(19) DANMARK

(10) **DK/EP 3313991 T3**

Patent- og Varemærkestyrelsen

(12) Oversættelse af europæisk patentskrift

(51) Int.Cl.: *C 12 N 9/64 (2006.01)* *A 61 K 38/48 (2006.01)* *A 61 K 48/00 (2006.01)* *C 07 K 14/745 (2006.01)* *C 12 N 15/52 (2006.01)* *C 12 N 15/864 (2006.01)* *C 12 N 15/86 (2006.01)*

(45) Oversættelsen bekendtgjort den: **2024-09-09**

(80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2024-07-17**

(86) Europæisk ansøgning nr.: **16815331.0**

(86) Europæisk indleveringsdag: **2016-06-23**

(87) Den europæiske ansøgnings publiceringsdag: **2018-05-02**

(86) International ansøgning nr.: **US2016039075**

(87) Internationalt publikationsnr.: **WO2016210170**

(30) Prioritet: **2015-06-23 US 201562183599 P** **2016-03-30 US 201662315453 P** **2016-05-18 US 201662338315 P** **2016-06-10 US 201662348781 P** **2016-06-13 US 201662349572 P**

(84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**

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(54) Benævnelse: **MODIFICERET FAKTOR IX OG SAMMENSÆTNINGER, FREMGANGSMÅDER OG ANVENDELSER TIL GENOVERFØRSEL TIL CELLER, ORGANER OG VÆV**

(56) Fremdragne publikationer:
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**WO-A2-01/98482**
**WO-A2-2015/013313**
**US-A1- 2014 271 550**
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# DESCRIPTION

Description

## Introduction

**[0001]** Genetic disorders, caused by absence or a defect in a desirable gene (loss of function) or expression of an undesirable or defective gene (gain of function) lead to a variety of diseases. One example of a loss of function genetic disorder is hemophilia, an inherited bleeding disorder caused by deficiency in either coagulation factor VIII (FVIII, hemophilia A) or factor IX (FIX, hemophilia B). One example of a gain of function genetic disorder is Huntington's disease, a disease caused by a pathologic "HTT" gene (encodes the huntingtin protein) that encodes a mutated protein that accumulates within and leads to gradual destruction of neurons, particularly in the basal ganglia and the cerebral cortex.

**[0002]** Current treatment for hemophilia consists in the intravenous administration of recombinant clotting factor either on demand, in case a bleeding occurs, or prophylactically. However, this therapeutic approach has several drawbacks such as the need for repeated infusions, the cost of the treatment, the risk of developing anti-therapeutic factor immune responses, and the risk of potentially fatal bleedings. These limitations have prompted the development of gene-based therapies for hemophilia. To this end, hemophilia is ideal for gene transfer based therapy as 1) the therapeutic window is very wide, as levels just above 1% of normal already can result in a change in phenotype from severe to moderate, and levels of 100% are not associated to any side effects; 2) tissue specific expression of the therapeutic transgene is not strictly required; and 3) there is a considerable experience in measuring the endpoints of therapeutic efficacy.

**[0003]** Currently, adeno-associated virus (AAV) vectors are recognized as the gene transfer vectors of choice since they have the best safety and efficacy profile for the delivery of genes *in vivo.* Of the AAV serotypes isolated so far, AAV2 and AAV8 have been used to target the liver of humans affected by severe hemophilia B.

## Summary

**[0004]** The present invention is defined by the claims as appended. Thus, in a first aspect, the present invention relates to a recombinant adeno-associated virus (rAAV) vector comprising a genome and a capsid, wherein the genome of said rAAV vector comprises a nucleic acid comprising a non-naturally occurring nucleotide sequence encoding human Factor IX protein, wherein said nucleotide sequence encodes the same human Factor IX protein that is encoded by the nucleotide sequence of SEQ ID NO: 10, and is at least 85% identical to the nucleotide sequence of SEQ ID NO: 10, and wherein the capsid of said rAAV vector comprises a VP1 protein comprising the amino acid sequence of SEQ ID NO:4.

**[0005]** In a preferred embodiment, said nucleotide sequence encoding human Factor IX protein is at least 90% identical to the nucleotide sequence of SEQ ID NO: 10.

**[0006]** In a further preferred embodiment, said non-naturally occurring nucleotide sequence encoding human Factor IX protein has a reduced number of CpG di-nucleotides compared to the wild-type sequence encoding human Factor IX.

**[0007]** In a further preferred embodiment, said nucleic acid further comprises at least one element selected from the group consisting of: an adeno-associated virus (AAV) inverted terminal repeat (ITR), an expression control element operably linked with said nucleotide sequence encoding human Factor IX protein, a polynucleotide stuffer, and a transcription terminator.

**[0008]** In a further preferred embodiment, said expression control element confers expression in liver and comprises a promoter and optionally an enhancer.

**[0009]** In a further preferred embodiment, said nucleic acid comprises an ITR from the AAV2 serotype, a promoter and enhancer that confers expression in liver operably linked with said nucleotide sequence encoding human Factor IX protein, a polyadenylation sequence, a transcription terminator, and optionally a second AAV2 ITR, wherein the AAV2 ITR is positioned 5' of the promoter and enhancer or 3' of the transcription terminator.

**[0010]** In a further preferred embodiment, said nucleotide sequence encoding human Factor IX protein is interrupted by an intron.

**[0011]** In a further preferred embodiment, said nucleic acid further comprises a polynucleotide stuffer. In a further preferred embodiment, said promoter is a human alpha1-antitrypsin (AAT) promoter and said enhancer is an apolipoprotein E (ApoE) HCR-1 or HCR-2 enhancer.

**[0012]** In a further preferred embodiment, said promoter comprises the nucleotide sequence of SEQ ID NO:15.

**[0013]** In a further preferred embodiment, said enhancer comprises the nucleotide sequence of SEQ ID NO:14.

**[0014]** In a further preferred embodiment, said AAV2 ITR comprises either of the AAV2 ITR sequences as found in the nucleotide sequence of SEQ ID NO:26.

**[0015]** In a further preferred embodiment, said intron comprises the nucleotide sequence of

SEQ ID NO: 17.

**[0016]** In a further preferred embodiment, the nucleotide sequence encoding human Factor IX protein and the intron comprise the nucleotide sequence of SEQ ID NO:25.

**[0017]** In a further preferred embodiment, the rAAV vector of the invention comprises in order an ApoE HCR-1 enhancer, an AAT promoter, the non-naturally occurring nucleotide sequence encoding human Factor IX protein, a polyadenylation sequence, an AAV2 ITR positioned 5' of the enhancer or 3' of the polyadenylation sequence, and optionally a second AAV2 ITR in the opposite position.

**[0018]** In a further preferred embodiment, the nucleotide sequence of said ApoE HCR-1 enhancer comprises nucleotides 152-472 of SEQ ID NO: 12, the nucleotide sequence of said AAT promoter comprises nucleotides 482-878 of SEQ ID NO: 12, the nucleotide sequence encoding human Factor IX protein comprises nucleotides 908-995 and 2434-3731 of SEQ ID NO:12, the nucleotide sequence of said polyadenylation sequence comprises nucleotides 3820-4047 of SEQ ID NO: 12, and the nucleotide sequence of said AAV2 ITR comprises either of the AAV2 ITR sequences as found in the nucleotide sequence of SEQ ID NO:26.

**[0019]** In a further preferred embodiment, the rAAV vector of the invention comprises nucleotides 142-4096 of SEQ ID NO: 12.

**[0020]** In certain even more preferred embodiments, said nucleotide sequence encoding human Factor IX protein is interrupted by an intron.

**[0021]** In a further preferred embodiment, said intron comprises the nucleotide sequence of SEQ ID NO: 17.

**[0022]** In a further preferred embodiment, the nucleotide sequence encoding human Factor IX protein and the intron comprise the nucleotide sequence of SEQ ID NO:25.

**[0023]** In a further preferred embodiment, the genome of said rAAV vector is single stranded.

**[0024]** In a further preferred embodiment, said nucleotide sequence encoding human Factor IX protein is at least 90% identical to the nucleotide sequence of SEQ ID NO: 10.

**[0025]** In a second aspect, the present invention relates to a pharmaceutical composition comprising the rAAV vector of any of the preceding claims for use in the treatment of hemophilia B.

**<u>Brief Description of the Drawing</u>**

**[0026]**

Figure 1 shows the amino acid sequence of Rh74 VP1.

Figure 2 shows the amino acid sequence or Rh74 VP2.

Figure 3 shows the amino acid sequence of Rh74 VP3.

Figure 4 shows the amino acid sequence of capsid variant 4-1 VP1 protein.

Figure 5 shows the amino acid sequence of capsid variant 15-1.

Figure 6 shows the amino acid sequence of capsid variant 15-2.

Figure 7 shows the amino acid sequence of capsid variant 15-3/15-5.

Figure 8 shows the amino acid sequence of capsid variant 15-4.

Figure 9 shows the amino acid sequence of capsid variant 15-6.

Figure 10 shows the nucleic acid sequence of FIX39.

Figure 11 shows the nucleic acid sequence of FIX19.

Figure 12A shows the sequence of the FIX39 plasmid.

Figure 12B shows the sequence of the phFIX39v2 plasmid.

Figure 13 shows a map of the FIX39 plasmid.

Figure 14 shows the Intron A nucleic acid sequence.

Figure 15 shows the nucleic acid sequence of FIX39 + Intron A.

Figure 16 shows transduction efficiency of the AAV-4-1 capsid variant (SEQ ID NO:4) analyzed in an *in vitro* setting.

Figure 17 shows levels of hFIX in plasma of wild-type mice following intravenous injection at week 8 of life with either $1 \times 10^{11}$ or $1 \times 10^{12}$ vg/kg of AAV-FIX39-Padua (square/circle) and AAV-FIX19-Padua (diamond/hexagon). Human FIX plasma levels were assayed by ELISA and represent multiple measurements, obtained by serial bleeding, on the same group of animals during the course of the study (n=5 mice in each cohort). Error bars denote standard error of the mean.

Figure 18 shows circulating levels of human FIX in mouse plasma 24 hours following hydrodynamic tail vein injection of 5 µg of pFIX19-Padua or pFIX39-Padua plasmids. P=0.3337.

Figure 19 shows a data summary of four human hemophilia B patients administered a single infusion of an AAV-FIX Padua variant (FIX39) bearing vector in accordance with the invention, and the FIX activity (%) over the ensuing evaluation periods (183, 102, 69 and 50 days, respectively).

Figure 20A shows the FIX activity (%) data of the first human hemophilia B patient administered the single infusion of AAV-FIX Padua variant (FIX39) bearing vector, over the 183 day evaluation period.

Figure 20B shows liver function test (ALT, AST and LDH enzymes) data of the first human hemophilia B patient administered the single infusion of AAV-FIX Padua variant (FIX39) bearing vector, over the 183 day evaluation period. The plotted LDH values (LDH[1]) have been divided by 10 in order to be shown with the ALT and AST values.

Figure 21A shows the FIX activity (%) data of the second human hemophilia B patient administered the single infusion of AAV-FIX Padua variant (FIX39) bearing vector, over the 102 day evaluation period.

Figure 21B shows liver function test (ALT, AST and LDH enzymes) data of the second human hemophilia B patient administered the single infusion of AAV-FIX Padua variant (FIX39) bearing vector, over the 102 day evaluation period. The plotted LDH values (LDH[1]) have been divided by 10 in order to be shown with the ALT and AST values.

Figure 22A shows the FIX activity (%) data of the third human hemophilia B patient administered the single infusion of AAV-FIX Padua variant (FIX39) bearing vector, over the 69 day evaluation period.

Figure 22B shows liver function test (ALT, AST and LDH enzymes) data of the third human hemophilia B patient administered the single infusion of AAV-FIX Padua variant (FIX39) bearing vector, over the 69 day evaluation period. The plotted LDH values (LDH[1]) have been divided by 10 in order to be shown with the ALT and AST values.

Figure 23A shows the FIX activity (%) data of the fourth human hemophilia B patient administered the single infusion of AAV-FIX Padua variant (FIX39) bearing vector, over the 50 day evaluation period.

Figure 23B shows liver function test (ALT, AST and LDH enzymes) data of the fourth human hemophilia B patient administered the single infusion of AAV-FIX Padua variant (FIX39) bearing vector, over the 50 day evaluation period. The plotted LDH values (LDH[1]) have been divided by 10 in order to be shown with the ALT and AST values.

Figure 24A shows low immunogenicity profile of AAV-FIX39-Padua in human subjects.

Figure 24B shows a comparative immunogenicity profile of AAV-FIX39-Padua and AAV8-FIX19 in human subjects.

## Detailed Description

**[0027]** The invention is based, at least in part, on development of modified nucleic acid sequences encoding proteins, such as human FIX protein. In various embodiments, a modified nucleic acid has a reduced number of CpG (cytosine-guanine) di-nucleotides compared to a reference nucleic acid sequence encoding Factor IX, such as a native (wild-type) sequence encoding human Factor IX. Such modified nucleic acids having a reduced number of CpG di-nucleotides compared to a reference Factor IX encoding nucleic acid (*e.g.,* a native sequence encoding human Factor IX) may be included within expression vectors (*e.g.*, vector genomes) or plasmids.

**[0028]** The invention also includes compositions, such as compositions including a modified nucleic acid sequence encoding human FIX, whereby it is understood that only such compositions as defined in accordance with the second aspect of the invention are covered by the claimed invention. In such compositions, a modified nucleic acid may have a reduced number of CpG di-nucleotides relative to a reference sequence such as a native (wild-type) sequence encoding human Factor IX. Compositions also include expression vectors (*e.g.*, viral vectors/vector genomes) and plasmids that include such modified nucleic acid sequences encoding human FIX protein having a reduced number of CpG di-nucleotides.

**[0029]** A nucleic acid sequence encoding human FIX protein may have 1-5 fewer CpG di-nucleotides than native sequence encoding human Factor IX; or may have 5-10 fewer CpG di-nucleotides than native (wild-type) sequence encoding human Factor IX; or may have 10-15 fewer CpG di-nucleotides than native (wild-type) sequence encoding human Factor IX; or may have 15-20 fewer CpG di-nucleotides than native (wild-type) sequence encoding human Factor IX; or may have 20-25 fewer CpG di-nucleotides than native (wild-type) sequence encoding human Factor IX; or may have 25-30 fewer CpG di-nucleotides than native (wild-type) sequence encoding human Factor IX; or may have 30-40 fewer CpG di-nucleotides than native (wild-type) sequence encoding human Factor IX; or may have 40-55 fewer CpG di-nucleotides than native (wild-type) sequence encoding human Factor IX; or is completely devoid of any CpG di-nucleotides.

**[0030]** Modified nucleic acids encoding Factor IX, such as FIX with a reduced number of CpG dinucleotide, may further include one or more additional cis elements. Representative cis elements include, without limitation, expression control elements, introns, ITRs, stop codons, polyA sequences, and/or filler polynucleotide sequences. Such cis-acting elements can also be modified. For example cis acting elements such as expression control elements, introns, ITRs, poly-A sequences, and/or filler polynucleotide sequences can have a reduced number of CpG di-nucleotides. One or more cis acting elements, such as expression control elements, introns, ITRs, poly-A sequences, and/or filler polynucleotide sequences, may be devoid of CpG di-nucleotides. One or more cis acting elements such as expression control elements, introns, ITRs, poly-A sequences, and/or filler polynucleotide sequences may have 1-5 fewer CpG di-nucleotides than a reference cis-acting element; or may have5-10 fewer CpG di-nucleotides than a reference cis-acting element; or may have 10-15 fewer CpG di-nucleotides than a reference cis-acting element; or may have 15-20 fewer CpG di-nucleotides than a reference cis-acting element; or may have 20-25 fewer CpG di-nucleotides than a reference cis-acting element; or may have 25-30 fewer CpG di-nucleotides than a reference cis-acting element; or may have 30-40 fewer CpG di-nucleotides than a reference cis-acting element; or may have 40-55 fewer CpG di-nucleotides than a reference cis element; or may be devoid of any CpG di-nucleotides.

**[0031]** Described herein are broadly also viral vectors that include a modified nucleic acid sequence encoding human FIX protein, such as FIX with a reduced number of CpG dinucleotides, whereby only such viral vectors are embraced by the claimed invention as defined by the appended claims. Generally, a vector may include a lenti- or parvo-viral vector, such as an adeno-viral vector. A modified nucleic acid sequence encoding human FIX protein, such as FIX with a reduced number of CpG dinucleotides, may be comprised in an adeno-associated virus (AAV) vector.

**[0032]** Adeno-associated virus (AAV) vectors may include capsids derived from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, Rh10, Rh74 and AAV-2i8, as well as variants (*e.g.*, capsid variants, such as amino acid insertions, additions and substitutions) thereof. As will be appreciated by one of ordinary skill in the art, AAV capsids may include a VP1 protein and two shorter proteins, called VP2 and VP3, that are essentially amino-terminal truncations of VP1. Depending on the capsid and other factors known to those of ordinary skill, the three capsid proteins VP1, VP2 and VP3 are typically present in a capsid at a ratio approximating 1:1:10, respectively, although this ratio, particularly of VP3, can vary significantly.

**[0033]** AAV variants include AAV-Rh74 variants, for example AAV capsid variants of the Rh74 VP1 capsid sequence (SEQ ID NO: 1; Figure 1), including but not limited to variants 4-1, 15-1, 15-2, 15-3/15-5, 15-4 and 15-6 described in Table 1. Rh74 VP2 and Rh74 VP3 amino acid sequences are provided in SEQ ID NO:2 (Figure 2) and SEQ ID NO:3 (Figure 3), respectively.

