d'acide aminé dans un cadre de lecture ouvert de type sauvage codant pour les protéines de capside.

**2.** Molécule d'acide nucléique selon la revendication 1, dans laquelle les protéines de capside d'AAV comprennent la séquence d'acides aminés de : SEQ ID NO : 22.

**3.** Molécule d'acide nucléique selon la revendication 1 ou la revendication 2, dans laquelle le second codon code pour l'alanine.

**4.** Molécule d'acide nucléique selon la revendication 1 ou la revendication 2, dans laquelle le second codon est choisi dans le groupe consistant en GCT, GCC, GCA, GCG et GGU, de préférence dans laquelle le codon est GCT.

**5.** Construction d'acide nucléique comprenant une molécule d'acide nucléique selon l'une quelconque des revendications 1 à 4, dans laquelle la séquence nucléotidique du cadre de lecture codant pour les protéines de capside de virus adéno-associé (AAV) est liée de manière fonctionnelle à des séquences de commande d'expression pour une

expression dans une cellule d'insecte.

**6.** Construction d'acide nucléique selon la revendication 5, dans laquelle la séquence nucléotidique du cadre de lecture est liée de manière fonctionnelle à un promoteur choisi dans le groupe consistant en : promoteur polyédrique, promoteur p10, promoteur 4xHsp27 EcRE+Hsp70 minimal, promoteur deltaE1, promoteur E1.

**7.** Construction d'acide nucléique selon la revendication 5 ou la revendication 6, dans laquelle la construction est un vecteur compatible avec un insecte, de préférence un vecteur baculoviral.

**8.** Construction d'acide nucléique selon l'une quelconque des revendications 5 à 7, dans laquelle la molécule d'acide nucléique comprend un cadre de lecture ouvert choisi dans le groupe consistant en : SEQ ID NO : 51, 69, 42, 47, 48 et 50, de préférence SEQ ID NO:51.

**9.** Cellule d'insecte comprenant une construction d'acide nucléique selon l'une quelconque des revendications 5 à 8.

**10.** Cellule d'insecte selon la revendication 9, dans laquelle la cellule d'insectes comprend en outre :

(a) une deuxième séquence nucléotidique comprenant au moins une séquence nucléotidique de répétition terminale inversée (ITR) d'AAV ;
(b) une troisième séquence nucléotidique comprenant une séquence codante Rep78 ou Rep68 liée de manière fonctionnelle à des séquences de commande d'expression pour une expression dans une cellule d'insecte ;
(c) facultativement, une quatrième séquence nucléotidique comprenant une séquence codante Rep52 ou Rep40 liée de manière fonctionnelle à des séquences de commande d'expression pour une expression dans une cellule d'insecte.

**11.** Cellule d'insecte selon la revendication 10, dans laquelle la cellule d'insecte comprend :

(a) une première construction d'acide nucléique selon l'une quelconque des revendications 5 à 8, dans laquelle la première construction d'acide nucléique comprend en outre les troisième et quatrième séquences nucléotidiques telles que définies dans (b) et (c) de la revendication 10 ; et,
(b) une seconde construction d'acide nucléique comprenant la deuxième séquence nucléotidique telle que définie dans (a) de la revendication 10, dans laquelle la seconde construction d'acide nucléique est de préférence un vecteur compatible avec une cellule d'insecte, plus préférablement un vecteur baculoviral.

**12.** Cellule d'insecte selon l'une quelconque des revendications 10 à 11, dans laquelle la deuxième séquence nucléotidique comprend en outre au moins une séquence nucléotidique codant pour un produit génique d'intérêt (pour une expression dans une cellule de mammifère) et dans laquelle la au moins une séquence nucléotidique codant pour un produit génique d'intérêt est incorporée dans le génome d'un sérotype 5 d'AAV produit dans la cellule d'insecte, de préférence dans laquelle la deuxième séquence nucléotidique comprend deux séquences nucléotidiques ITR d'AAV et dans laquelle la au moins une séquence nucléotidique codant pour un produit génique d'intérêt est située entre les deux séquences nucléotidiques ITR d'AAV.

**13.** Cellule d'insecte selon la revendication 10, dans laquelle la première séquence nucléotidique, la deuxième séquence nucléotidique, la troisième séquence nucléotidique et facultativement la quatrième séquence nucléotidique sont intégrées de manière stable dans le génome de la cellule d'insecte.