**Table 1.AAV capsid variants**

| Variant | Amino Acid Substitutions and Indicated Positions in Rh74 VP1 Capsid | Sequence Identifier | Figure |
|---|---|---|---|
| 4-1 | G195A-L199V- S201P-G202N | SEQ ID NO:4 | Figure 4 |
| 15-1 | G195A-L199V-S201P-G202N<br>K(137/259/333/530/552/569/38/51/77/169/547)R | SEQ ID NO:5 | Figure 5 |
| 15-2 | G195A-L199V- S201P-G202N<br>K(137/259/333/530/552/569/38/51/77/163/169)R | SEQ ID NO:6 | Figure 6 |
| 15-3/15-5 | G195A-L199V- S201P-G202N<br>K(137/259/333/530/552/569/38/51/77/163/547)R<br>(variant 15-3)<br>G195A-L199V- S201P-G202N | SEQ ID NO:7 | Figure 7 |

| Variant | Amino Acid Substitutions and Indicated Positions in Rh74 VP1 Capsid | Sequence Identifier | Figure |
|---|---|---|---|
| | G195A-L199V- S201P-G202N<br>K(137/259/333/530/552/569/38/51/77/547/163)R<br>(variant 15-5) | | |
| 15-4 | G195A-L199V- S201P-G202N<br>K(137/259/333/530/552/569/38/51/77/163/668)R | SEQ ID NO:8 | Figure 8 |
| 15-6 | G195A-L199V- S201P-G202N<br>K(137/259/333/530/552/569/38/51/77/547/688)R | SEQ ID NO:9 | Figure 9 |

**[0034]** 4-1 variant (SEQ ID NO:4) has an alanine, a valine, a proline, and an asparagine substitution at amino acid positions 195, 199, 201 and 202, respectively, of VP1 capsid. The 4-1 variant VP1 capsid amino acid sequence, with substituted residues a, v, p and n, underlined and in bold is shown in Figure 4 (SEQ ID NO:4). For variant 4-1, the VP2 sequence consists of SEQ ID NO:27, and the VP3 sequence consists of SEQ ID NO:3, respectively.

**[0035]** 15-1, 15-2, 15-3, 15-4, 1-5 and 15-6 variants also have an alanine, a valine, a proline, and an asparagine substitution at amino acid positions 195, 199, 201 and 202, respectively, of VP1 capsid. In addition, these variants have multiple arginine substitutions of lysine at various positions. The 15-1 variant VP1 capsid amino acid sequence (SEQ ID NO:5) is shown in Figure 5; the 15-2 variant VP1 capsid amino acid sequence (SEQ ID NO:6) is shown in Figure 6; the 15-3/15-5 variant VP1 capsid amino acid sequence (SEQ ID NO:7) is shown in Figure 7; the 15-4 variant VP1 capsid amino acid sequence (SEQ ID NO:8) is shown in Figure 8; and the 15-6 variant VP1 capsid amino acid sequence (SEQ ID NO:9) is shown in Figure 9. Examples of capsids include, but are not limited to, those described in United States patent publication no. 2015/0023924.

**[0036]** Accordingly, lenti- and parvo-viral vectors such as AAV vectors and viral vector variants such as AAV variants (*e.g.*, capsid variants such as 4-1, 15-1, 15-2, 15-3/15-5, 15-4 and 15-6) that include (encapsidate or package) vector genome including modified nucleic acid sequence encoding human FIX protein, such as FIX with a reduced number of CpG dinucleotides, are provided.

**[0037]** In exemplary studies, AAV-Rh74 mediated gene transfer/delivery produced protein expression levels that were significantly higher than several other serotypes. In particular, AAV-Rh74 vector and capsid variants (*e.g.*, 4-1) target genes for delivery to the liver with efficiency at least comparable to the gold standard for liver transduction, AAV8, in hemophilia B dogs and/or in mice and/or macaques.

**[0038]** As set forth herein, viral vectors such as lenti- and parvo-virus vectors, including AAV serotypes and variants provide a means for delivery of polynucleotide sequences into cells *ex vivo*, *in vitro* and *in vivo,* which can encode proteins such that the cells express the encoded proteins. For example, a recombinant AAV vector can include a heterologous polynucleotide encoding a desired protein or peptide (*e.g.*, Factor IX). Vector delivery or administration to a subject (*e.g.*, mammal) therefore provides encoded proteins and peptides to the subject. Thus, viral vectors such as lenti- and parvo-virus vectors, including AAV serotypes and variants such as capsid variants (*e.g.*, 4-1) can be used to transfer/deliver heterologous polynucleotides for expression, and optionally for treating a variety of diseases.

**[0039]** A recombinant vector (*e.g.*, AAV) may be a parvovirus vector. Parvoviruses are small viruses with a single-stranded DNA genome. "Adeno-associated viruses" (AAV) are in the parvovirus family.

**[0040]** Parvoviruses including AAV are viruses useful as gene therapy vectors as they can penetrate cells and introduce nucleic acid/genetic material so that the nucleic acid/genetic material may be stably maintained in cells. In addition, these viruses can introduce nucleic acid/genetic material into specific sites, for example, such as a specific site on chromosome 19. Because AAV are not associated with pathogenic disease in humans, AAV vectors are able to deliver heterologous polynucleotide sequences (*e.g.*, therapeutic proteins and agents) to human patients without causing substantial AAV pathogenesis or disease.

**[0041]** AAV and AAV variants (*e.g.,* capsid variants such as 4-1) serotypes *(e.g.,* VP1, VP2, and/or VP3 sequences) may or may not be distinct from other AAV serotypes, including, for example, AAV1-AAV11, Rh74 or Rh10 (*e.g.*, distinct from VP1, VP2, and/or VP3 sequences of any of AAV1-AAV11, Rh74 or Rh10 serotypes).

**[0042]** As used herein, the term "serotype" is a distinction used to refer to an AAV having a capsid that is serologically distinct from other AAV serotypes. Serologic distinctiveness is determined on the basis of the lack of cross-reactivity between antibodies to one AAV as compared to another AAV. Such cross-reactivity differences are usually due to differences in capsid protein sequences/antigenic determinants (*e.g.*, due to VP1, VP2, and/or VP3 sequence differences of AAV serotypes). Despite the possibility that AAV variants including capsid variants may not be serologically distinct from a reference AAV or other AAV serotype, they differ by at least one nucleotide or amino acid residue compared to the reference or other AAV serotype.

**[0043]** Under the traditional definition, a serotype means that the virus of interest has been tested against serum specific for all existing and characterized serotypes for neutralizing activity and no antibodies have been found that neutralize the virus of interest. As more naturally occurring virus isolates of are discovered and/or capsid mutants generated, there may or may not be serological differences with any of the currently existing serotypes. Thus, in cases where the new virus (*e.g.,* AAV) has no serological difference, this new virus (*e.g.,* AAV) would be a subgroup or variant of the corresponding serotype. In many cases, serology testing for neutralizing activity has yet to be performed on mutant viruses with capsid sequence modifications to determine if they are of another serotype according to the traditional definition of serotype. Accordingly, for the sake of convenience and to avoid repetition, the term "serotype" broadly refers to both serologically distinct viruses (*e.g.,* AAV) as well as viruses (*e.g.,* AAV) that are not serologically distinct that may be within a subgroup or a variant of a given serotype.

**[0044]** Recombinant vector (*e.g.,* AAV) plasmids, vector (*e.g.,* AAV) genomes, as well as methods and uses thereof, include any viral strain or serotype. As a non-limiting example, a recombinant vector (*e.g.,* AAV) plasmid or vector (*e.g.,* AAV) genome can be based upon any AAV genome, such as AAV-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11, -rh74, -rh10 or AAV-2i8, for example. Such vectors can be based on the same of strain or serotype (or subgroup or variant), or be different from each other. As a non-limiting example, a recombinant vector (*e.g.*, AAV) plasmid or vector (*e.g.*, AAV) genome based upon one serotype genome can be identical to one or more of the capsid proteins that package the vector. In addition, a recombinant vector (*e.g.*, AAV) plasmid or vector (*e.g.,* AAV) genome can be based upon an AAV (*e.g.,* AAV2) serotype genome distinct from one or more of the capsid proteins that package the vector, in which case at least one of the three capsid proteins could be a AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, Rh10, Rh74 or AAV-2i8 or variant such as AAV-Rh74 variant (*e.g.*, capsid variants such as 4-1, 15-1, 15-2, 15-3/15-5, 15-4 and 15-6), for example.

**[0045]** AAV vectors therefore include gene/protein sequences identical to gene/protein sequences characteristic for a particular serotype. As used herein, an "AAV vector related to AAV1" refers to one or more AAV proteins (*e.g.,* VP1, VP2, and/or VP3 sequences) that has substantial sequence identity to one or more polynucleotides or polypeptide sequences that comprise AAV1. Analogously, an "AAV vector related to AAV8" refers to one or more AAV proteins (*e.g.*, VP1, VP2, and/or VP3 sequences) that has substantial sequence identity to one or more polynucleotides or polypeptide sequences that comprise AAV8. An "AAV vector related to AAV-Rh74" refers to one or more AAV proteins (*e.g.*, VP1, VP2, and/or VP3 sequences) that has substantial sequence identity to one or more polynucleotides or polypeptide sequences that comprise AAV-Rh74. (*see*, *e.g.*, VP1, VP2, VP3 of Figures 1-3). Such AAV vectors related to another serotype, e.g., AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, Rh10, Rh74 or AAV-2i8, can therefore have one or more distinct sequences from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, Rh10, Rh74 and AAV-2i8, but can exhibit substantial sequence identity to one or more genes and/or proteins, and/or have one or more functional characteristics of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, Rh10, Rh74 or AAV-2i8 (*e.g.*, such as cell/tissue tropism). Exemplary non-limiting AAV-Rh74 and related AAV variants such as AAV-Rh74 or related AAV such as AAV-Rh74 variants (*e.g.*, capsid variants such as 4-1, 15-1, 15-2, 15-3/15-5, 15-4 and 15-6) sequences include VP1, VP2, and/or VP3 set forth herein, for example, in Figures 1-9.

**[0046]** An AAV vector related to a reference serotype may have a polynucleotide, polypeptide or subsequence thereof that includes or consists of a sequence at least 80% or more (*e.g.,* 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, etc.) identical to one or more AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, Rh10, Rh74 or AAV-2i8 ( *e.g.,* such as AAV-Rh74 VP1, VP2, and/or VP3 sequences set forth in Figures 1-9).

**[0047]** Methods and uses include AAV sequences (polypeptides and nucleotides), AAV-Rh74 sequences (polypeptides and nucleotides) and subsequences thereof that exhibit less than 100% sequence identity to a reference AAV serotype such as AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, Rh10, or AAV-2i8, for example, AAV-Rh74 gene or protein sequence (*e.g.*, VP1, VP2, and/or VP3 sequences set forth in Figures 1-9), but are distinct from and not identical to known AAV genes or proteins, such as AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, Rh10, Rh74 or AAV-2i8, genes or proteins, etc. An AAV polypeptide or subsequence thereof may include or consist of a sequence at least 80% or more identical, *e.g.*, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, etc., i.e. up to 100% identical to any reference AAV sequence or subsequence thereof, such as AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, Rh10, Rh74 or AAV-2i8 (*e.g.*, VP1, VP2 and/or VP3 sequences set forth in Figures 1-9). An AAV variant may have one, two, three or four of the four amino acid substitutions (*e.g.*, capsid variant 4-1, 15-1, 15-2, 15-3/15-5, 15-4 and 15-6).

**[0048]** Recombinant vectors (*e.g.*, AAV), including AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, Rh10, Rh74 or AAV-2i8 and variant, related, hybrid and chimeric sequences, can be constructed using recombinant techniques that are known to the skilled artisan, to include one or more heterologous polynucleotide sequences (transgenes) flanked with one or more functional AAV ITR sequences. Such vectors can have one or more of the wild type AAV genes deleted in whole or in part, for example, a rep and/or cap gene, but retain at least one functional flanking ITR sequence, as necessary for the rescue, replication, and packaging of the recombinant vector into an AAV vector particle. An AAV vector genome would therefore include sequences required in cis for replication and packaging (*e.g.*, functional ITR sequences)

**[0049]** The terms "polynucleotide" and "nucleic acid" are used interchangeably herein to refer to all forms of nucleic acid, oligonucleotides, including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Polynucleotides include genomic DNA, cDNA and antisense DNA, and spliced or unspliced mRNA, rRNA tRNA and inhibitory DNA or RNA (RNAi, *e.g.*, small or short hairpin (sh)RNA, microRNA (miRNA), small or short interfering (si)RNA, trans-splicing RNA, or antisense RNA). Polynucleotides include naturally occurring, synthetic, and intentionally modified or altered polynucleotides (*e.g.*, having reduced CpG dinucleotides). Polynucleotides can be single, double, or triplex, linear or circular, and can be of any length. In discussing polynucleotides, a sequence or structure of a particular polynucleotide may be described herein according to the convention of providing the sequence in the 5' to 3' direction.

**[0050]** A "heterologous" polynucleotide refers to a polynucleotide inserted into a vector (*e.g.*, AAV) for purposes of vector mediated transfer/delivery of the polynucleotide into a cell. Heterologous polynucleotides are typically distinct from vector (*e.g.*, AAV) nucleic acid, i.e., are non-native with respect to viral (*e.g.*, AAV) nucleic acid. Once transferred/delivered into the cell, a heterologous polynucleotide, contained within the vector, can be expressed (*e.g.*, transcribed, and translated if appropriate). Alternatively, a transferred/delivered heterologous polynucleotide in a cell, contained within the vector, need not be expressed. Although the term "heterologous" is not always used herein in reference to polynucleotides, reference to a polynucleotide even in the absence of the modifier "heterologous" is intended to include heterologous polynucleotides in spite of the omission. An example of a heterologous sequence would be a Factor IX encoding nucleic acid, for example, a modified nucleic acid encoding Factor IX, such as a nucleic acid having reduced CpG dinucleotides relative to a reference nucleic acid sequence.

**[0051]** The "polypeptides," "proteins" and "peptides" encoded by the "polynucleotide sequences," include full-length native sequences, as with naturally occurring proteins, as well as functional subsequences, modified forms or sequence variants so long as the subsequence, modified form or variant retains some degree of functionality of the native full-length protein. Such polypeptides, proteins and peptides encoded by the polynucleotide sequences can be but are not required to be identical to the endogenous protein that is defective, or whose expression is insufficient, or deficient in the treated mammal.

**[0052]** Lenti- and parvo-viral vectors, such as an adeno-viral vector and AAV vectors, including AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, Rh10, Rh74 or AAV-2i8 and related AAV variants such as AAV-Rh74 variants (*e.g.*, capsid variants such as 4-1, 15-1, 15-2, 15-3/15-5, 15-4 and 15-6) can be used to introduce/deliver polynucleotides stably or transiently into cells and progeny thereof. The term "transgene" is used herein to conveniently refer to such a heterologous polynucleotide that is intended or has been introduced into a cell or organism. Transgenes include any polynucleotide, such as a gene that encodes a polypeptide or protein (*e.g.*, Factor IX).

**[0053]** For example, in a cell having a transgene, the transgene has been introduced/transferred by way of vector, such as AAV, "transduction" or "transfection" of the cell. The terms "transduce" and "transfect" refer to introduction of a molecule such as a polynucleotide into a cell or host organism.

**[0054]** A cell into which the transgene has been introduced is referred to as a "transduced cell." Accordingly, a "transduced" cell (*e.g.*, in a mammal, such as a cell or tissue or organ cell), means a genetic change in a cell following incorporation of an exogenous molecule, for example, a polynucleotide or protein (*e.g.*, a transgene) into the cell. Thus, a "transduced" cell is a cell into which, or a progeny thereof in which an exogenous molecule has been introduced, for example. The cell(s) can be propagated and the introduced protein expressed, or nucleic acid transcribed. For gene therapy uses and methods, a transduced cell can be in a subject.

**[0055]** The introduced polynucleotide may or may not be integrated into nucleic acid of the recipient cell or organism. If an introduced polynucleotide becomes integrated into the nucleic acid (genomic DNA) of the recipient cell or organism it can be stably maintained in that cell or organism and further passed on to or inherited by progeny cells or organisms of the recipient cell or organism. Finally, the introduced nucleic acid may exist in the recipient cell or host organism only transiently.

**[0056]** Cells that may be transduced include a cell of any tissue or organ type, of any origin (*e.g.*, mesoderm, ectoderm or endoderm). Non-limiting examples of cells include liver (*e.g.*, hepatocytes, sinusoidal endothelial cells), pancreas (*e.g.*, beta islet cells), lung, central or peripheral nervous system, such as brain (*e.g.*, neural, glial or ependymal cells) or spine, kidney, eye (*e.g.*, retinal, cell components), spleen, skin, thymus, testes, lung, diaphragm, heart (cardiac), muscle or psoas, or gut (*e.g.*, endocrine), adipose tissue (white, brown or beige), muscle (*e.g.*, fibroblasts), synoviocytes, chondrocytes, osteoclasts, epithelial cells, endothelial cells, salivary gland cells, inner ear nervous cells or hematopoietic (*e.g.*, blood or lymph) cells. Additional examples include stem cells, such as pluripotent or multipotent progenitor cells that develop or differentiate into liver (*e.g.*, hepatocytes, sinusoidal endothelial cells), pancreas (*e.g.*, beta islet cells), lung, central or peripheral nervous system, such as brain (*e.g.*, neural, glial or ependymal cells) or spine, kidney, eye (retinal, cell components), spleen, skin, thymus, testes, lung, diaphragm, heart (cardiac), muscle or psoas, or gut (*e.g.*, endocrine), adipose tissue (white, brown or beige), muscle (*e.g.*, fibroblasts), synoviocytes, chondrocytes, osteoclasts, epithelial cells, endothelial cells, salivary gland cells, inner ear nervous cells or hematopoietic (*e.g.*, blood or lymph) cells.