**14.** Virion AAV5, comprenant dans son génome au moins une séquence nucléotidique codant pour un produit génique d'intérêt, la au moins une séquence nucléotidique n'étant pas une séquence nucléotidique d'AAV native, et dans lequel la protéine de capside VP1 d'AAV comprend, de la terminaison N à la terminaison C

(i) un premier résidu d'acide aminé, qui est codé par un codon d'initiation de traduction, de préférence choisi dans le groupe consistant en CTG, ACG, TTG et GTG ;
(ii) une séquence d'acides aminés de la protéine de capside VP1 d'AAV, la séquence n'étant dépourvue que du résidu d'acide aminé qui est codé par le codon d'initiation de traduction VP1 ;

dans lequel un second résidu d'acide aminé est inséré après le premier résidu d'acide aminé codant pour un résidu d'acide aminé choisi dans le groupe consistant en alanine, glycine, valine, acide aspartique et acide glutamique, et, facultativement, un ou plusieurs résidus d'acide aminé supplémentaires suivant le second résidu d'acide aminé.

**15.** Procédé de production d'un AAV5 dans une cellule d'insecte, comprenant l'étape suivante consistant à : (a) cultiver une cellule d'insecte telle que définie dans l'une quelconque des revendications 9 à 13 dans des conditions telles que l'AAV est produit ; et facultativement (b) récupérer l'AAV.

**16.** Virus AAV5 selon la revendication 14, dans lequel le produit génique d'intérêt code pour une protéine du Facteur IX ou du Facteur VIII.

Fig. 1

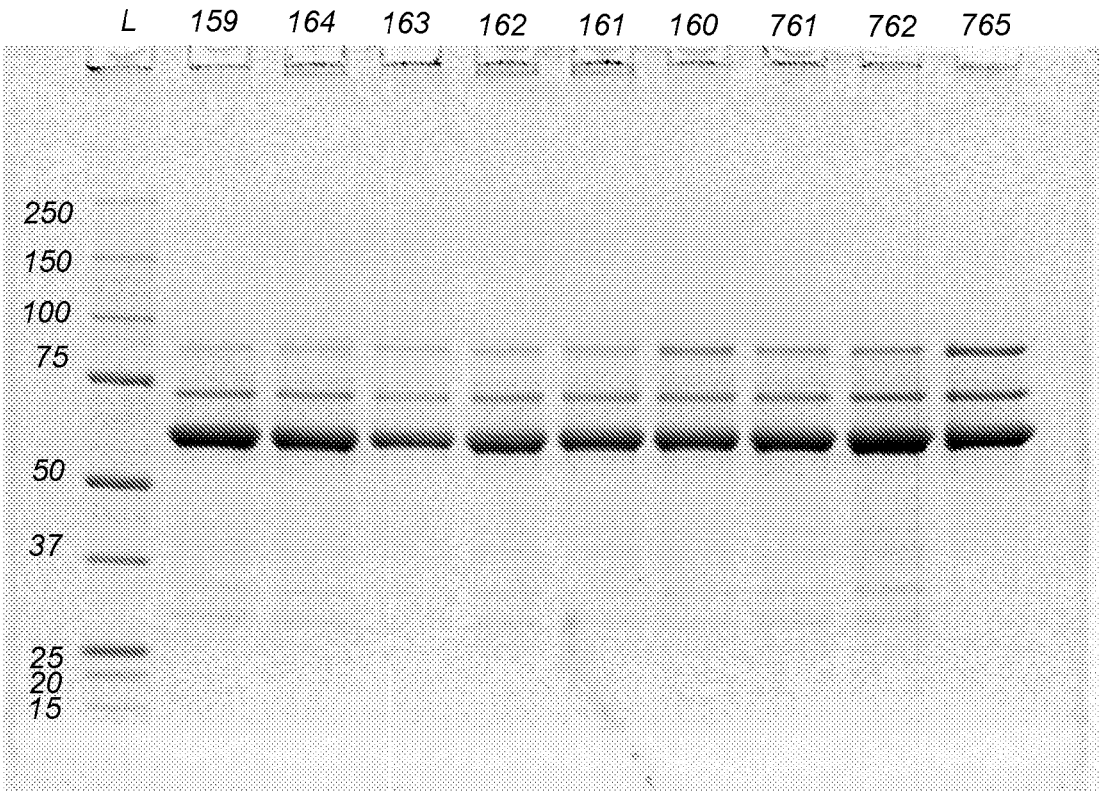


Fig. 2

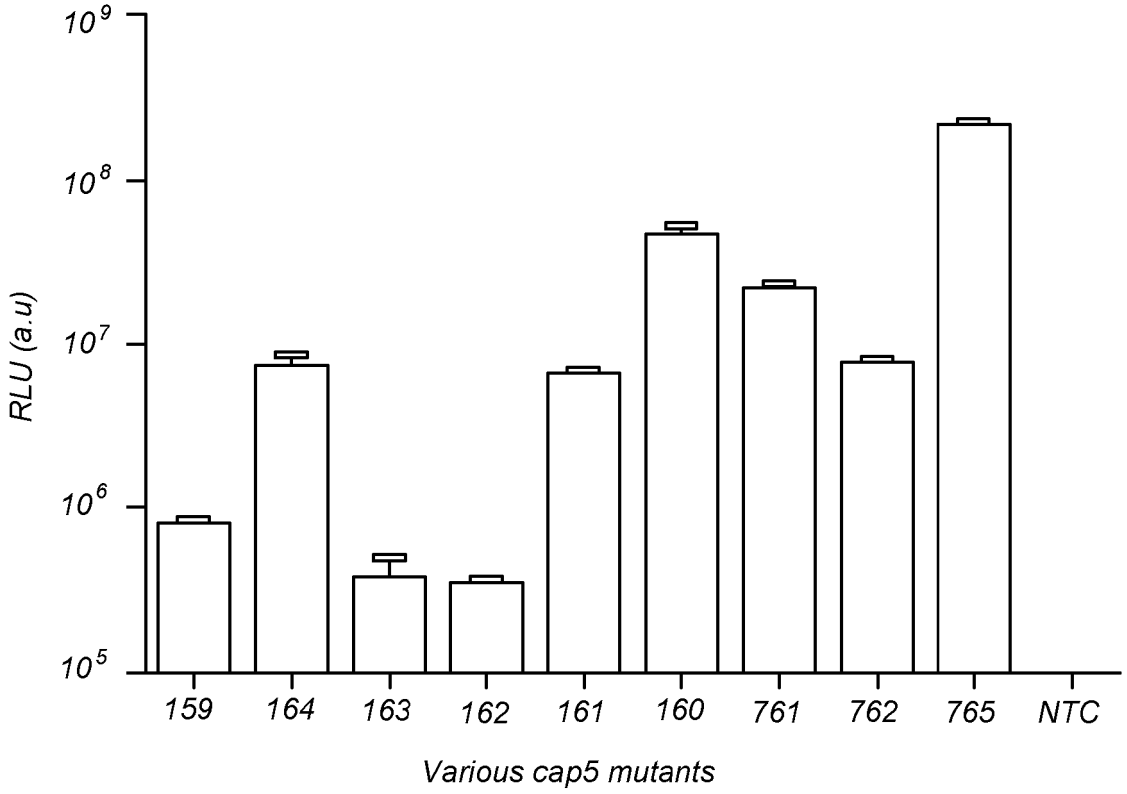

$10^9$
$10^8$
$10^7$
$10^6$
$10^5$
RLU (a.u)
159
164
163
162
161
160
761
762
765
NTC
Various cap5 mutants