**[0057]** A "therapeutic molecule" may be a peptide or protein that may alleviate or reduce symptoms that result from an absence or defect in a protein in a cell or subject. Alternatively, a "therapeutic" peptide or protein encoded by a transgene may be one that confers a benefit to a subject, *e.g.*, to correct a genetic defect, to correct a gene (expression or functional) deficiency.

**[0058]** Non-limiting examples of heterologous polynucleotides encoding gene products (*e.g.*, therapeutic proteins) include those that may be used in the treatment of a disease or disorder including, but not limited to, blood clotting disorders such as hemophilia A, hemophilia B, thalassemia, and anemia.

**[0059]** Non-limiting examples of mammalian non-human Factor IX sequences are described in Yoshitake et al., 1985, supra; Kurachi et al., 1995, supra; Jallat et al., 1990, supra; Kurachi et al., 1982, Proc. Natl. Acad. Sci. USA 79:6461-6464; Jaye et al., 1983, Nucl. Acids Res. 11:2325-2335; Anson et al., 1984, EMBO J. 3: 1053-1060; Wu et al., 1990, Gene 86:275-278; Evans et al., Proc Natl Acad Sci USA 86:10095 (1989), Blood 74:207-212; Pendurthi et al., 1992, Thromb. Res. 65:177-186; Sakar et al., 1990, Genomics 1990, 6:133-143; and, Katayama et al., 1979, Proc. Natl. Acad. Sci. USA 76:4990-4994.

**[0060]** Polynucleotides, polypeptides and subsequences thereof include modified and variant forms. As used herein, the terms "modify" or "variant" and grammatical variations thereof, mean that a polynucleotide, polypeptide or subsequence thereof deviates from a reference sequence. Modified and variant sequences may therefore have substantially the same, greater or less activity or function than a reference sequence, but at least retain partial activity or function of the reference sequence. In particular embodiments, a modified nucleic acid encodes Factor IX, and has been modified to reduce the number of CpG dinucleotides compared to a reference Factor IX encoding nucleic acid (*e.g.*, wild-type Factor IX sequence, such as a human or other mammalian Factor IX gene sequence).

**[0061]** Variants also include gain and loss of function variants. For example, wild type human Factor IX DNA sequences, which protein variants or mutants retain activity, or are therapeutically effective, or are comparably or even more therapeutically active than invariant human Factor IX. One example of a naturally occurring human Factor IX variant, called the "Padua", human Factor IX has a L (leucine) at position 338 instead of an R (arginine). The Padua FIX has greater catalytic and coagulant activity compared to human Factor IX lacking the Padua mutation. Changing residue 338 in Human Factor IX from arginine to alanine causes an increase in catalytic activity (Chang et al., J. Biol. Chem., 273:12089-94 (1998)). Collagen IV may serve to trap Factor IX, meaning that when introduced into the muscle tissue of a mammal some of the Factor IX is not available for participation in blood coagulation because it is retained in the interstitial spaces in the muscle tissue. A mutation in the sequence of Factor IX that results in a protein with reduced binding to collagen IV (e.g., loss of function) is a useful mutant, for example, for treatment of hemophilia. Such a mutant Factor IX gene may encode a human FIX protein with the amino acid alanine in place of lysine in the fifth amino acid position from the beginning of the mature protein.

**[0062]** Modifications include one or more nucleotide or amino acid substitutions (*e.g.*, 1-3, 3-5, 5-10, 10-15, 15-20, 20-25, 25-30, 30-40, 40-50, 50-100, or more nucleotides or residues), such as substituting a CpG for an alternative dinucleotide in a transgene (*e.g.*, a Factor IX encoding gene, such as FIX encoding gene with a reduced number of CpG dinucleotides). An amino acid substitution may be a conservative amino acid substitution in a capsid sequence. An amino acid substitution may be an arginine for a lysine residue (*e.g.*, one or more arginine substitution of a lysine as set forth in any of 4-1, 15-1, 15-2, 15-3/15-5, 15-4 and/or 15-6). Further modifications include additions (*e.g.*, insertions or 1-3, 3-5, 5-10, 10-15, 15-20, 20-25, 25-30, 30-40, 40-50, 50-100, or more nucleotides or residues) and deletions *(e.g.,* subsequences or fragments) of a reference sequence. A modified or variant sequence may retain at least part of a function or an activity of unmodified sequence. Such modified forms and variants can have the same, less than, or greater, but at least a part of, a function or activity of a reference sequence, for example, as described herein.

**[0063]** A variant can have one or more non-conservative or a conservative amino acid sequence differences or modifications, or both. A "conservative substitution" is the replacement of one amino acid by a biologically, chemically or structurally similar residue. Biologically similar means that the substitution does not destroy a biological activity. Structurally similar means that the amino acids have side chains with similar length, such as alanine, glycine and serine, or a similar size. Chemical similarity means that the residues have the same charge or are both hydrophilic or hydrophobic. Particular examples include the substitution of one hydrophobic residue, such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, serine for threonine, and the like. Particular examples of conservative substitutions include the substitution of a hydrophobic residue such as isoleucine, valine, leucine or methionine for another, the substitution of a polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. For example, conservative amino acid substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. A "conservative substitution" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid.

**[0064]** Accordingly, gene and protein variants (*e.g.*, of polynucleotides encoding proteins described herein) may retain one or more biological activities (*e.g.*, function in blood clotting, etc.). Variants can differ from a reference sequence, such as naturally occurring polynucleotides, proteins or peptides. Such variants of polynucleotides, proteins or polypeptides include proteins or polypeptides which have been or may be modified using recombinant DNA technology such that the polynucleotide, protein or polypeptide possesses altered or additional properties.

**[0065]** At the nucleotide sequence level, a naturally and non-naturally occurring variant gene will typically be at least about 50% identical, more typically about 70% identical, even more typically about 80% identical to the reference gene. Thus, for example, a FIX gene with a reduced number of CpG dinucleotides may have 80% or more identity to wild-type FIX gene, or 80-85%, 85-90%, 90-95%, or more identity to wild-type FIX gene, *e.g.*, 96%, 97%, 98%, or 99% or more identity to wild-type FIX gene.

**[0066]** At the amino acid sequence level, a naturally and non-naturally occurring variant protein will typically be at least about 70% identical, more typically about 80% identical, even more typically about 90% or more identity to the reference protein, although substantial regions of non-identity are permitted in non-conserved regions (*e.g.*, less, than 70% identical, such as less than 60%, 50% or even 40%). The sequences may have at least 60%, 70%, 75% or more identity (*e.g.,* 80%, 85% 90%, 95%, 96%, 97%, 98%, 99% or more identity) to a reference sequence.

**[0067]** The term "identity," "homology" and grammatical variations thereof, mean that two or more referenced entities are the same, when they are "aligned" sequences. Thus, by way of example, when two polypeptide sequences are identical, they have the same amino acid sequence, at least within the referenced region or portion. Where two polynucleotide sequences are identical, they have the same polynucleotide sequence, at least within the referenced region or portion. The identity can be over a defined area (region or domain) of the sequence. An "area" or "region" of identity refers to a portion of two or more referenced entities that are the same. Thus, where two protein or nucleic acid sequences are identical over one or more sequence areas or regions they share identity within that region. An "aligned" sequence refers to multiple polynucleotide or protein (amino acid) sequences, often containing corrections for missing or additional bases or amino acids (gaps) as compared to a reference sequence

**[0068]** The identity can extend over the entire length or a portion of the sequence. The length of the sequence sharing the percent identity may be 2, 3, 4, 5 or more contiguous polynucleotide or amino acids, *e.g.*, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, etc. contiguous polynucleotides or amino acids. The length of the sequence sharing identity may be 21 or more contiguous polynucleotide or amino acids, *e.g.*, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, etc. contiguous polynucleotides or amino acids. The length of the sequence sharing identity may be 41 or more contiguous polynucleotide or amino acids, *e.g.*42, 43, 44, 45, 45, 47, 48, 49, 50, etc., contiguous polynucleotides or amino acids. The length of the sequence sharing identity may be 50 or more contiguous polynucleotide or amino acids, *e.g.*, 50-55, 55-60, 60-65, 65-70, 70-75, 75-80, 80-85, 85-90, 90-95, 95-100, 100-110, etc. contiguous polynucleotide or amino acids.

**[0069]** The terms "homologous" or "homology" mean that two or more referenced entities share at least partial identity over a given region or portion. "Areas, regions or domains" of homology or identity mean that a portion of two or more referenced entities share homology or are the same. Thus, where two sequences are identical over one or more sequence regions they share identity in these regions. "Substantial homology" means that a molecule is structurally or functionally conserved such that it has or is predicted to have at least partial structure or function of one or more of the structures or functions (*e.g.*, a biological function or activity) of the reference molecule, or relevant/corresponding region or portion of the reference molecule to which it shares homology.

**[0070]** The extent of identity (homology) between two sequences can be ascertained using a computer program and/or mathematical algorithm. Such algorithms that calculate percent sequence identity (homology) generally account for sequence gaps and mismatches over the comparison region or area. For example, a BLAST (*e.g.*, BLAST 2.0) search algorithm (*see, e.g.*, Altschul et al., J. Mol. Biol. 215:403 (1990), publicly available through NCBI) has exemplary search parameters as follows: Mismatch -2; gap open 5; gap extension 2. For polypeptide sequence comparisons, a BLASTP algorithm is typically used in combination with a scoring matrix, such as PAM100, PAM 250, BLOSUM 62 or BLOSUM 50. FASTA (*e.g.*, FASTA2 and FASTA3) and SSEARCH sequence comparison programs are also used to quantitate extent of identity (Pearson et al., Proc. Natl. Acad. Sci. USA 85:2444 (1988); Pearson, Methods Mol Biol. 132:185 (2000); and Smith et al., J. Mol. Biol. 147:195 (1981)). Programs for quantitating protein structural similarity using Delaunay-based topological mapping have also been developed (Bostick et al., Biochem Biophys Res Commun. 304:320 (2003)).

**[0071]** Polynucleotides include additions and insertions, for example, one or more heterologous domains. An addition (*e.g.*, heterologous domain) can be a covalent or noncovalent attachment of any type of molecule to a composition. Typically additions and insertions (*e.g.,* a heterologous domain) confer a complementary or a distinct function or activity.

**[0072]** Additions and insertions include chimeric and fusion sequences, which is a polynucleotide or protein sequence having one or more molecules not normally present in a reference native (wild type) sequence covalently attached to the sequence. The terms "fusion" or "chimeric" and grammatical variations thereof, when used in reference to a molecule means that a portions or part of the molecule contains a different entity distinct (heterologous) from the molecule- as they do not typically exist together in nature. That is, for example, one portion of the fusion or chimera, includes or consists of a portion that does not exist together in nature, and is structurally distinct.

**[0073]** The term "vector" refers to a plasmid, virus (*e.g.*, AAV vector), or other vehicle that can be manipulated by insertion or incorporation of a polynucleotide. Such vectors can be used for genetic manipulation (*i.e.*, "cloning vectors"), to introduce/transfer polynucleotides into cells, and to transcribe or translate the inserted polynucleotide in cells. A vector nucleic acid sequence generally contains at least an origin of replication for propagation in a cell and optionally additional elements, such as a heterologous polynucleotide sequence, expression control element (*e.g.*, a promoter, enhancer), intron, ITR(s), selectable marker (*e.g.,* antibiotic resistance), poly-Adenine (also referred to as poly-adenylation) sequence.

**[0074]** A viral vector is derived from or based upon one or more nucleic acid elements that comprise a viral genome. Particular viral vectors include lenti- and parvo-virus vectors, such as adeno-associated virus (AAV) vectors.

**[0075]** As used herein, the term "recombinant," as a modifier of viral vector, such as recombinant lenti- or parvo-virus (*e.g.*, AAV) vectors, as well as a modifier of sequences such as recombinant polynucleotides and polypeptides, means that the compositions (*e.g.*, AAV or sequences) have been manipulated (*i.e.*, engineered) in a fashion that generally does not occur in nature. A particular example of a recombinant vector, such as an AAV vector would be where a polynucleotide that is not normally present in the wild-type viral (*e.g.*, AAV) genome is inserted within the viral genome. For example, an example of a recombinant polynucleotide would be where a heterologous polynucleotide (*e.g.*, gene) encoding a protein is cloned into a vector, with or without 5', 3' and/or intron regions that the gene is normally associated within the viral (*e.g.*, AAV) genome. Although the term "recombinant" is not always used herein in reference to vectors, such as viral and AAV vectors, as well as sequences such as polynucleotides and polypeptides, recombinant forms of viral, AAV, and sequences including polynucleotides and polypeptides, are expressly included in spite of any such omission.

**[0076]** A recombinant viral "vector" or "AAV vector" is derived from the wild type genome of a virus, such as AAV by using molecular methods to remove the wild type genome from the virus (*e.g.*, AAV), and replacing with a non-native nucleic acid, such as a heterologous polynucleotide sequence (*e.g.*, modified nucleic acid sequence encoding human FIX, such as FIX with a reduced number of CpG dinucleotides). Typically, for AAV one or both inverted terminal repeat (ITR) sequences of AAV genome are retained in the AAV vector. A "recombinant" viral vector (*e.g.*, AAV) is distinguished from a viral (*e.g.*, AAV) genome, since all or a part of the viral genome has been replaced with a non-native sequence with respect to the viral (*e.g.*, AAV) genomic nucleic acid such as a heterologous polynucleotide sequence (*e.g.*, modified nucleic acid sequence encoding human FIX, such as FIX with a reduced number of CpG dinucleotides). Incorporation of a non-native sequence (*e.g.*, modified nucleic acid sequence encoding human FIX, such as FIX with a reduced number of CpG dinucleotides) therefore defines the viral vector (*e.g.*, AAV) as a "recombinant" vector, which in the case of AAV can be referred to as a "rAAV vector."

**[0077]** A recombinant vector (*e.g.*, lenti-, parvo-, AAV) sequence can be packaged - referred to herein as a "particle" - for subsequent infection (transduction) of a cell, *ex vivo*, *in vitro* or *in vivo*. Where a recombinant vector sequence is encapsidated or packaged into an AAV particle, the particle can also be referred to as a "rAAV." Such particles include proteins that encapsidate or package the vector genome. Particular examples include viral envelope proteins, and in the case of AAV, capsid proteins.

**[0078]** For a recombinant plasmid, a vector "genome" refers to the portion of the recombinant plasmid sequence that is ultimately packaged or encapsidated to form a viral (*e.g.*, AAV) particle. In cases where recombinant plasmids are used to construct or manufacture recombinant vectors, the vector genome does not include the portion of the "plasmid" that does not correspond to the vector genome sequence of the recombinant plasmid. This non vector genome portion of the recombinant plasmid is referred to as the "plasmid backbone," which is important for cloning and amplification of the plasmid, a process that is needed for propagation and recombinant virus production, but is not itself packaged or encapsidated into virus (*e.g.*, AAV) particles.

**[0079]** Thus, a vector "genome" refers to the portion of the vector plasmid that is packaged or encapsidated by virus (*e.g.*, AAV), and which contains a heterologous polynucleotide sequence. The non vector genome portion of the recombinant plasmid is the "plasmid backbone" that is important for cloning and amplification of the plasmid, *e.g.*, has a selectable marker, such as Kanamycin, but is not itself packaged or encapsidated by virus (*e.g.*, AAV).

**[0080]** Amounts of rAAV that encapsidate/package vector genomes can be determined, for example, by quantitative PCR. This assay measures the physical number of packaged vector genomes by real-time quantitative polymerase chain reaction and can be performed at various stages of the manufacturing/prufication process, for example, on bulk AAV vector and final product.

**[0081]** Recombinant vector sequences are manipulated by insertion or incorporation of a polynucleotide. A vector plasmid generally contains at least an origin of replication for propagation in a cell and one or more expression control elements.

**[0082]** Vector sequences including AAV vectors can include one or more "expression control elements." Typically, expression control elements are nucleic acid sequence(s) that influence expression of an operably linked polynucleotide. Control elements, including expression control elements such as promoters and enhancers, present within a vector are included to facilitate proper heterologous polynucleotide transcription and if appropriate translation (*e.g.*, a promoter, enhancer, splicing signal for introns, maintenance of the correct reading frame of the gene to permit in-frame translation of mRNA and, stop codons etc.). Such elements typically act in cis, referred to as a "cis acting" element, but may also act in trans.

**[0083]** Expression control can be effected at the level of transcription, translation, splicing, message stability, etc. Typically, an expression control element that modulates transcription is juxtaposed near the 5' end (*i.e.*, "upstream") of a transcribed polynucleotide (*e.g.*, of a modified nucleic acid encoding Factor IX, such as FIX with a reduced number of CpG dinucleotides). Expression control elements can also be located at the 3' end (*i.e.*, "downstream") of the transcribed sequence or within the transcript (*e.g.*, in an intron). Expression control elements can be located adjacent to or at a distance away from the transcribed sequence (*e.g.*, 1-10, 10-25, 25-50, 50-100, 100 to 500, or more nucleotides from the polynucleotide), even at considerable distances. Nevertheless, owing to the polynucleotide length limitations of certain vectors, such as AAV vectors, such expression control elements will typically be within 1 to 1000 nucleotides from the transcribed polynucleotide.