Fig. 3

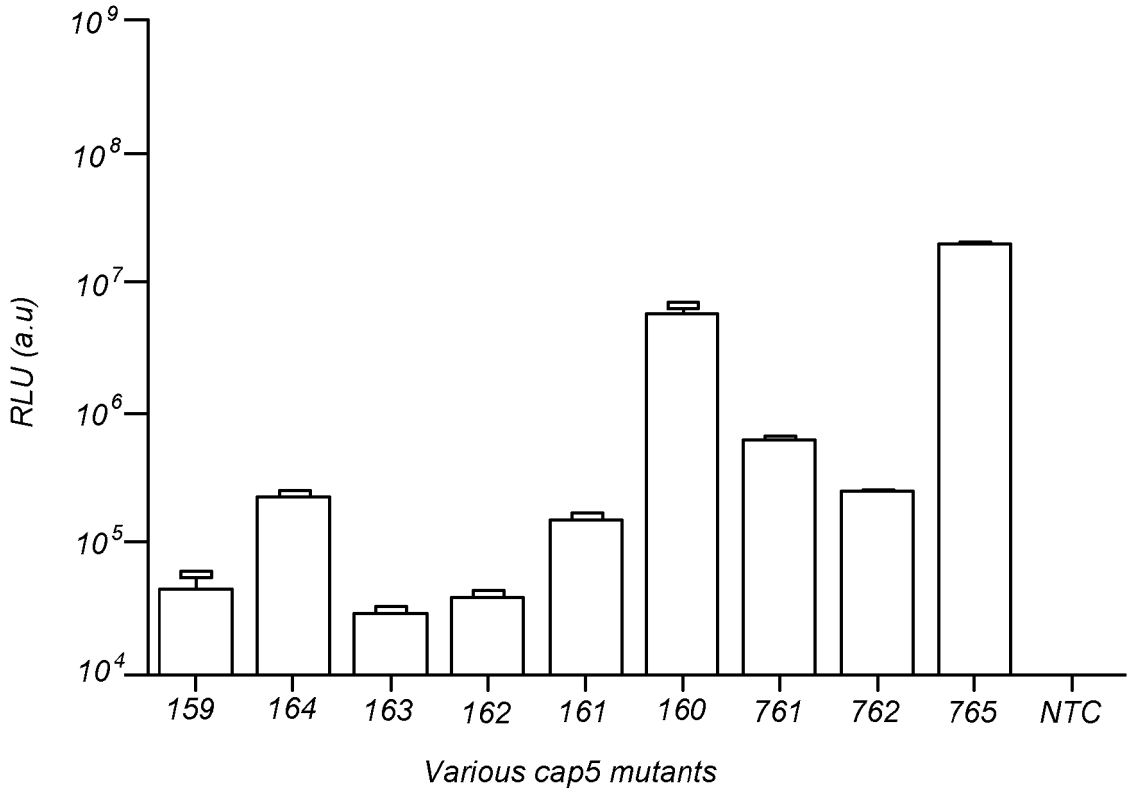

$10^9$
$10^8$
$10^7$
$10^6$
$10^5$
$10^4$
RLU (a.u)
159
164
163
162
161
160
761
762
765
NTC
Various cap5 mutants

Fig. 4

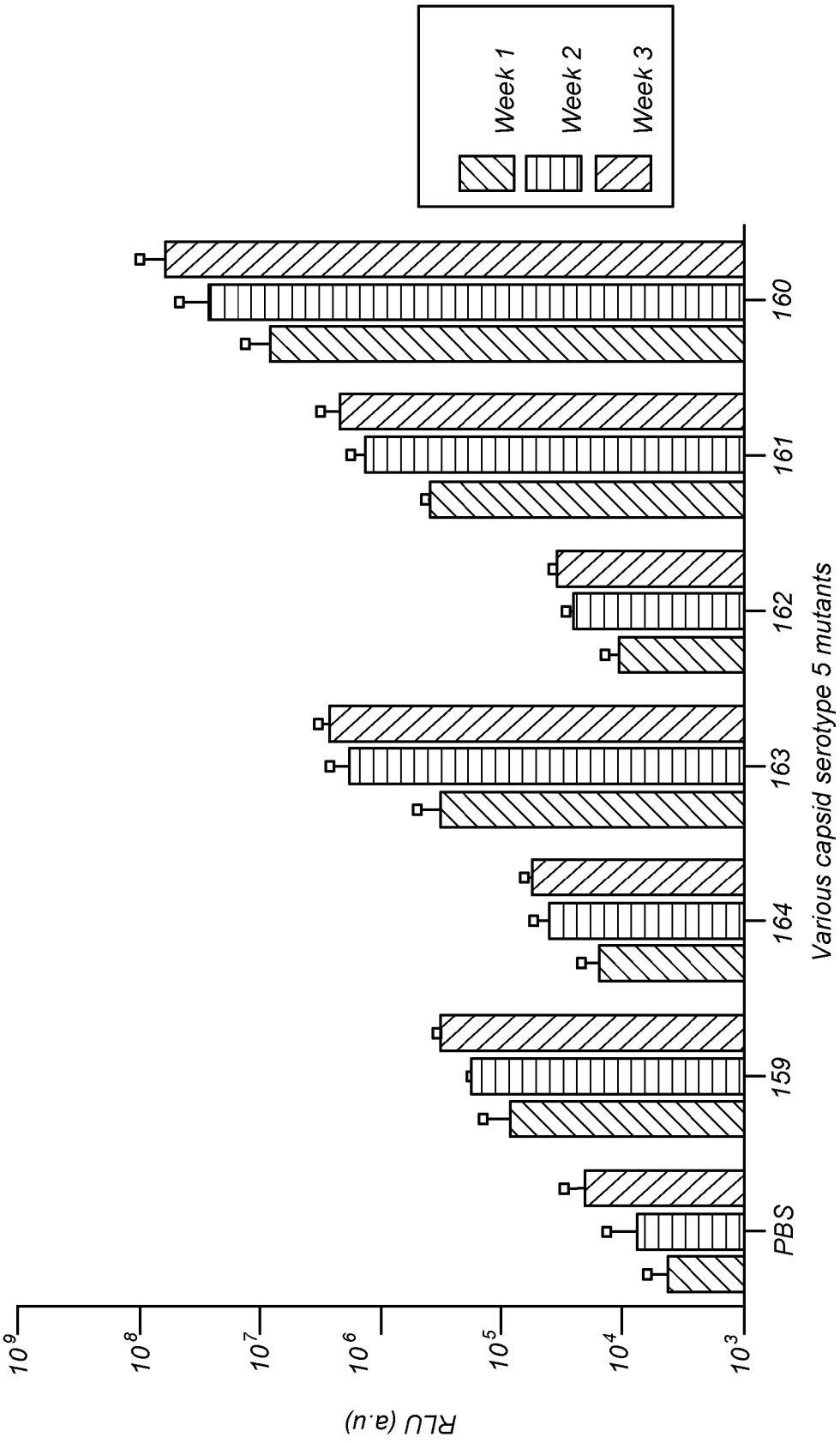

Week 1
Week 2
Week 3
RLU (a.u)
$10^9$
$10^8$
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
PBS
159
164
163
162
161
160
Various capsid serotype 5 mutants

Fig. 5

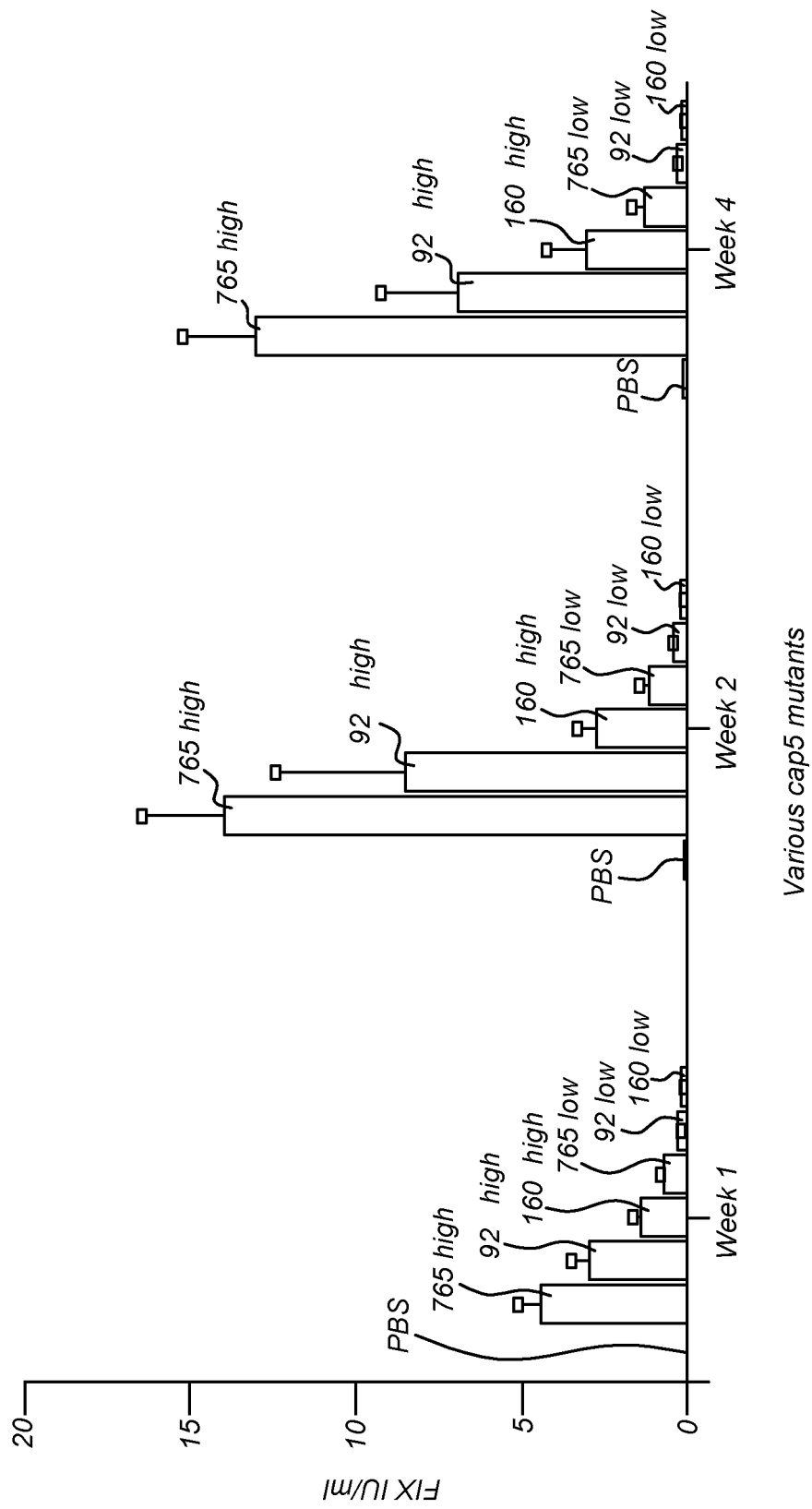

FIX IU/ml
0
5
10
15
20
PBS
765 high
92 high
160 high
765 low
92 low
160 low
Week 1
Week 2
Week 4
Various cap5 mutants