**[0084]** Functionally, expression of operably linked heterologous polynucleotide is at least in part controllable by the element (*e.g.*, promoter) such that the element modulates transcription of the polynucleotide and, as appropriate, translation of the transcript. A specific example of an expression control element is a promoter, which is usually located 5' of the transcribed sequence. Another example of an expression control element is an enhancer, which can be located 5', 3' of the transcribed sequence, or within the transcribed sequence.

**[0085]** A "promoter" as used herein can refer to a nucleic acid (*e.g.*, DNA) sequence that is located adjacent to a polynucloetide sequence that encodes a recombinant product. A promoter is typically operatively linked to an adjacent sequence, *e.g.,* heterologous polynucleotide (*e.g.*, modified nucleic acid encoding Factor IX). A promoter typically increases an amount expressed from a heterologous polynucleotide as compared to an amount expressed when no promoter exists.

**[0086]** An "enhancer" as used herein can refer to a sequence that is located adjacent to the heterologous polynucleotide. Enhancer elements are typically located upstream of a promoter element but also function and can be located downstream of or within a DNA sequence (*e.g.*, a heterologous polynucleotide). Hence, an enhancer element can be located 100 base pairs, 200 base pairs, or 300 or more base pairs upstream or downstream of a heterologous polynucleotide. Enhancer elements typically increase expressed of a heterologous polynucleotide above increased expression afforded by a promoter element.

**[0087]** Expression control elements (*e.g.*, promoters) include those active in a particular tissue or cell type, referred to herein as a "tissue-specific expression control elements/promoters." Tissue-specific expression control elements are typically active in specific cell or tissue (*e.g.*, liver, brain, central nervous system, spinal cord, eye, retina, bone, muscle, lung, pancreas, heart, kidney cell, etc.). Expression control elements are typically active in these cells, tissues or organs because they are recognized by transcriptional activator proteins, or other regulators of transcription, that are unique to a specific cell, tissue or organ type.

**[0088]** Examples of promoters active in skeletal muscle include promoters from genes encoding skeletal α-actin, myosin light chain 2A, dystrophin, muscle creatine kinase, as well as synthetic muscle promoters with activities higher than naturally-occurring promoters (*see, e.g.*, Li, et al., Nat. Biotech. 17:241-245 (1999)). Examples of promoters that are tissue-specific for liver are the human alpha 1-antitrypsin (hAAT) promoter; albumin, Miyatake, et al. J. Virol., 71:5124-32 (1997); hepatitis B virus core promoter, Sandig, et al., Gene Ther. 3:1002-9 (1996); alpha-fetoprotein (AFP), Arbuthnot, et al., Hum. Gene. Ther., 7:1503-14 (1996)], bone (osteocalcin, Stein, et al., Mol. Biol. Rep., 24:185-96 (1997); bone sialoprotein, Chen, et al., J. Bone Miner. Res. 11 :654-64 (1996)), lymphocytes (CD2, Hansal, et al., J. Immunol., 161:1063-8 (1998); immunoglobulin heavy chain; T cell receptor a chain), neuronal (neuron-specific enolase (NSE) promoter, Andersen, et al., Cell. Mol. Neurobiol., 13:503-15 (1993); neurofilament light-chain gene, Piccioli, et al., Proc. Natl. Acad. Sci. USA, 88:5611-5 (1991); the neuron-specific vgf gene, Piccioli, et al., Neuron, 15:373-84 (1995); among others. An example of an enhancer active in liver is apolipoprotein E (apoE) HCR-1 and HCR-2 (Allan et al., J. Biol. Chem., 272:29113-19 (1997)).

**[0089]** Expression control elements also include ubiquitous or promiscuous promoters/enhancers which are capable of driving expression of a polynucleotide in many different cell types. Such elements include, but are not limited to the cytomegalovirus (CMV) immediate early promoter/enhancer sequences, the Rous sarcoma virus (RSV) promoter/enhancer sequences and the other viral promoters/enhancers active in a variety of mammalian cell types, or synthetic elements that are not present in nature (*see, e.g.*, Boshart et al, Cell, 41:521-530 (1985)), the SV40 promoter, the dihydrofolate reductase promoter, the cytoplasmic β-actin promoter and the phosphoglycerol kinase (PGK) promoter.

**[0090]** Expression control elements also can confer expression in a manner that is regulatable, that is, a signal or stimuli increases or decreases expression of the operably linked heterologous polynucleotide. A regulatable element that increases expression of the operably linked polynucleotide in response to a signal or stimuli is also referred to as an "inducible element" (*i.e.,* is induced by a signal). Particular examples includea hormone (*e.g.,* steroid) inducible promoter. A regulatable element that decreases expression of the operably linked polynucleotide in response to a signal or stimuli is referred to as a "repressible element" (*i.e.,* the signal decreases expression such that when the signal, is removed or absent, expression is increased). Typically, the amount of increase or decrease conferred by such elements is proportional to the amount of signal or stimuli present; the greater the amount of signal or stimuli, the greater the increase or decrease in expression. Particular examples include zinc-inducible sheep metallothionine (MT) promoter; the steroid hormone-inducible mouse mammary tumor virus (MMTV) promoter; the T7 polymerase promoter system (WO 98/10088); the tetracycline-repressible system (Gossen, et al., Proc. Natl. Acad. Sci. USA, 89:5547-5551 (1992)); the tetracycline-inducible system (Gossen, et al., Science. 268:1766-1769 (1995); see also Harvey, et al., Curr. Opin. Chem. Biol. 2:512-518 (1998)); the RU486-inducible system

(Wang, et al., Nat. Biotech. 15:239-243 (1997) and Wang, et al., Gene Ther. 4:432-441 (1997)]; and the rapamycin-inducible system (Magari, et al., J. Clin. Invest. 100:2865-2872 (1997); Rivera, et al., Nat. Medicine. 2: 1028-1032 (1996)). Other regulatable control elements which may be useful in this context are those which are regulated by a specific physiological state, e.g., temperature, acute phase, development.

**[0091]** Expression control elements also include the native elements(s) for the heterologous polynucleotide. A native control element (*e.g.*, promoter) may be used when it is desired that expression of the heterologous polynucleotide should mimic the native expression. The native element may be used when expression of the heterologous polynucleotide is to be regulated temporally or developmentally, or in a tissue-specific manner, or in response to specific transcriptional stimuli. Other native expression control elements, such as introns, polyadenylation sites or Kozak consensus sequences may also be used.

**[0092]** As used herein, the term "operable linkage" or "operably linked" refers to a physical or functional juxtaposition of the components so described as to permit them to function in their intended manner. In the example of an expression control element in operable linkage with a nucleic acid, the relationship is such that the control element modulates expression of the nucleic acid. More specifically, for example, two DNA sequences operably linked means that the two DNAs are arranged (cis or trans) in such a relationship that at least one of the DNA sequences is able to exert a physiological effect upon the other sequence.

**[0093]** Accordingly, modified nucleic acid sequences encoding human FIX protein, and vectors and plasmids, including viral vectors such as lenti- and parvovirus vectors, including AAV vectors, as well as compositions thereof, can include additional nucleic acid elements. These elements include, without limitation one or more copies of an AAV ITR sequence, an expression control (*e.g.*, promoter/enhancer) element, a transcription termination signal or stop codon, 5' or 3' untranslated regions (*e.g.*, polyadenylation (polyA) sequences) which flank a polynucleotide sequence, or an intron, such as all or a portion of intron I of genomic human Factor IX (SEQ ID NO: 13).

**[0094]** Nucleic acid elements further include, for example, filler or stuffer polynucleotide sequences, for example to improve packaging and reduce the presence of contaminating nucleic acid, e.g., to reduce packaging of the plasmid backbone. AAV vectors typically accept inserts of DNA having a defined size range which is generally about 4 kb to about 5.2 kb, or slightly more. Thus, for shorter sequences, inclusion of a stuffer or filler in the insert fragment in order to adjust the length to near or at the normal size of the virus genomic sequence acceptable for AAV vector packaging into virus particle. A filler/stuffer nucleic acid sequence may be an untranslated (non-protein encoding) segment of nucleic acid. In an AAV vector, a heterologous polynucleotide sequence may have a length less than 4.7 kb and the filler or stuffer polynucleotide sequence has a length that when combined (*e.g.*, inserted into a vector) with the heterologous polynucleotide sequence has a total length between about 3.0-5.5kb, or between about 4.0-5.0Kb, or between about 4.3-4.8Kb.

**[0095]** An intron can also function as a filler or stuffer polynucleotide sequence in order to achieve a length for AAV vector packaging into a virus particle. Introns and intron fragments (*e.g.* portion of intron I of FIX) that function as a filler or stuffer polynucleotide sequence also can enhance expression. Inclusion of an intron element may enhance expression compared with expression in the absence of the intron element (Kurachi et al., 1995, supra).

**[0096]** The use of introns is not limited to the inclusion of Factor IX intron I sequences, but also include other introns, which introns may be associated with the same gene (*e.g.*, where the nucleic acid encodes Factor IX, the intron is derived from an intron present in Factor IX genomic sequence) or associated with a completely different gene or other DNA sequence. Accordingly, other untranslated (non-protein encoding) regions of nucleic acid, such as introns found in genomic sequences from cognate (related) genes (the heterologous polynucleotide sequence encodes all or a portion of same protein encoded by the genomic sequence) and non-cognate (unrelated) genes (the heterologous polynucleotide sequence encodes a protein that is distinct from the protein encoded by the genomic sequence) can also function as filler or stuffer polynucleotide sequences.

**[0097]** A "portion of intron I" as used herein, is meant region of intron I having a nucleotide length of from about 0.1 kb to about 1.7 kb, which region enhances expression of Factor IX, typically by about 1.5-fold or more on a plasmid or viral vector template when compared with expression of FIX in the absence of a portion of intron I. A more specific portion is a 1.3 kb portion of intron I. A non-limiting example of a sequence of Factor IX intron I is intron A, a chimera composed of the 5' part and the 3' part of FIX first intron as set forth in SEQ ID NO:13.

**[0098]** Expression control elements, ITRs, poly A sequences, filler or stuffer polynucleotide sequences can vary in length. An expression control element, ITR, poly A, or a filler or stuffer polynucleotide sequence may be a sequence between about 1-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-75, 75-100, 100-150, 150-200, 200-250, 250-300, 300-400, 400-500, 500-750, 750-1,000, 1,000-1,500, 1,500-2,000, or 2,000-2,500 nucleotides in length.

**[0099]** An AAV vector may comprise an AAV capsid comprising the 4-1 capsid variant VP1 protein (SEQ ID NO:4) and a genome for expressing a heterologous gene in a transduced mammalian cell.

**[0100]** The capsid of this vector may further comprise the VP2 and VP3 proteins from the 4-1 capsid variant (SEQ ID NO:27 and SEQ ID NO:3, respectively). The VP1 protein and VP2 proteins may be in a stoichiometric ratio of approximately 1:1 (or some other ratio), and the VP3 protein may be in a ratio to either VP1 or VP2, or both VP1 and VP2, in an approximate ratio of about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, 15:1, 16:1, 17:1, 18:1, 19:1, 20:1, or some other ratio.

**[0101]** A genome of an AAV vector, including without limitation one having a capsid comprising the 4-1 capsid variant proteins (VP1, VP2, VP3), may comprise a heterologous nucleic acid sequence encoding human Factor IX (FIX) protein. FIX protein may be wild type, contain a substitution mutation or other mutation that alters the protein's activity. The mutation may increase FIX catalytic activity and/or activity of the protein as a procoagulant. In accordance with the invention, the FIX protein is the Padua FIX protein, with an Arg to Ala substitution at the amino acid corresponding to position 338 of FIX protein. A gene encoding human FIX (including FIX Padua) may be codon optimized, for example, by reducing or even eliminating CpG dinucleotides. Other types of codon optimization are possible as well.

**[0102]** A genome of the AAV vector may further comprise inverted terminal repeats (ITRs) from AAV2 positioned at the left and right ends (i.e., 5' and 3' termini, respectively) of the genome. A left ITR may comprise or consist of nucleotides 1-141 from SEQ ID NO:12 (disclosed herein as SEQ ID NO: 13), and a right ITR may comprise or consist of nucleotides 4097-4204 from SEQ ID NO:12 (disclosed herein as SEQ ID NO:20). Each ITR may be separated from other elements in the vector genome by a nucleic acid sequence of variable length.

**[0103]** A genome of the AAV may vector further comprise an expression control element, including a promoter, and optionally an enhancer. An AAV vector genome may comprise both a promoter and an enhancer, which may be constituitive, inducible, or tissue specific. A promoter, or an enhancer, or both may be tissue specific. Both enhancer and promoter may be active in hepatocytes, compared to certain other cell types. An enhancer may be all or a portion of the human ApoE HCR-1 enhancer, and a promoter may be all or a portion of the human alpha-1 antitrypsin (AAT) promoter. An AAV vector genome may include a human ApoE HCR-1 enhancer comprising or consisting of nucleotides 152-472 from SEQ ID NO:12 (disclosed herein as SEQ ID NO:14), and may include a human AAT promoter comprising or consisting of nucleotides 482-878 from SEQ ID NO: 12 (disclosed herein as SEQ ID NO:15). An ApoE HCR-1 enhancer may be positioned 5' of an AAT promoter, and the sequences may be contiguous, or separated by another nucleotide sequence. An enhancer and promoter may be positioned 5' of a nucleic acid sequence coding Factor IX, and may be continguously joined to the first exon of a Factor IX gene, or may be separated therefrom by 5' untranslated sequence (UTR) from a human Factor IX gene, or some other sequence serving as a spacer. A 5' UTR sequence may comprise or consist of nucleotides 879-907 from SEQ ID NO:12.

**[0104]** Gene encoding FIX, including naturally occurring FIX Padua, may include one or more introns present in a human Factor IX genomic sequence. All introns may be excluded, an example of which is disclosed as the nucleic acid sequence of SEQ ID NO: 10 and referred to herein as the coding sequence for "FIX39." If present, an intron can behave as a stuffer or filler sequence as described herein. The entire gene can be codon-optimized to deplete or eliminate CpG dinucleotides.

**[0105]** A gene encoding human Factor IX used in an AAV vector may comprise or consist of nucleotides 908-3731 from SEQ ID NO:12, which encodes the FIX Padua and is codon-optimized to eliminate CpG dinucleotides. This sequence includes an exon 1 (nucleotides 908-995 from SEQ ID NO:12), a first intron (sometimes known as intron I; nucleotides 996-2433 from SEQ ID NO:12), and exons 2-8 (nucleotides 2434-3731 from SEQ ID NO:12).

**[0106]** A gene encoding Factor IX may be followed at its 3' end by 3' UTR sequence from a human Factor IX gene (such as without limitation nucleotides 3732-3779 from SEQ ID NO: 12 and/or by a polyadenylate (polyA) sequence from a Factor IX gene, or another gene. The polyA sequence can be from the bovine growth hormone (bGH) gene, and can comprise or consist of nucleotides 3820-4047 from SEQ ID NO:12. A 3'UTR can be variably spaced from the polyA sequence by an intervening sequence of nucleotides.

**[0107]** The elements described above can be combined into one AAV vector genome. An AAV vector can have a genome comprising, in 5' to 3' order, a left AAV ITR, the ApoE HCR-1 enhancer (or portion thereof), the hAAT promoter (or portion thereof), a portion of human Factor IX 5'UTR, nucleic acid encoding human Factor IX Padua (including optionally one or more introns, such as intron I), a portion of human Factor IX 3' UTR, a polyA sequence from bGH (or portion thereof), and at the right an AAV2 ITR. A left AAV2 ITR may have the nucleic acid sequence of SEQ ID NO: 13; an ApoE HCR-1 enhancer may have the nucleic acid sequence of SEQ ID NO:14; a hAAT promoter may have the nucleic acid sequence of SEQ ID NO:15; a 5' UTR may have the nucleic acid sequence of SEQ ID NO: 16; a gene encoding FIX Padua (including intron I) may encode the FIX protein encoded by nucleic acid sequence of SEQ ID NO: 10; the 3' UTR may have a nucleic acid sequence of SEQ ID NO: 18; a polyA region may have the nucleic acid sequence of SEQ ID NO: 19; and a right AAV2 ITR may have a nucleic acid sequence of SEQ ID NO:20.

**[0108]** A genome of an AAV vector may comprise or consist of nucleotides 1-4204 from SEQ ID NO: 12, or a sequence that is at least 95%, 96%, 97%, 98%, or 99% identical thereto. A capsid may comprise the 4-1 VP1 capsid protein variant (SEQ ID NO:4) and the corresponding VP2 and VP2 capsid proteins. In a particular AAV vector, referred to herein as "AAV-FIX39-Padua," the vector may include a capsid formed from 4-1 capsid variant proteins (VP1, VP2, VP3), and a single-stranded genome comprising a nucleic acid sequence corresponding to nucleotides 1-4204 from SEQ ID NO: 12.

**[0109]** AAV "empty capsids" as used herein do not contain a vector genome (hence, the term "empty"), in contrast to "genome containing capsids" which contain an AAV vector genome. Empty capsids are virus-like particles in that they react with one or more antibodies that reacts with the intact (genome containing AAV vector) virus.