*Fig. 6*

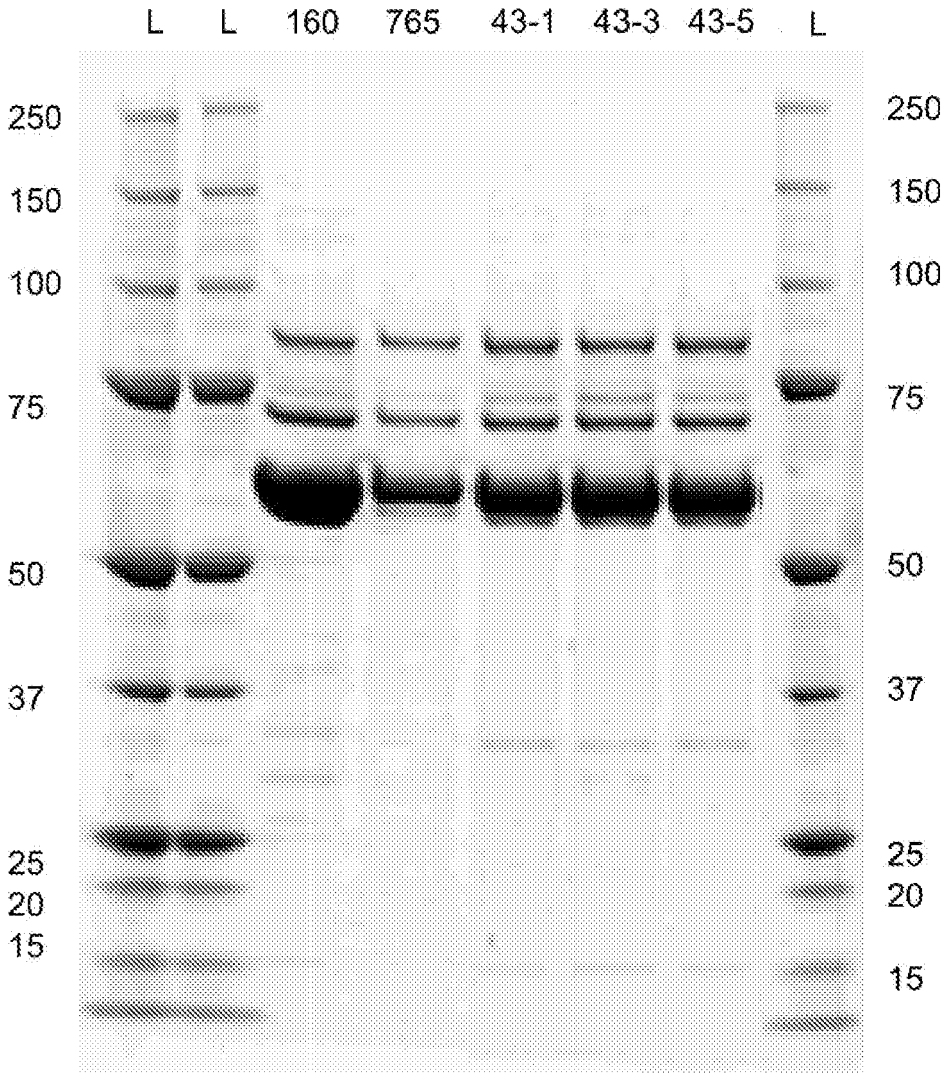

L
L
160
765
43-1
43-3
43-5
L
250
150
100
75
50
37
25
20
15
250
150
100
75
50
37
25
20
15

*Fig. 7a*

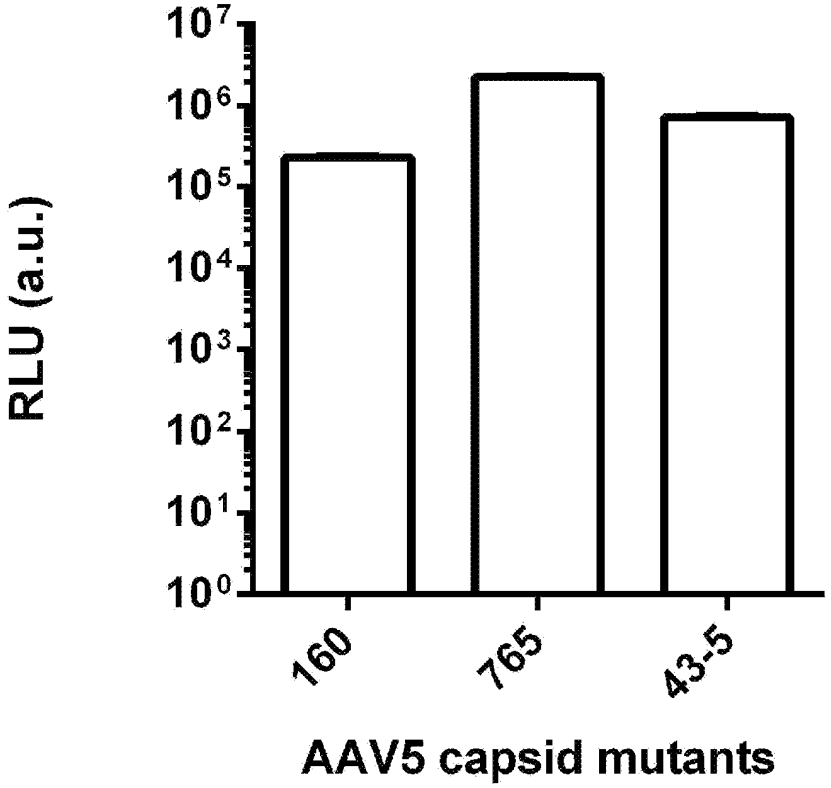
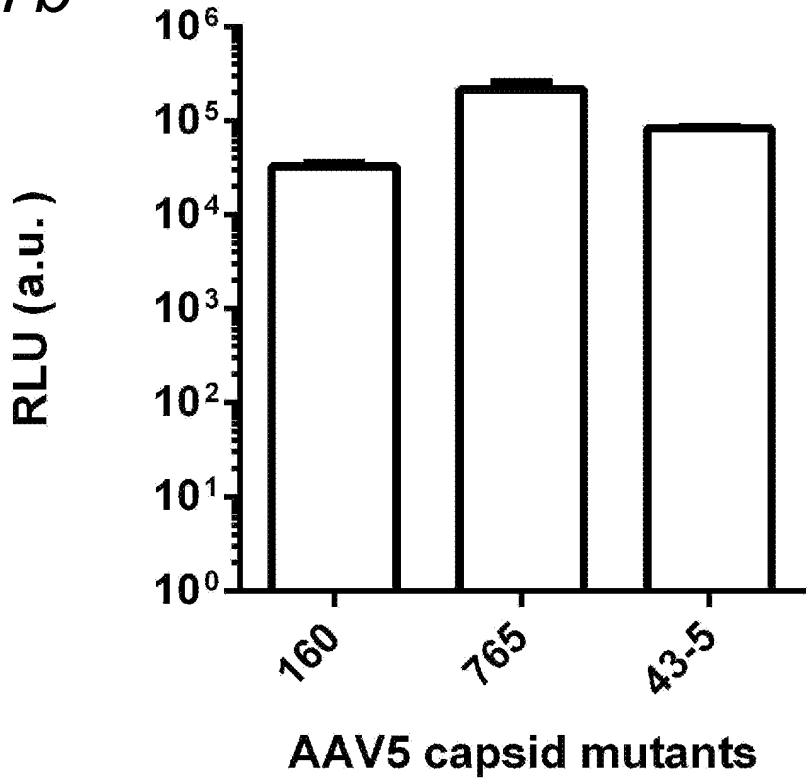

RLU (a.u.)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
160
765
43-5
AAV5 capsid mutants

*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

## REFERENCES CITED IN THE DESCRIPTION

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### Patent documents cited in the description


- US 20030148506 A **[0005]**
- US 20040197895 A **[0005]**
- WO 2007046703 A **[0006] [0010] [0099]**
- WO 2009014445 A **[0008] [0010]**
- CA 921213752 **[0030]**
- US 4745051 A **[0054]**
- US 2003148506 A **[0054] [0059] [0071]**
- WO 03074714 A **[0054]**
- WO 2009154452 A **[0059]**
- WO 2011122950 A **[0059]**
- WO 0100220 A **[0065] [0082]**
- WO 200914445 A **[0090]**
- WO 200636502 A **[0104]**
- WO 2007046703 A2 **[0108]**


### Non-patent literature cited in the description


- **URABE et al.** *Hum. Gene Ther.,* 2002, vol. 13, 1935-1943 **[0005] [0010]**
- **KOHLBRENNER et al.** *Mol. Ther,* 2005, vol. 12, 1217-25 **[0007]**
- **URABE et al.** *J. Virol.,* 2006, vol. 80 (4), 1874-1885 **[0009]**
- **HENIKOFF ; HENIKOFF.** *PNAS,* 1992, vol. 89, 915-919 **[0030]**
- Parvoviridae: The Viruses and Their Replication. **KENNETH I. BERNS.** Fields Virology. 1996 **[0034]**
- **SCHAFFER ; MAHESHRI.** *Proceedings of the 26th Annual International Conference of the IEEE EMBS San Francisco, CA,* 01 September 2004, 3520-3523 **[0048]**
- **ASURI et al.** *Molecular Therapy,* 2012, vol. 20 (2), 329-3389 **[0048]**
- **LISOWSKI et al.** *Nature,* 2014, vol. 506 (7488), 382-386 **[0048]**
- **SAMBROOK ; RUSSELL.** Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, 2001 **[0052]**
- **SUMMERS ; SMITH.** A Manual of Methods for Baculovirus Vectors and Insect Culture Procedures. *Texas Agricultural Experimental Station Bull,* 1986 **[0054]**
- **LUCKOW. et al.** *In Prokop et al., Cloning and Expression of Heterologous Genes in Insect Cells with Baculovirus Vectors' Recombinant DNA Technology and Applications,* 1991 **[0054]**
- **KING, L. A ; R. D. POSSEE.** The baculovirus expression system. Chapman and Hall, 1992 **[0054]**
- **O'REILLY, D. R. ; L. K. MILLER ; V. A. LUCKOW.** *Baculovirus Expression Vectors: A Laboratory Manual,* 1992 **[0054]**
- **W. H. FREEMAN ; RICHARDSON, C. D.** *Baculovirus Expression Protocols, Methods in Molecular Biology,* 1995, vol. 39 **[0054]**
- **WU et al.** *Molecular Therapy,* 2010, vol. 18 (1), 80-86 **[0063]**
- **DONG et al.** *Molecular Therapy,* 2010, vol. 18 (1), 87-92 **[0063]**
- **KAPRANOV et al.** *Human Gene Therapy,* 2012, vol. 23, 46-55 **[0063]**
- **LAI et al.** *Molecular Therapy,* 2010, vol. 18 (1), 75-79 **[0063]**
- **ELBASHIR et al.** *Nature,* 2001, vol. 411, 494-98 **[0064]**
- **CAPLEN et al.** *Proc. Natl. Acad. Sci. USA,* 2001, vol. 98, 9742-47 **[0064]**
- **SAMBROOK ; RUSSEL.** Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, 2001 **[0065]**
- **CLAIR et al.** *Antimicrob. Agents Chemother.,* 1987, vol. 31, 844-849 **[0065]**
- **PARR et al.** *Nat. Med,* 1997, vol. 3, 1145-9 **[0067]**
- **HAGENBAUGH et al.** *J Exp Med,* 1997, vol. 185, 2101-10 **[0067]**
- **TRATSCHIN et al.** *Mol. Cell Biol.,* 1985, vol. 5 (11), 3251-3260 **[0068]**
- **GRIMM et al.** *Hum. Gene Ther,* 1999, vol. 10 (15), 2445-2450 **[0068]**
- **JENNINGS et al.** *Arthritis Res,* 2001, vol. 3, 1 **[0068]**
- **DAVIDSON et al.** *Proc. Natl. Acad. Sci. USA,* 2000, vol. 97 (7), 3428-3432 **[0068]**
- **CHLORINI et al.** *J. Vir.,* 1997, vol. 71, 6823-33 **[0069]**
- **SRIVASTAVA et al.** *J. Vir.,* 1983, vol. 45, 555-64 **[0069]**
- **CHLORINI et al.** *J. Vir.,* 1999, vol. 73, 1309-1319 **[0069]**
- **RUTLEDGE et al.** *J. Vir,* 1998, vol. 72, 309-319 **[0069]**
- **WU et al.** *J. Vir.,* 2000, vol. 74, 8635-47 **[0069]**
- **BANTEL-SCHAAL et al.** *J. Virol.,* 1999, vol. 73 (2), 939-947 **[0071]**

- **E. HARLOW ; D. LANE.** Using Antibodies. Cold Spring Harbor Laboratory Press, 1999 **[0079]**
- **MUYLDERMANS.** *Biotechnol,* 2001, vol. 74, 277-302 **[0084]**
- **URABE et al.** *Human Gene Therapy,* 2002, vol. 13 (16), 1935-1943 **[0087]**
- **KOHLBRENNER et al.** *Molecular Therapy,* 2005, vol. 12 (6), 1217-1225 **[0088]**
- **URABE et al.** *Journal of Virology,* 2006, vol. 80 (4), 1874-1885 **[0088]**
- **KOZAK.** *Nucleic Acid Research,* 1987, vol. 15 (20), 8125-8148 **[0099]**
- **HERMONAT et al.** *Journal of Virology,* 1984, vol. 51 (2), 329-339 **[0107]**
- **TRATSCHIN et al.** *Journal of Virology,* 1984, vol. 51 (3), 611-619 **[0107]**