**[0110]** Empty capsids can be included in AAV vector preparations. If desired, AAV empty capsids can be added to AAV vector preparations, or administered separately to a subject in accordance with herein described, yet unclaimed methods.
It is generally to be understood that any references to methods of treatment by therapy or surgery in the present description are to be interpreted as references to pharmaceutical compositions of the present invention for use in those methods.

**[0111]** Although not wishing to be bound by theory, AAV empty capsids are believed to bind to or react with antibodies against the AAV vectors, thereby functioning as a decoy to reduce inactivation of the AAV vector. Such a decoy acts to absorb antibodies directed against the AAV vector thereby increasing or improving AAV vector transgene transduction of cells (introduction of the transgene), and in turn increased cellular expression of the transcript and/or encoded protein.

**[0112]** Empty capsids can be generated and purified at a quality and their quantities determined. For example, empty capsid titer can be measured by spectrophotometry by optical density at 280nm wavelength (based on Sommer et al., Mol. Ther. 2003 Jan;7(1): 122-8).

**[0113]** Empty-AAV or empty capsids are sometimes naturally found in AAV vector preparations. Such natural mixtures can be used in accordance with the invention, or if desired be manipulated to increase or decrease the amount of empty capsid and/or vector. For example, the amount of empty capsid can optionally be adjusted to an amount that would be expected to reduce the inhibitory effect of antibodies that react with an AAV vector that is intended to be used for vector-mediated gene transduction in the subject. The use of empty capsids is described in US Publication 2014/0336245.

**[0114]** AAV empty capsids may be formulated with AAV vectors and/or administered to a subject. AAV empty capsids may be formulated with less than or an equal amount of vector (e.g., about 1.5 to 100-fold AAV vectors to AAV empty capsids, or about a 1:1 ratio of AAV vectors to AAV empty capsids). AAV vectors may be formulated with an excess of AAV empty capsids (e.g., greater than 1 fold AAV empty capsids to AAV vectors, e.g., 1.5 to 100-fold AAV empty capsids to AAV vectors). Optionally, subjects with low to negative titer AAV NAb can receive lower amounts of empty capsids (1 to 10 fold AAV empty capsids to AAV vectors, 2-6 fold AAV empty capsids to AAV vectors, or about 4-5 fold AAV empty capsids to AAV vectors).

**[0115]** Pharmaceutical compositions comprising AAV vectors including those comprising the 4-1 capsid variant proteins (VP1, VP2 and VP3), may comprise an excess of empty capsids greater than the concentration of AAV vectors (i.e., those containing a vector genome) in the composition. A ratio of empty capsids to AAV vectors can be about 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9.0, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10 to 1, or some other ratio.

**[0116]** Empty capsids may comprise the same VP1, VP2, and VP3 capsid proteins that are present in the AAV vectors. Empty capsids may comprise VP1, VP2 and VP3 proteins having a different amino acid sequence than those found in the AAV vectors. Typically, although not necessarily, if the capsid proteins of the empty capsids and capsids of the AAV vectors are not identical in sequence, they will be of the same serotype.

**[0117]** A composition may comprise an AAV vector described herein as AAV-FIX39-Padua (or one having the same capsid and a genome sequence at least 95%, 96%, 97%, 98% or 99%

identical thereto) and optionally an excess of empty capsids comprising the same capsid proteins, wherein the ratio of empty capsids to the AAV vector is about 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9.0, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10 to 1, or some other ratio. The ratio in the composition of AAV-FIX39-Padua to empty capsids may be about 1:5. Compositions comprising AAV-FIX39-Padua and empty capsids may be administered to a human subject having hemophilia B, including severe, moderate, or mild hemophilia B.

**[0118]** A "selectable marker gene" refers to a gene that when expressed confers a selectable phenotype, such as antibiotic resistance (*e.g.*, kanamycin), on a transduced cell. A "reporter" gene is one that provides a detectable signal. A non-limiting example of a reporter gene is the luciferase gene.

**[0119]** Nucleic acid, polynucleotides, expression vectors (*e.g.*, vector genomes), plasmids, including modified forms can be made using various standard cloning, recombinant DNA technology, via cell expression or *in vitro* translation and chemical synthesis techniques. Purity of polynucleotides can be determined through sequencing, gel electrophoresis and the like. For example, nucleic acids can be isolated using hybridization or computer-based database screening techniques. Such techniques include, but are not limited to: (1) hybridization of genomic DNA or cDNA libraries with probes to detect homologous nucleotide sequences; (2) antibody screening to detect polypeptides having shared structural features, for example, using an expression library; (3) polymerase chain reaction (PCR) on genomic DNA or cDNA using primers capable of annealing to a nucleic acid sequence of interest; (4) computer searches of sequence databases for related sequences; and (5) differential screening of a subtracted nucleic acid library.

**[0120]** The term "isolated," when used as a modifier of a composition, means that the compositions are made by the hand of man or are separated, completely or at least in part, from their naturally occurring *in vivo* environment. Generally, isolated compositions are substantially free of one or more materials with which they normally associate with in nature, for example, one or more protein, nucleic acid, lipid, carbohydrate, cell membrane. The term "isolated" does not exclude combinations produced by the hand of man, for example, a recombinant vector (*e.g.*, rAAV) sequence, or virus particle that packages or encapsidates a vector genome and a pharmaceutical formulation. The term "isolated" also does not exclude alternative physical forms of the composition, such as hybrids/chimeras, multimers/oligomers, modifications (*e.g.*, phosphorylation, glycosylation, lipidation) or derivatized forms, or forms expressed in host cells produced by the hand of man.

**[0121]** Methods and uses described herein, but not covered by the claimed invention, provide a means for delivering (transducing) heterologous polynucleotides (transgenes) into host cells, including dividing and/or non-dividing cells. The recombinant vector (*e.g.*, rAAV) sequences, vector genomes, recombinant virus particles, methods, uses and pharmaceutical formulations of the invention are additionally useful in a method of delivering, administering or providing a nucleic acid, or protein to a subject in need thereof, as a method of treatment. In this manner, the nucleic acid is transcribed and the protein may be produced *in vivo* in a subject. The subject may benefit from or be in need of the nucleic acid or protein because the subject has a deficiency of the nucleic acid or protein, or because production of the nucleic acid or protein in the subject may impart some therapeutic effect, as a method of treatment or otherwise.

**[0122]** In general, recombinant lenti- or parvo-virus vector (*e.g.*, AAV) sequences, vector genomes, recombinant virus particles, methods and uses may be used to deliver any heterologous polynucleotide (transgene) with a biological effect to treat or ameliorate one or more symptoms associated with any disorder related to insufficient or undesirable gene expression. Recombinant lenti- or parvo-virus vector (*e.g.*, AAV) sequences, plasmids, vector genomes, recombinant virus particles, methods and uses may be used to provide therapy for various disease states.

**[0123]** Nucleic acids, vectors, recombinant vectors (*e.g.*, rAAV), vector genomes, and recombinant virus particles, methods and uses permit the treatment of genetic diseases. In general, disease states fall into two classes: deficiency states, usually of enzymes, which are generally inherited in a recessive manner, and unbalanced states, at least sometimes involving regulatory or structural proteins, which are inherited in a dominant manner. For deficiency state diseases, gene transfer could be used to bring a normal gene into affected tissues for replacement therapy, as well as to create animal models for the disease using antisense mutations. For unbalanced disease states, gene transfer could be used to create a disease state in a model system, which could then be used in efforts to counteract the disease state. The use of site-specific integration of nucleic acid sequences to correct defects is also possible.

**[0124]** Illustrative examples of disease states include, but are not limited to: blood coagulation disorders such as hemophilia A, hemophilia B, thalassemia, and anemia.

**[0125]** Treatment methods and uses described herein, but not claimed include nucleic acids, vectors, recombinant vectors (*e.g.*, rAAV), vector genomes, and recombinant virus particles. Methods and uses of the invention are broadly applicable to providing, or increasing or stimulating, gene expression or function, *e.g.*, gene addition or replacement. It is generally to be understood that any references to methods of treatment by therapy or surgery in the present description are to be interpreted as references to pharmaceutical compositions of the present invention for use in those methods.

**[0126]** A method or use may include: (a) providing a modified nucleic acid encoding Factor IX, such as FIX with a reduced number of CpG dinucleotides, such as in a vector or a vector genome, wherein the modified nucleic acid sequence is operably linked to an expression control element conferring transcription of said sequence; and (b) administering an amount of the modified nucleic acid to the mammal such that Factor IX is expressed in the mammal.

**[0127]** A method or use may include delivering or transferring a modified nucleic acid encoding Factor IX sequence, such as FIX with a reduced number of CpG dinucleotides, into a mammal or a cell of a mammal, by administering a viral (*e.g.*, AAV) particle or plurality of viral (*e.g.*, AAV) particles (*e.g.*, such as capsid variants (*e.g.*, 4-1)) comprising a vector genome, the vector genome comprising the modified nucleic acid encoding Factor IX, such as FIX with a reduced number of CpG dinucleotides (and optionally an ITR, intron, poly A, a filler/stuffer polynucleotide sequence) to a mammal or a cell of a mammal, thereby delivering or transferring the modified nucleic acid encoding Factor IX into the mammal or cell of the mammal.

**[0128]** In such methods and uses disclosed herein, but not claimed, expression of the nucleic acid may provide a therapeutic benefit to the mammal (*e.g.*, human). Expression of Factor IX may provide a therapeutic benefit to the mammal (*e.g.*, human), such as a mammal that has hemophilia B. A filler/stuffer polynucleotide sequence may be included in the vector sequence such that the combined length with the modified nucleic acid encoding Factor IX, such as FIX with a reduced number of CpG dinucleotides, has a total length of between about 3.0Kb - 5.5Kb, or between about 4.0Kb - 5.0Kb, or between about 4.3Kb - 4.8kb.

**[0129]** Methods and uses described herein, but not claimed,include treatment methods, which result in any therapeutic or beneficial effect. Such methods and uses may be inhibiting, decreasing or reducing one or more adverse (*e.g.*, physical) symptoms, disorders, illnesses, diseases or complications caused by or associated with the disease. For a bleeding disorder such as hemophilia, a therapeutic or beneficial effect includes, but is not limited to, reduced bruising, reduced blood clotting time, reduced bleeding episodes (duration, severity, frequency). For example, reduced duration, severity or frequency of joint or cerebral (brain) bleeding episodes. For a bleeding disorder such as hemophilia, a therapeutic or beneficial effect also includes, but is not limited to, reduced dosage of a supplemental clotting factor protein (*e.g.*, Factor IX protein) or elimination of administration of a supplemental clotting factor protein (*e.g.*, Factor IX protein).

**[0130]** A therapeutic or beneficial effect of treatment is therefore any objective or subjective measurable or detectable improvement or benefit provided to a particular subject. A therapeutic or beneficial effect can but need not be complete ablation of all or any particular adverse symptom, disorder, illness, or complication of a disease. Thus, a satisfactory clinical endpoint is achieved when there is an incremental improvement or a partial reduction in an adverse symptom, disorder, illness, or complication caused by or associated with a disease, or an inhibition, decrease, reduction, suppression, prevention, limit or control of worsening or progression of one or more adverse symptoms, disorders, illnesses, or complications caused by or associated with the disease, over a short or long duration (hours, days, weeks, months, etc.).

**[0131]** Compositions, such as nucleic acids, vectors, recombinant vectors (*e.g.*, rAAV), vector genomes, and recombinant virus particles including vector genomes, can be administered in a sufficient or effective amount to a subject in need thereof. An "effective amount" or "sufficient amount" refers to an amount that provides, in single or multiple doses, alone or in combination, with one or more other compositions (therapeutic agents such as a drug), treatments, protocols, or therapeutic regimens agents, a detectable response of any duration of time (long or short term), an expected or desired outcome in or a benefit to a subject of any measurable or detectable degree or for any duration of time (*e.g.*, for minutes, hours, days, months, years, or cured).

**[0132]** One skilled in the art can determine whether administration of a single rAAV/vector dose is sufficient or whether are to administer multiple doses of rAAV/vector. For example, if FIX levels decrease below a pre-determined level (e.g., less than the minimum that provides a therapeutic benefit), one skilled in the art can determine if appropriate to administer additional doses of rAAV/vector.

**[0133]** The dose to achieve a therapeutic effect, *e.g.*, the dose in vector genomes/per kilogram of body weight (vg/kg), will vary based on several factors including, but not limited to: route of administration, the level of heterologous polynucleotide expression required to achieve a therapeutic effect, the specific disease treated, any host immune response to the viral vector, a host immune response to the heterologous polynucleotide or expression product (protein), and the stability of the protein expressed. One skilled in the art can determine a rAAV/vector genome dose range to treat a patient having a particular disease or disorder based on the aforementioned factors, as well as other factors. Generally, doses will range from at least $1\times10^{8}$, or more, for example, $1\times10^{9}$, $1\times10^{10}$, $1\times10^{11}$, $1\times10^{12}$, $1\times10^{13}$ or $1\times10^{14}$, or more, vector genomes per kilogram (vg/kg) of the weight of the subject, to achieve a therapeutic effect.

**[0134]** A therapeutically effective dose of an AAV vector, including, for example, AAV-FIX39-Padua, or one having the same capsid and a genome sequence at least 95%, 96%, 97%, 98% or 99% identical thereto, may be one that is sufficient, when administered to a subject, for example, a human, with hemophilia B or other deficiency of Factor IX activity, to convert severe hemophilia B to moderate or mild hemophilia B, or even to result in an apparently disease-free state. A therapeutically effective dose of an AAV vector may be one that is sufficient to allow a human subject with hemophilia B to forego Factor IX replacement therapy entirely, or reduce the frequency with which replacement FIX is administered to maintain adequate hemostasis. As understood by those of skill in the art, factor replacement therapy is the current standard of care for hemophilia B, but requires frequent injections of recombinantly produced human Factor IX to compensate for the patient's inability to produce sufficient levels of functional clotting factor.

**[0135]** It is generally accepted that severe hemophilia B is characterized by frequent bleeding (for example, at least once or twice per week), often spontaneously (without preceding trauma), into a subject's muscles or joints. Less than 1% of FIX activity found in healthy humans is associated with severe hemophilia B. It is generally accepted that human subjects with moderate hemophilia B bleed less frequently than those with severe hemophilia B, for example, about once per month, but will bleed for a longer time than normal after surgery, trauma, or dental work. It is generally accepted that human subjects with moderate disease do not often bleed spontaneously. FIX activity 1%-5% of normal is associated with moderate hemophilia B. Generally, human subjects with mild hemophilia B bleed excessively, if at all, only as a result of surgery or major trauma. Generally, mild hemophilia is associated with 6%-40% of normal FIX activity. Generally, individuals who are considered healthy, having no symptoms of hemophilia B, have a range of about 50% to 150% of normal FIX activity. Additional information can be found in Fijnvandraat, et al., Diagnosis and management of hemophilia, Br. Med. J., 344:36-40 (2012).

**[0136]** Factor IX activity can be measured in a variety of ways known to those of skill in the art. For example, one exemplary non-limiting assay is the one-stage activated partial thromboplastin time (APTT) assay to determine FIX clotting activity in a plasma sample obtained from a subject. FIX activity is frequently expressed in international units (IU), where 1 IU is defined as the FIX clotting activity present in 1 ml of pooled plasma from normal donors. Using this convention, severe hemophilia B is associated with less than 0.01 IU/ml FIX levels, moderate disease is associated with 0.02-0.05 IU/ml FIX levels, mild disease is associated with 0.06-0.40 IU/ml FIX levels, and being disease-free is associated with 0.50-1.50 IU/ml FIX levels.

**[0137]** As will be appreciated by one of skill in the art, a Factor IX variant, such as naturally occurring FIX Padua variant, that has higher catalytic activity compared to wild type human FIX, can produce a given level of FIX activity (e.g., 1 IU/ml) at a lower concentration of active protein compared to "non-Padua" FIX.

**[0138]** A therapeutically effective dose of an AAV vector, including, for example, AAV-FIX39-Padua, or one having the same capsid and a genome sequence at least 95%, 96%, 97%, 98% or 99% identical thereto, may be one that is sufficient, when administered to a subject, for example, a human, with severe, moderate or mild hemophilia B, to achieve plasma FIX activity that is about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, or 50%, or more of normal FIX activity. A therapeutically effective dose may be one that achieves 1% or greater FIX activity in a subject otherwise lacking such activity, for example, from 1.5-10%, 10-15%, 15-20%, 20-25%, 25-30% or greater FIX activity in a subject.

**[0139]** With respect to treating a subject with hemophilia B, a therapeutically effective dose of an AAV vector including, for example, AAV-FIX39-Padua, or one having the same capsid and a genome sequence at least 98% or 99% identical thereto, may be at least $1\times10^{10}$ vector genomes (vg) per kilogram (vg/kg) of the weight of the subject, or between about $1\times10^{10}$ to $1\times10^{11}$ vg/kg of the weight of the subject, or between about $1\times10^{11}$ to $1\times10^{12}$vg/kg (e.g., about $1\times10^{11}$ to $2\times10^{11}$ vg/kg or about $2\times10^{11}$ to $3\times10^{11}$ vg/kg or about $3\times10^{11}$ to $4\times10^{11}$ vg/kg or about $4\times10^{11}$ to $5\times10^{11}$ vg/kg or about $5\times10^{11}$ to $6\times10^{11}$ vg/kg or about $6\times10^{11}$ to $7\times10^{11}$ vg/kg or about $7\times10^{11}$ to $8\times10^{11}$ vg/kg or about $8\times10^{11}$ to $9\times10^{11}$ vg/kg or about $9\times10^{11}$ to $1\times10^{12}$ vg/kg) of the weight of the subject, or between about $1\times10^{12}$ to $1\times10^{13}$ vg/kg of the weight of the subject, to achieve a desired therapeutic effect. Additional doses can be in a range of about $5\times10^{10}$ to $1\times10^{10}$ vector genomes (vg) per kilogram (vg/kg) of the weight of the subject, or in a range of about $1\times10^{10}$ to $5\times10^{11}$ vg/kg of the weight of the subject, or in a range of about $5\times10^{11}$ to $1\times10^{12}$ vg/kg of the weight of the subject, or in a range of about $1\times10^{12}$ to $5\times10^{13}$ vg/kg of the weight of the subject, to achieve a desired therapeutic effect. A therapeutically effective dose of an AAV vector may be about 2.0 $\times10^{11}$ vg/kg, 2.1 $\times10^{11}$ vg/kg, 2.2 $\times10^{11}$ vg/kg, 2.3 $\times10^{11}$ vg/kg, 2.4 $\times10^{11}$ vg/kg, 2.5 $\times10^{11}$ vg/kg, 2.6 $\times10^{11}$ vg/kg, 2.7 $\times10^{11}$ vg/kg, 2.8 $\times10^{11}$ vg/kg, 2.9 $\times10^{11}$ vg/kg, 3.0 $\times10^{11}$ vg/kg, 3.1 $\times10^{11}$ vg/kg, 3.2 $\times10^{11}$ vg/kg, 3.3 $\times10^{11}$ vg/kg, 3.4 $\times10^{11}$ vg/kg, 3.5 $\times10^{11}$ vg/kg, 3.6 $\times10^{11}$ vg/kg, 3.7 $\times10^{11}$ vg/kg, 3.8 $\times10^{11}$ vg/kg, 3.9 $\times10^{11}$ vg/kg, 4.0 $\times10^{11}$ vg/kg, 4.1 $\times10^{11}$ vg/kg, 4.2 $\times10^{11}$ vg/kg, 4.3 $\times10^{11}$ vg/kg, 4.4 $\times10^{11}$ vg/kg, 4.5 $\times10^{11}$ vg/kg, 4.6 $\times10^{11}$ vg/kg, 4.7 $\times10^{11}$ vg/kg, 4.8 $\times10^{11}$ vg/kg, 4.9 $\times10^{11}$ vg/kg, 5.0 $\times10^{11}$ vg/kg, 5.1 $\times10^{11}$ vg/kg, 5.2 $\times10^{11}$ vg/kg, 5.3 $\times10^{11}$ vg/kg, 5.4 $\times10^{11}$ vg/kg, 5.5 $\times10^{11}$ vg/kg, 5.6 $\times10^{11}$ vg/kg, 5.7 $\times10^{11}$ vg/kg, 5.8 $\times10^{11}$ vg/kg, 5.9 $\times10^{11}$ vg/kg, 6.0 $\times10^{11}$ vg/kg, 6.1 $\times10^{11}$ vg/kg, 6.2 $\times10^{11}$ vg/kg, 6.3 $\times10^{11}$ vg/kg, 6.4 $\times10^{11}$ vg/kg, 6.5 $\times10^{11}$ vg/kg, 6.6 $\times10^{11}$ vg/kg, 6.7 $\times10^{11}$ vg/kg, 6.8 $\times10^{11}$ vg/kg, 6.9 $\times10^{11}$ vg/kg, 7.0 $\times10^{11}$ vg/kg, 7.1 $\times10^{11}$ vg/kg, 7.2 $\times10^{11}$ vg/kg, 7.3 $\times10^{11}$ vg/kg, 7.4 $\times10^{11}$ vg/kg, 7.5 $\times10^{11}$ vg/kg, 7.6 $\times10^{11}$ vg/kg, 7.7 $\times10^{11}$ vg/kg, 7.8 $\times10^{11}$ vg/kg, 7.9 $\times10^{11}$ vg/kg, or 8.0 $\times10^{11}$ vg/kg, or some other dose. An AAV vector can be AAV-FIX39-Padua, or an AAV vector having the same capsid and a genome sequence at least 95%, 96%, 97%, 98% or 99% identical thereto, and may be administered to a subject in a pharmaceutically acceptable composition alone, or with empty capsids of the same capsid type at an empty to vector ratio of about 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, or some other ratio.

**[0140]** The doses of an "effective amount" or "sufficient amount" for treatment (*e.g.*, to ameliorate or to provide a therapeutic benefit or improvement) typically are effective to provide a response to one, multiple or all adverse symptoms, consequences or complications of the disease, one or more adverse symptoms, disorders, illnesses, pathologies, or complications, for example, caused by or associated with the disease, to a measurable extent, although decreasing, reducing, inhibiting, suppressing, limiting or controlling progression or worsening of the disease is a satisfactory outcome.

**[0141]** An effective amount or a sufficient amount can but need not be provided in a single administration, may require multiple administrations, and, can but need not be, administered alone or in combination with another composition (*e.g.*, agent), treatment, protocol or therapeutic regimen. For example, the amount may be proportionally increased as indicated by the need of the subject, type, status and severity of the disease treated or side effects (if any) of treatment. In addition, an effective amount or a sufficient amount need not be effective or sufficient if given in single or multiple doses without a second composition (*e.g.*, another drug or agent), treatment, protocol or therapeutic regimen, since additional doses, amounts or duration above and beyond such doses, or additional compositions (*e.g.*, drugs or agents), treatments, protocols or therapeutic regimens may be included in order to be considered effective or sufficient in a given subject. Amounts considered effective also include amounts that result in a reduction of the use of another treatment, therapeutic regimen or protocol, such as administration of recombinant clotting factor protein for treatment of a clotting disorder (*e.g.*, hemophilia A or B).

**[0142]** An effective amount or a sufficient amount need not be effective in each and every subject treated, nor a majority of treated subjects in a given group or population. An effective amount or a sufficient amount means effectiveness or sufficiency in a particular subject, not a group or the general population. As is typical for such methods, some subjects will exhibit a greater response, or less or no response to a given treatment method or use.

**[0143]** The term "ameliorate" means a detectable or measurable improvement in a subject's disease or symptom thereof, or an underlying cellular response. A detectable or measurable improvement includes a subjective or objective decrease, reduction, inhibition, suppression, limit or control in the occurrence, frequency, severity, progression, or duration of the disease, or complication caused by or associated with the disease, or an improvement in a symptom or an underlying cause or a consequence of the disease, or a reversal of the disease.

**[0144]** Thus, a successful treatment outcome can lead to a "therapeutic effect," or "benefit" of decreasing, reducing, inhibiting, suppressing, limiting, controlling or preventing the occurrence, frequency, severity, progression, or duration of a disease, or one or more adverse symptoms or underlying causes or consequences of the disease in a subject. Treatment methods and uses affecting one or more underlying causes of the disease or adverse symptoms are therefore considered to be beneficial. A decrease or reduction in worsening, such as stabilizing the disease, or an adverse symptom thereof, is also a successful treatment outcome.

**[0145]** A therapeutic benefit or improvement therefore need not be complete ablation of the disease, or any one, most or all adverse symptoms, complications, consequences or underlying causes associated with the disease. Thus, a satisfactory endpoint is achieved when there is an incremental improvement in a subject's disease, or a partial decrease, reduction, inhibition, suppression, limit, control or prevention in the occurrence, frequency, severity, progression, or duration, or inhibition or reversal of the disease (*e.g.*, stabilizing one or more symptoms or complications), over a short or long duration of time (hours, days, weeks, months, etc.). Effectiveness of a method or use, such as a treatment that provides a potential therapeutic benefit or improvement of a disease, can be ascertained by various methods, such as blood clot formation time, etc.

**[0146]** A therapeutically effective dose of an AAV vector may be one that is sufficient, when administered to a human subject with hemophilia B, to result in FIX activity above a certain level for a sustained period of time. An effective dose of an AAV vector may result in at least 1% normal FIX activity in human subjects with hemophilia B for a sustained period of at least 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 12 months, 13 months, 14 months, 15 months, 16 months, 17 months, 1.5 years, 2 years, 2.5 years, 3 years, 3.5 years, 4 years, 4.5 years, 5 years, 5.5 years, 6 years, 6.5 years, 7 years, 7.5 years, 8 years, 8.5 years, 9 years, 9.5 years, 10 years, or more. An effective dose of an AAV vector may result in at least 5% normal FIX activity for a sustained period of at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or 17 months, or at least 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10 years, or more. An effective dose of an AAV vector may result in at least 10% normal FIX activity for a sustained period of at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or 17 months, or at least 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10 years, or more. An effective dose of an AAV vector may result in at least 15% normal FIX activity for a sustained period of at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or 17 months, or at least 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10 years, or more. An effective dose of an AAV vector may result in at least 20% normal FIX activity for a sustained period of at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or 17 months, or at least 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10 years, or more. An effective dose of an AAV vector may result in at least 25% normal FIX activity for a sustained period of at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or 17 months, or at least 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10 years, or more. An effective dose of an AAV vector may result in at least 30% normal FIX activity for a sustained period of at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or 17 months, or at least 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10 years, or more. An effective dose of an AAV vector may result in at least 35% normal FIX activity for a sustained period of at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or 17 months, or at least 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10 years, or more. , An effective dose of an AAV vector may result in at least 40% normal FIX activity for a sustained period of at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or 17 months, or at least 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10 years, or more. , An effective dose of an AAV vector may result in at least 45% normal FIX activity for a sustained period of at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or 17 months, or at least 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10 years, or more. The AAV vector can be AAV-FIX39-Padua, or an AAV vector having the same capsid and a genome sequence at least 95%, 96%, 97%, 98% or 99% identical thereto, and may be administered to a subject in a pharmaceutically acceptable composition alone, or with empty capsids of the same capsid type at an empty to vector ratio of about 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, or some other ratio.

**[0147]** A therapeutically effective dose of an AAV vector may be one that is sufficient, when administered to a human subject with severe or moderate hemophilia B, to result in FIX activity that is at least 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, or 45%, of normal for a sustained period of at least 6 months. The dose of AAV-FIX39-Padua, or an AAV vector having the same capsid and a genome sequence at least 95%, 96%, 97%, 98% or 99% identical thereto, may be about 5.0 $\times 10^{11}$ vg/kg, which may be administered in a pharmaceutically acceptable composition alone, or with empty capsids of the same capsid type at an empty to vector ratio of about 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, or some other ratio.

**[0148]** It may be seen that in some human subjects that have received a therapeutically effective dose of an AAV vector, including for example, AAV-FIX39-Padua, or an AAV vector having the same capsid and a genome sequence at least 95%, 96%, 97%, 98% or 99% identical thereto, that FIX activity attributable to the vector declines over an extended period of time (e.g., months or years) to a level that is no longer deemed sufficient (for example, where the subject exhibits symptoms and/or FIX activity characteristic of moderate or severe hemophilia B). In such circumstances, the subject can be dosed again with the same type of AAV vector as in the initial treatment. If the subject has developed an immune reaction to the initial vector, the patient may be dosed with an AAV vector designed to express FIX in target cells, but having a capsid of a different or variant serotype that is less immunoreactive compared to the first AAV vector.

**[0149]** A therapeutically effective dose of an AAV vector may be one that is sufficient, when administered to a human subject with hemophilia B, to reduce or even eliminate the subject's need for recombinant human Factor IX replacement therapy to maintain adequate hemostasis. Thus,a therapeutically effective dose of an AAV vectormay be one which can reduce the frequency with which an average human subject having moderate or severe hemophilia B needs FIX replacement therapy to maintain adequate hemostasis by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%. A therapeutically effective dose of an AAV vector may be one which can reduce the dose of recombinant human Factor IX that an average human subject having moderate or severe hemophilia B needs to maintain adequate hemostasis by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%. , The AAV vector may be AAV-FIX39-Padua, or an AAV vector having the same capsid and a genome sequence at least 95%, 96%, 97%, 98% or 99% identical thereto, which may be administered to a subject in a pharmaceutically acceptable composition alone, or with empty capsids of the same capsid type at an empty to vector ratio of about 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, or some other ratio.

**[0150]** A therapeutically effective dose of an AAV vector may be one that is sufficient, when administered to a human subject with severe hemophilia B, to reduce or even eliminate spontaneous bleeding into the joints. Thus, a therapeutically effective dose of an AAV vector may be one which can reduce the frequency of spontaneous bleeding into the joints of a human subject with severe hemophilia B by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%, compared to the average untreated human subject with severe hemophilia B. Bleeding into the joints can be detected using magnetic resonance imaging or ultrasonography of the joints, or other techniques familiar to those of skill in the art. The AAV vector may be AAV-FIX39-Padua, or an AAV vector having the same capsid and a genome sequence at least 95%, 96%, 97%, 98% or 99% identical thereto, and may be administered to a subject in a pharmaceutically acceptable composition alone, or with empty capsids of the same capsid type at an empty to vector ratio of about 2:1, 3: 1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, or some other ratio.

**[0151]** Prior efforts to develop AAV vectors to treat hemophilia have been unsuccessful, at least in part it is believed, because of a robust immune response to AAV capsid in prior clinical trials. (See, for example, Nathwani, et al., NEJM 2011;365(25):2357-2365; and Manno, et al., Nat Med 2006;12(3):342-347). A clinical trial underway has demonstrated that AAV vectors can produce a high level of FIX activity in human subjects with severe hemophilia B, while resulting in no or minimal immune response even as much as 6 months after the AAV vectors were administered (Example 5). Thus, a therapeutically effective dose of an AAV vector may be one that when administered to a subject with severe or moderate hemophilia B results in FIX activity adequate to maintain hemostasis, while producing no or minimal immune response over a significant period of time. The immune response may be an innate immune response, a humoral immune response, or a cellular immune response, or even all three types of immune response. The immune response may be against the capsid, vector genome, and/or Factor IX protein produced from transduced cells.

**[0152]** A therapeutically effective dose of an AAV vector may result in FIX activity adequate to maintain hemostasis in a subject with hemophilia B, while producing no or minimal humoral (i.e., antibody) immune response against the capsid, genome and/or Factor IX protein produced from transduced cells. The antibody response to a virus, or virus-like particles such as AAV vectors, can be determined by measuring antibody titer in a subject's serum or plasma using techniques familiar to those of skill in the field of immunology. Antibody titer to any component of an AAV vector, such as the capsid proteins, or a gene product encoded by the vector genome and produced in transduced cells, such as Factor IX Padua (or other FIX variant), can be measured using such techniques. Antibody titers are typically expressed as a ratio indicating the dilution before which antibody signal is no longer detectable in the particular assay being used to detect the presence of the antibody. Different dilution factors can be used, for example, 2-fold, 5-fold, 10-fold, or some other dilution factor. Any suitable assay for the presence of an antibody can be used, for example and without limitation, ELISA, FACS, or a reporter gene assay, such as described in WO 2015/006743. Use of other assays is also possible according to the knowledge of those skilled in the art. Antibody titers can be measured at different times after initial administration of an AAV vector.

**[0153]** A therapeutically effective dose of an AAV vector may result in at least 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, or more FIX activity in subjects with hemophilia B, while producing an antibody titer against the capsid, genome and/or Factor IX protein (such as FIX Padua) produced from transduced cells that is not greater than 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:11, 1:12, 1:13, 1:14, 1:15, 1:20, 1:30, 1:40, 1:50, 1:60, 1:70, 1:80, 1:90, 1:100, 1:200, 1:300, 1:400, 1:500, or more, when determined at 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 12 months, 18 months, 2 years, 3 years, 4 years, 5 years, or a longer period after the subjects were administered the AAV vector. An AAV vector may result in at least 20% FIX activity in a subject with severe hemophilia B while inducing an antibody titer against the capsid and/or Factor IX produced by transduced cells that is not greater than 1:2, 1:3 or 1:4, both at 6 months after the AAV vector was administered. The AAV vector can be AAV-FIX39-Padua, or an AAV vector having the same capsid and a genome sequence at least 95%, 96%, 97%, 98% or 99% identical thereto, and may be administered to a subject in a pharmaceutically acceptable composition alone, or with empty capsids of the same capsid type at an empty to vector ratio of about 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, or some other ratio.

**[0154]** As noted above, prior trials using AAV-mediated gene therapy for hemophilia B triggered a self-limiting immune response that prevented the therapy from being effective for a significant period of time without need for high doses of steroids to cause immunosuppression. An important factor appears to have been a cellular immune response that eliminated the liver cells that had been transduced with the AAV vectors under study. This effect was detectable from both an elevation of liver enzymes, suggesting liver damage, and the presence of capsid-specific T cells in the subjects.

**[0155]** A therapeutically effective dose of an AAV vector may result in FIX activity adequate to maintain hemostasis in a subject with hemophilia B, while producing no or minimal cellular immune response against the capsid and/or Factor IX protein produced from transduced cells. A cellular immune response can be determined in at least two ways: assaying for T cell activity specific for capsid proteins or Factor IX, and testing for the presence of elevated liver enzyme levels that indicate damage to hepatocytes.

**[0156]** Cellular immune response may be determined by assaying for T cell activity specific for capsid proteins and/or the Factor IX protein produced by the transduced liver cells. Different assays for T cell response are known in the art. T cell response may be determined by collecting peripheral blood mononuclear cells (PBMC) from a subject that was previously treated with an AAV vector for treating hemophilia B. The cells are then incubated with peptides derived from the VP1 capsid protein used in the vector, and/or the Factor IX protein, such as FIX Padua, produced by the transduced liver cells. T cells that specifically recognize the capsid protein or Factor IX protein will be stimulated to release cytokines, such as interferon gamma or another cytokine, which can then be detected and quantified using the ELISPOT assay, or another assay familiar to those of skill in the art. (See, e.g., Manno, et al., Nat Med 2006;12(3):342-347). T cell response can be monitored before and at different times after a subject has received a dose of an AAV vector for treating hemophilia B, for example, weekly, monthly, or some other interval. Thus, a therapeutically effective dose of an AAV vector may result in FIX activity adequate to maintain hemostasis in a subject with hemophilia B (for example, FIX activity of at least 1%, 5%, 10%, 20%, 30%, or more), while causing a T cell response as measured using ELISPOT that is not greater than 10, 20, 30, 40, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, or more, spot-forming units per 1 million PBMCs assayed when measured weekly, monthly, or some other interval after the AAV vector is administered, or at 2 weeks, 1 month, 2 months, 3 months, 6 months, 9 months, 1 year, 2 years, or some different time after the AAV vector is administered. , The ELISPOT assay may be designed to detect interferon gamma (or some other cytokine) production stimulated by peptides from the AAV vector capsid protein or Factor IX protein (including FIX Padua, or a different variant) produced by transduced liver cells. The AAV vector can be AAV-FIX39-Padua, or an AAV vector having the same capsid and a genome sequence at least 95%, 96%, 97%, 98% or 99% identical thereto, andmay be administered to a subject in a pharmaceutically acceptable composition alone, or with empty capsids of the same capsid type at an empty to vector ratio of about 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, or some other ratio.

**[0157]** As a proxy for the cellular immune response against transduced hepatocytes, the presence of greater-than-normal liver enzymes can be assayed using standard methods. While not wishing to be bound by theory, it is believed that T cells specific for certain AAV vectors, such as those used in prior clinical trials, can attack and kill transduced hepatocytes, which transiently releases liver enymes into the circulation. Exemplary liver enzymes include alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH), but other enzymes indicactive of liver damage can also be monitored. A normal level of these enzymes in the circulation is typically defined as a range that has an upper level, above which the enzyme level is considered elevated, and therefore indicactive of liver damage. A normal range depends in part on the standards used by the clinical laboratory conducting the assay. A therapeutically effective dose of an AAV vector may result in FIX activity adequate to maintain hemostasis in a subject with hemophilia B (for example, FIX activity of at least 1%, 5%, 10%, 20%, 30%, or more), while causing an elevated circulating liver enzyme level, such as that of ALT, AST, or LDH, which is not greater than 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000%, 1500%, 2000% of the upper limit of normal (ULN) value of their respective ranges, on average, or at the highest level measured in multiple samples drawn from the same subject under treatment at different times (e.g., at weekly or monthly intervals) after administration of the AAV vector. The AAV vector can be AAV-FIX39-Padua, or an AAV vector having the same capsid and a genome sequence at least 95%, 96%, 97%, 98% or 99% identical thereto, and may be administered to a subject in a pharmaceutically acceptable composition alone, or with empty capsids of the same capsid type at an empty to vector ratio of about 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, or some other ratio.

**[0158]** In prior clinical trials using AAV vectors to treat hemophilia B, the investigators needed to co-administer an immunosuppressant drug, such as a steroid, to prevent the subjects receiving treatment from mounting an immune response that would eliminate the transduced cells producing the Factor IX protein. Due to the attenuated immune response seen in subjects undergoing experimental treatment with certain AAV vectors, however, co-administration of immunosuppressing drugs may not be necessary. Thus, a therapeutically effective dose of an AAV vector may be one that is sufficient to maintain adequate hemostasis in a subject with severe or moderate hemophilia B, without need for co-administration (before, contemporaneously, or after) of an immunosuppressant drug (such as a steroid or other immunosuppressant). Because an immune response is not predictable in all subjects, however, the methods herein of treatment for hemophilia B include AAV vectors that are co-administered with an immunosuppressant drug. Co-administration of an immunosuppressant drug can occur before, contemporaneously with, or after AAV vectors are administered to a subject having hemophilia B. An immunosuppressant drug may be administered to a subject for a period of days, weeks, or months after being administered an AAV vector for treating hemophilia B. Exemplary immunosuppressing drugs include steroids (e.g., without limitation, prednisone or prednisolone) and non-steroidal immunosuppressants, such as cyclosporin, rapamycin, and others. What drug doses and time course of treatment are required to effect sufficient immunosuppression will depend on factors unique to each subject undergoing treatment, but determining dose and treatment time are within the skill of those ordinarily skilled in the art. An immunosuppressant may need to be administered more than one time.

**[0159]** A therapeutically effective dose of an AAV vector may result in a consistent elevation of FIX activity when administered to a population of human subjects with severe or moderate hemophilia B. Consistency can be determined by calculating variability of response in a population of human subjects using statistical methods such the mean and standard deviation (SD), or another statistical technique familiar to those of skill in the art. A therapeutically effective dose of an AAV vector, when administered to a population of human subjects with severe or moderate hemophilia B may result, at 3 months, 6 months, 9 months, 12 months, 15 months, 18 months, 21 months, or 21 months after administration, in a mean FIX activity of 1-5% with a SD of less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1; a mean FIX activity of 2.5-7.5% with a SD of less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1; a mean FIX activity of 5-10% with a SD of less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1;a mean FIX activity of 7.5-12.5% with a SD of less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1; a mean FIX activity of 10-15% with a SD of less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1; a mean FIX activity of 12.5-17.5% with a SD of less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1; a mean FIX activity of 15-20% with a SD of less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1; a mean FIX activity of 17.5-22.5% with a SD of less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1;a mean FIX activity of 20-25% with a SD of less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1; a mean FIX activity of 22.5-27.5% with a SD of less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1; a mean FIX activity of 25-30% with a SD of less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1; a mean FIX activity of 27.5-32.5% with a SD of less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1; a mean FIX activity of 30-35% with a SD of less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1; a mean FIX activity of 32.5-37.5% with a SD of less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1; a mean FIX activity of 35-40% with a SD of less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1; a mean FIX activity of 37.5-42.5% with a SD of less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1; a mean FIX activity of 40-45% with a SD of less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1; a mean FIX activity of 42.5-47.5% with a SD of less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1; or a mean FIX activity of 45-50% with a SD of less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1. The AAV vector can be AAV-FIX39-Padua, or an AAV vector having the same capsid and a genome sequence at least 95%, 96%, 97%, 98% or 99% identical thereto, and may be administered to a subject in a pharmaceutically acceptable composition alone, or with empty capsids of the same capsid type at an empty to vector ratio of about 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, or some other ratio.

**[0160]** Methods and uses described herein can be combined with any compound, agent, drug, treatment or other therapeutic regimen or protocol having a desired therapeutic, beneficial, additive, synergistic or complementary activity or effect. Exemplary combination compositions and treatments include second actives, such as, biologics (proteins), agents and drugs. Such biologics (proteins), agents, drugs, treatments and therapies can be administered or performed prior to, substantially contemporaneously with or following any other method or use of the invention, for example, a therapeutic method of treating a subject for a blood clotting disease.

**[0161]** The compound, agent, drug, treatment or other therapeutic regimen or protocol can be administered as a combination composition, or administered separately, such as concurrently or in series or sequentially (prior to or following) delivery or administration of a nucleic acid, vector, recombinant vector (*e.g.*, rAAV), vector genome, or recombinant virus particle. The invention therefore provides combinations in which a method or use of the invention is in a combination with any compound, agent, drug, therapeutic regimen, treatment protocol, process, remedy or composition, set forth herein or known to one of skill in the art. The compound, agent, drug, therapeutic regimen, treatment protocol, process, remedy or composition can be administered or performed prior to, substantially contemporaneously with or following administration of a nucleic acid, vector, recombinant vector (*e.g.*, rAAV), vector genome, or recombinant virus particle of the invention, to a subject.

**[0162]** A combination composition may include one or more immunosuppressive agents. A method may include administering or delivering one or more immunosuppressive agents to the mammal. A combination composition may include AAV-FIX particles and one or more immunosuppressive agents. A method may include administering or delivering AAV-FIX particles to a mammal and administering an immunosuppressive agent to the mammal. The skilled artisan can determine appropriate need or timing of such a combination composition with one or more immunosuppressive agents and administering the immunosuppressive agent to the mammal.

**[0163]** Methods and uses also include, among other things, methods and uses that result in a reduced need or use of another compound, agent, drug, therapeutic regimen, treatment protocol, process, or remedy. For example, for a blood clotting disease, a method or use has a therapeutic benefit if in a given subject a less frequent or reduced dose or elimination of administration of a recombinant clotting factor protein to supplement for the deficient or defective (abnormal or mutant) endogenous clotting factor in the subject. Thus, methods and uses of reducing need or use of another treatment or therapy aredescribed.

**[0164]** The invention is useful in animals including human and veterinary medical applications. Suitable subjects therefore include mammals, such as humans, as well as non-human mammals. The term "subject" refers to an animal, typically a mammal, such as humans, non-human primates (apes, gibbons, gorillas, chimpanzees, orangutans, macaques), a domestic animal (dogs and cats), a farm animal (poultry such as chickens and ducks, horses, cows, goats, sheep, pigs), and experimental animals (mouse, rat, rabbit, guinea pig). Human subjects include fetal, neonatal, infant, juvenile and adult subjects. Subjects include animal disease models, for example, mouse and other animal models of blood clotting diseases and others known to those of skill in the art.

**[0165]** Subjects appropriate for treatment include those having or at risk of producing an insufficient amount or having a deficiency in a functional gene product (protein), or produce an aberrant, partially functional or non-functional gene product (protein), which can lead to disease. Subjects appropriate for treatment in accordance with the invention also include those having or at risk of producing an aberrant, or defective (mutant) gene product (protein) that leads to a disease such that reducing amounts, expression or function of the aberrant, or defective (mutant) gene product (protein) would lead to treatment of the disease, or reduce one or more symptoms or ameliorate the disease. Target subjects therefore include subjects having aberrant, insufficient or absent blood clotting factor production, such as hemophiliacs (*e.g.*, hemophilia B).

**[0166]** Subjects appropriate for treatment in accordance with the invention further include those previously or currently treated with supplemental protein (e.g., recombinant blood clotting factor such as FIX to treat hemophilia). Subjects appropriate for treatment in accordance with the invention moreover include those that have not developed a substantial or detectable immune response against FIX protein, or amounts of inhibitory antibodies against FIX protein that would interfere with or block FIX based gene therapy.

**[0167]** Human pediatric subjects that are determined to have hemophilia B (e.g., by genotyping), but have not yet exhibited any of the symptoms of hemophilia B, can be treated prophylactically with an AAV vector to prevent any such symptoms from occurring in the first place orfrom being as severe as they otherwise would have been in the absence of treatment. Human subjects treated prophylactically in this way may be at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 months old, or older, when they are administered an AAV vector to produce and maintain FIX activity adequate to maintain hemostasis, and thus prevent or reduce the severity of one or more symptoms of hemophilia B. The AAV vector can be AAV-FIX39-Padua, or an AAV vector having the same capsid and a genome sequence at least 95%, 96%, 97%, 98% or 99% identical thereto, and may be administered to a subject in a pharmaceutically acceptable composition alone, or with empty capsids of the same capsid type at an empty to vector ratio of about 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, or some other ratio.

**[0168]** Administration or *in vivo* delivery to a subject can be performed prior to development of an adverse symptom, condition, complication, etc. caused by or associated with the disease. For example, a screen (*e.g.,* genetic) can be used to identify such subjects as candidates for invention compositions, methods and uses. Such subjects therefore include those screened positive for an insufficient amount or a deficiency in a functional gene product (protein), or that produce an aberrant, partially functional or non-functional gene product (protein).

**[0169]** Methods and uses described herein include delivery and administration systemically, regionally or locally, or by any route, for example, by injection or infusion. Such delivery and administration include parenterally, *e.g.* intravascularly, intravenously, intramuscularly, intraperitoneally, intradermally, subcutaneously, or transmucosal. Exemplary administration and delivery routes include intravenous (i.v.), intraperitoneal (i.p.), intrarterial, subcutaneous, intra-pleural, intubation, intrapulmonary, intracavity, iontophoretic, intraorgan, intralymphatic.

**[0170]** Alternatively or in addition, AAV vector can be delivered to the liver via the portal vein. ,A catheter introduced into the femoral artery can be used to deliver AAV vectors to liver via the hepatic artery. Non-surgical means can also be employed, such as endoscopic retrograde cholangiopancreatography (ERCP), to deliver AAV vectors directly to the liver, thereby bypassing the bloodstream and AAV neutralizing antibodies. Other ductal systems, such as the ducts of the submandibular gland, can also be used as portals for delivering AAV vectors into a subject that develops or has preexisting anti-AAV antibodies.

**[0171]** Doses can vary and depend upon whether the type, onset, progression, severity, frequency, duration, or probability of the disease to which treatment is directed, the clinical endpoint desired, previous or simultaneous treatments, the general health, age, gender, race or immunological competency of the subject and other factors that will be appreciated by the skilled artisan. The dose amount, number, frequency or duration may be proportionally increased or reduced, as indicated by any adverse side effects, complications or other risk factors of the treatment or therapy and the status of the subject. The skilled artisan will appreciate the factors that may influence the dosage and timing required to provide an amount sufficient for providing a therapeutic or prophylactic benefit.

**[0172]** Methods and uses of the invention as disclosed herein can be practiced within 1-2, 2-4, 4-12, 12-24 or 24-72 hours after a subject has been identified as having the disease targeted for treatment, has one or more symptoms of the disease, or has been screened and is identified as positive as set forth herein even though the subject does not have one or more symptoms of the disease. Of course, methods and uses of the invention can be practiced 1-7, 7-14, 14-21, 21-48 or more days, months or years after a subject has been identified as having the disease targeted for treatment, has one or more symptoms of the disease, or has been screened and is identified as positive as set forth herein.

**[0173]** Nucleic acids, vectors, recombinant vectors (*e.g.*, rAAV), vector genomes, and recombinant virus particles and other compositions, agents, drugs, biologics (proteins) can be incorporated into pharmaceutical compositions, *e.g.*, a pharmaceutically acceptable carrier or excipient. Such pharmaceutical compositions are useful for, among other things, administration and delivery to a subject *in vivo* or *ex vivo.*

**[0174]** As used herein the term "pharmaceutically acceptable" and "physiologically acceptable" mean a biologically acceptable formulation, gaseous, liquid or solid, or mixture thereof, which is suitable for one or more routes of administration, *in vivo* delivery or contact. A "pharmaceutically acceptable" or "physiologically acceptable" composition is a material that is not biologically or otherwise undesirable, *e.g.*, the material may be administered to a subject without causing substantial undesirable biological effects. Thus, such a pharmaceutical composition may be used, for example in administering a viral vector or viral particle to a subj ect.

**[0175]** Such compositions include solvents (aqueous or non-aqueous), solutions (aqueous or non-aqueous), emulsions (*e.g.*, oil-in-water or water-in-oil), suspensions, syrups, elixirs, dispersion and suspension media, coatings, isotonic and absorption promoting or delaying agents, compatible with pharmaceutical administration or *in vivo* contact or delivery. Aqueous and non-aqueous solvents, solutions and suspensions may include suspending agents and thickening agents. Such pharmaceutically acceptable carriers include tablets (coated or uncoated), capsules (hard or soft), microbeads, powder, granules and crystals. Supplementary active compounds (*e.g.*, preservatives, antibacterial, antiviral and antifungal agents) can also be incorporated into the compositions.

**[0176]** Pharmaceutical compositions can be formulated to be compatible with a particular route of administration or delivery, as set forth herein or known to one of skill in the art. Thus, pharmaceutical compositions include carriers, diluents, or excipients suitable for administration by various routes.

**[0177]** Compositions suitable for parenteral administration comprise aqueous and non-aqueous solutions, suspensions or emulsions of the active compound, which preparations are typically sterile and can be isotonic with the blood of the intended recipient. Non-limiting illustrative examples include water, saline, dextrose, fructose, ethanol, animal, vegetable or synthetic oils.

**[0178]** Cosolvents and adjuvants may be added to the formulation. Non-limiting examples of cosolvents contain hydroxyl groups or other polar groups, for example, alcohols, such as isopropyl alcohol; glycols, such as propylene glycol, polyethyleneglycol, polypropylene glycol, glycol ether; glycerol; polyoxyethylene alcohols and polyoxyethylene fatty acid esters. Adjuvants include, for example, surfactants such as, soya lecithin and oleic acid; sorbitan esters such as sorbitan trioleate; and polyvinylpyrrolidone.

**[0179]** Pharmaceutical compositions and delivery systems appropriate for the compositions, methods and uses described herein are known in the art (*see*, *e.g.,* Remington: The Science and Practice of Pharmacy (2003) 20th ed., Mack Publishing Co., Easton, PA; Remington's Pharmaceutical Sciences (1990) 18th ed., Mack Publishing Co., Easton, PA; The Merck Index (1996) 12th ed., Merck Publishing Group, Whitehouse, NJ; Pharmaceutical Principles of Solid Dosage Forms (1993), Technonic Publishing Co., Inc., Lancaster, Pa.; Ansel and Stoklosa, Pharmaceutical Calculations (2001) 11th ed., Lippincott Williams & Wilkins, Baltimore, MD; and Poznansky et al., Drug Delivery Systems (1980), R. L. Juliano, ed., Oxford, N.Y., pp. 253-315).

**[0180]** A "unit dosage form" as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity optionally in association with a pharmaceutical carrier (excipient, diluent, vehicle or filling agent) which, when administered in one or more doses, is calculated to produce a desired effect (*e.g.*, prophylactic or therapeutic effect). Unit dosage forms may be within, for example, ampules and vials, which may include a liquid composition, or a composition in a freeze-dried or lyophilized state; a sterile liquid carrier, for example, can be added prior to administration or delivery *in vivo.* Individual unit dosage forms can be included in multi-dose kits or containers. Recombinant vector (*e.g.*, rAAV) sequences, vector genomes, recombinant virus particles, and pharmaceutical compositions thereof can be packaged in single or multiple unit dosage form for ease of administration and uniformity of dosage.

**[0181]** Also described, but not covered by the claimed invention, are kits with packaging material and one or more components therein. A kit typically includes a label or packaging insert including a description of the components or instructions for use *in vitro*, *in vivo*, or *ex vivo*, of the components therein. A kit can contain a collection of such components, *e.g.*, a nucleic acid, recombinant vector, virus (*e.g.*, AAV) vector, vector genome or virus particle and optionally a second active, such as another compound, agent, drug or composition.

**[0182]** A kit refers to a physical structure housing one or more components of the kit. Packaging material can maintain the components sterilely, and can be made of material commonly used for such purposes (*e.g.*, paper, corrugated fiber, glass, plastic, foil, ampules, vials, tubes, etc.).

**[0183]** Labels or inserts can include identifying information of one or more components therein, dose amounts, clinical pharmacology of the active ingredient(s) including mechanism of action, pharmacokinetics and pharmacodynamics. Labels or inserts can include information identifying manufacturer, lot numbers, manufacture location and date, expiration dates. Labels or inserts can include information identifying manufacturer information, lot numbers, manufacturer location and date. Labels or inserts can include information on a disease for which a kit component may be used. Labels or inserts can include instructions for the clinician or subject for using one or more of the kit components in a method, use, or treatment protocol or therapeutic regimen. Instructions can include dosage amounts, frequency or duration, and instructions for practicing any of the methods, uses, treatment protocols or prophylactic or therapeutic regimes described herein.

**[0184]** Labels or inserts can include information on any benefit that a component may provide, such as a prophylactic or therapeutic benefit. Labels or inserts can include information on potential adverse side effects, complications or reactions, such as warnings to the subject or clinician regarding situations where it would not be appropriate to use a particular composition. Adverse side effects or complications could also occur when the subject has, will be or is currently taking one or more other medications that may be incompatible with the composition, or the subject has, will be or is currently undergoing another treatment protocol or therapeutic regimen which would be incompatible with the composition and, therefore, instructions could include information regarding such incompatibilities.

**[0185]** Labels or inserts include "printed matter," *e.g.*, paper or cardboard, or separate or affixed to a component, a kit or packing material (*e.g.*, a box), or attached to an ampule, tube or vial containing a kit component. Labels or inserts can additionally include a computer readable medium, such as a bar-coded printed label, a disk, optical disk such as CD- or DVD-

ROM/RAM, DVD, MP3, magnetic tape, or an electrical storage media such as RAM and ROM or hybrids of these such as magnetic/optical storage media, FLASH media or memory type cards.

**[0186]** Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described herein.

**[0187]** In case of conflict, the specification, including definitions, will control.

**[0188]** As used herein, the singular forms "a", "and," and "the" include plural referents unless the context clearly indicates otherwise. Thus, for example, reference to "a nucleic acid" includes a plurality of such nucleic acids, reference to "a vector" includes a plurality of such vectors, and reference to "a virus" or "particle" includes a plurality of such virions/particles.

**[0189]** As used herein, all numerical values or numerical ranges include integers within such ranges and fractions of the values or the integers within ranges unless the context clearly indicates otherwise. Thus, to illustrate, reference to 80% or more identity, includes 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94% etc., as well as 81.1%, 81.2%, 81.3%, 81.4%, 81.5%, etc., 82.1%, 82.2%, 82.3%, 82.4%, 82.5%, etc., and so forth.

**[0190]** Reference to an integer with more (greater) or less than includes any number greater or less than the reference number, respectively. Thus, for example, a reference to less than 100, includes 99, 98, 97, etc. all the way down to the number one (1); and less than 10, includes 9, 8, 7, etc. all the way down to the number one (1).

**[0191]** As used herein, all numerical values or ranges include fractions of the values and integers within such ranges and fractions of the integers within such ranges unless the context clearly indicates otherwise. Thus, to illustrate, reference to a numerical range, such as 1-10 includes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, as well as 1.1, 1.2, 1.3, 1.4, 1.5, etc., and so forth. Reference to a range of 1-50 therefore includes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, etc., up to and including 50, as well as 1.1, 1.2, 1.3, 1.4, 1.5, etc., 2.1, 2.2, 2.3, 2.4, 2.5, etc., and so forth.

**[0192]** Reference to a series of ranges includes ranges which combine the values of the boundaries of different ranges within the series. Thus, to illustrate reference to a series of ranges, for example, of 1-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-75, 75-100, 100-150, 150-200, 200-250, 250-300, 300-400, 400-500, 500-750, 750-1,000, 1,000-1,500, 1,500-2,000, 2,000-2,500, 2,500-3,000, 3,000-3,500, 3,500-4,000, 4,000-4,500, 4,500-5,000, 5,500-6,000, 6,000-7,000, 7,000-8,000, or 8,000-9,000, includes ranges of 10-50, 50-100, 100-1,000, 1,000-3,000, 2,000-4,000, etc.

**[0193]** Accordingly, the following examples are intended to illustrate but not limit the scope of the invention claimed.

**Example 1. Vector Design/Preparation**

**[0194]** A novel Factor IX nucleic acid encoding a high specific activity human factor IX protein having the 338L Padua variant (Simioni P, et al., *N Engl J Med* 2009, 361:1671) was designed ("FIX 39-Padua"; SEQ ID No:10; Figure 10). FIX39-Padua is completely devoid of CpG dinucleotides in the FIX coding and intronic sequences. For comparative testing, FIX19 (Mingozzi et al. *Sci. Transl Med.* 2013) was prepared and modified to include the FIX Padua, to rule out any potential confounding effects resulting from the FIX Padua ("FIX 19-Padua"; SEQ ID NO:11; Figure 11).

**[0195]** A plasmid ("pAAV-ApoE_hAAT-FIX39"; 11125 bp; SEQ ID NO:12; Figure 12A) was synthesized and included the FIX39-Padua expression cassette and the elements described in Table 2. A map of pAAV-ApoE_hAAT-FIX39 is shown in Figure 13.

Table 2. pAAV-ApoE_hAAT-FIX39

| | |
|---|---|
| 5' AAV2 ITR | SEQ ID NO:13 |
| Enhancer (Hepatic Control Region) | SEQ ID NO:14 |
| hAAT promoter | SEQ ID NO:15 |
| 5' UTR | SEQ ID NO:16 |
| FIX39-Padua CDS | SEQ ID NO:10 (Figure10) |
| Intron A | SEQ ID NO:17 (Figure 14) |
| 3' UTR | SEQ ID NO:18 |
| polyA | SEQ ID NO:19 |
| 3' AAV2 ITR | SEQ ID NO:20 |
| Lambda stuffer | SEQ ID NO:21 |
| F1 origin of replication | SEQ ID NO:22 |
| Kanamycin resistance | SEQ ID NO:23 |
| pUC origin of replication | SEQ ID NO:24 |

**[0196]** The sequence of the FIX39-Padua coding sequence and intron A is set forth in SEQ ID NO:25 (Figure 15). A plasmid was also synthesized that included the FIX19-Padua CDS and the same regulatory elements, the same adeno-associated inverted terminal repeats (ITRs), and the same liver-specific ApoE/hAAT promoter as pAAV-ApoE_hAAT-FIX39.

**[0197]** AAV vector having the 4-1 capsid variant (SEQ ID NO:4) was prepared for the FIX39-Padua ("AAV-FIX39-Padua") and FIX19-Padua ("AAV-FIX19-Padua") transgenes using a triple transfection process followed by double cesium chloride gradient centrifugation (Ayuso E, Met al., Gene Ther 2010, 17:503). Vectors were titrated by quantitative PCR using a linearized plasmid as the standard. For the study described in Example 3, vector was diluted in PBS, 5% sorbitol, 0.001% F68 to a final volume of 200 μl per mouse, for tail vein injection.

**Example 2. *in vitro* AAV variant 4-1 transduction**

**[0198]** Primary hepatocytes from cynomolgus macaque and human origin were transduced with the 4-1 variant capsid (SEQ ID NO:4) expressing luciferase at four different multiplicities of infection (MOI) ranging from 500 to 62,500 vector genomes per cell. Seventy-two hours after transduction, luciferase expression was analyzed. As shown in Figure 16, the ratio of transduced human hepatocytes relative to non-human primate hepatocytes ranged from 0.8 to 1.5, depending on the MOI used. These data generated *in vitro* appear to be consistent with previous observations in vivo when comparing expression of coagulation factor IX in cynomolgus macaques and human subjects.

**Example 3. Potency Study**

**[0199]** A study was conducted to evaluate the potency of AAV-FIX39-Padua versus AAV-FIX19-Padua in mice. Groups of 5 mice were injected at 8-10 weeks of age with either $1\times10^{11}$ or $1\times10^{12}$ vg/kg of AAV-FIX39-Padua and AAV-FIX19-Padua. Following vector administration, blood was collected by retro-orbital bleeding using heparinized capillary tubes; plasma was isolated by centrifugation at 9000 rpm for 10 minutes at 4°C and stored frozen at -80°C until assayed.

**[0200]** Plasma collected was used to evaluate hFIX transgene expression. Human FIX levels in plasma were measured using an ELISA kit (Affinity Biologicals, Ancaster, ON, Canada).

**[0201]** Activity levels of human FIX were measured by activated partial thromboplastin time (aPTT) assay. The aPTT assay was performed by mixing sample plasma in a 1:1:1 volume-ratio with human FIX-deficient plasma (George King Biomedical, Inc) and aPTT reagent (Trinity Biotech), followed by a 180s incubation period at 37°C. Coagulation was initiated by addition of 25 mM calcium chloride. Time to clot formation was measured using a STart 4 coagulation instrument (Diagnostica Stago). A standard curve was generated with pooled normal plasma from George King starting at a 1:5 dilution in TBS pH 7.4 (48 μl + 192 μl) followed by serial 1:2 dilutions (120 μl +120 μl). The human standard curve was used to calculate the activity of each sample at week 17 after vector administration; activity in two untreated mice was also measured. FIX activity in untreated mice was averaged and then subtracted from the treated samples to calculate the extra (i.e. human) activity due to the FIX Padua protein.

**[0202]** As shown in Figure 17, AAV-FIX39-Padua and AAV-FIX19-Padua appear to express substantially equivalent levels of FIX.

**[0203]** Seventeen weeks after vector administration, human FIX activity was measured in those mice treated with a vector dose of $1\times10^{12}$ vg/kg. The activity-to-antigen ratio ranged between 5.2 and 7.5, with an average value of 6.4 for both FIX19-Padua and FIX39-Padua groups (Table 3).

Table 3. Human FIX activity values

| Animal ID | Antigen (% of normal) | Activity (% of normal) | Ratio |
|---|---|---|---|
| 01 - FIX19 | 53.9 | 352.7 | 6.5 |
| 02 - FIX19 | 95.6 | 631.8 | 6.6 |
| 03 - FIX19 | 120.6 | 882.3 | 7.3 |
| 04 - FIX19 | 132.9 | 797.1 | 6.0 |
| 05 - FIX19 | 105.2 | 599.7 | 5.7 |
| 06 - FIX39 | 163.1 | 1092.8 | 6.7 |
| 07 - FIX39 | 108.2 | 670.3 | 6.2 |
| 08 - FIX39 | 121.1 | 781.2 | 6.4 |
| 09 - FIX39 | 152.3 | 1147.8 | 7.5 |
| 10 - FIX39 | 134.1 | 702.1 | 5.2 |
| | **Average** | | |
| | **Antigen (% of normal)** | **Activity (% of normal)** | **Ratio** |
| AAV-FIX19-Padua | 101.7 | 652.7 | 6.4 |
| AAV-FIX39-Padua | 135.8 | 878.8 | 6.4 |

**[0204]** While these results suggest that the potency of both expression cassettes is substantially similar, the two constructs were also analyzed in the setting of plasmid hydrodynamic tail vein injection. The rationale for evaluating FIX levels derived of *in vivo* administration of naked DNA was to compare both expression cassettes without the potential interferencE of differences in AAV tittering, vector manufacturing, etc.

**[0205]** As shown in Figure 18, both naked expression cassettes were equally potent at driving FIX expression, confirming the data obtained in the AAV setting. These results indicate that the FIX19-Padua and FIX39-Padua expression cassettes have similar potency.

**Example 4. AAV-FIX39-Padua gene therapy**

**[0206]** A clinical study is being conducted to determine safety and kinetics of a single IV infusion of AAV-FIX39-Padua. The AAV 4-1 capsid variant used has been shown in preclinical studies to have good safety and efficacy, the ability to achieve sustained FIX activity levels of

-35% in NHPs at $1\times10^{12}$ vg/kg after 3 months of vector infusion; and cross reacting neutralizing antibodies (Ab) to the AAV 4-1 capsid variant are approximately 10% less prevalent than AAV8. The design of the study is provided in Table 4.

Table 4. AAV-FIX39-Padua Clinical Study Design

| | |
|---|---|
| Safety and Tolerability of AAVFIX39- Padua | Clinically significant in vital signs, lab values and clinical assessments (including number of bleeds and QoL) from baseline |
| Kinetics of AAVFIX39-Padua | Transgene FIX activity levels and antigen levels at peak and |
| steady-state | |
| Dosing | Starting, Middle and Highest Dose Cohorts will each include 2-5 subjects |
| Design | Open-label, non-randomized, dose escalation |
| Participating countries | USA and potentially Europe, Japan and Canada |
| Sample size | Up to 15 subjects |
| Eligibility | Ages Eligible for Study: 18 Years and older |
| | Genders Eligible for Study: Male |
| | Accepts Healthy Volunteers: No |
| Inclusion Criteria | Able to provide informed consent and comply with requirements of the study |
| | Males ≥18 y.o. with confirmed diagnosis of hemophilia B (≤2 IU/dL or ≤2% endogenous factor IX) |
| | Received ≥50 exposure days to factor IX products |
| | A minimum of an average of 4 bleeding events per year requiring episodic treatment of factor IX infusions or prophylactic factor IX infusions |
| | No measurable factor IX inhibitor as assessed by the central laboratory and have no prior history of inhibitors to factor IX protein |
| | Agree to use reliable barrier contraception until 3 consecutive samples are negative for vector sequences |
| | |
| Exclusion Criteria | Evidence of active hepatitis B or C |
| | Currently on antiviral therapy for hepatitis B or C |
| | Have significant underlying liver disease |
| | Have serological evidence* of HIV-1 or HIV-2 with CD4 counts ≤200/mm3 (* subjects who are HIV+ and stable with CD4 count >200/mm3 and undetectable viral load are eligible to enroll) |
| | Have detectable antibodies reactive with 4-1 variant AAV capsid (SEQ ID NO:4) |

| | |
|---|---|
| | Participated in a gene transfer trial within the last 52 weeks or an investigational drug within the last 12 weeks |
| | Unable or unwilling to comply with study |
| | assessments |
| Screening Visit | Eligibility evaluation |
| | AAV NAb titer is the major screen failure (highly recommend referring subjects to CHOP's AAV NAb titer protocol for phone screening) |
| Day 0 Visit | FIX product incremental recovery then vector infusion |
| Follow-up Visits (~17 visits) | Safety and kinetic evaluations |
| End-of Study Visit (at week 52) | Final safety evaluation |

**Example 5. Clinical Results**

**[0207]** Four subjects with hemophilia B were administered a single IV infusion of AAV-FIX39-Padua vector. The first two subjects, ages 23 and 18 respectively, had no prior history of liver disease, while the third, age 47, had a history of HCV infection but had cleared spontaneously. All four subjects had been screened for neutralizing antibodies to the novel AAV capsid and found to be negative.

**[0208]** Subjects were infused intravenously with $5 \times 10^{11}$ vg/kg of AAV-FIX39-Padua vector over a period of ~1 hour. The total AAV-FIX39-Padua vector administered to each subject is shown in Figures 20-23, which had been combined with the indicated amount of AAV empty capsids.

**[0209]** Figures 19-23 show study results, with AAV-FIX39-Padua vector administered at day 0. The results show increased Factor IX production in all four subjects, as reflected by increased FIX activity throughout the study evaluation period.

**[0210]** The initial increase in FIX activity from day 0 to about day 3 is due to administration of 100 IU/Kg Alprolix™ or BeneFIX™, which are recombinant FIX-Fc fusion protein having an approximate half-life of about 82 hours. Factor IX activity attributable to the AAV-FIX39 Padua vector begins at about day 6-8 after AAV vector infusion.

**[0211]** As summarized in Figure 19 and shown for each individual subject in Figures 20, 21A, 22A and 23A, Factor IX activity gradually increased and appeared stable throughout the 183, 102, 69 and 50 day evaluation periods for all four subjects. These data indicate that a single infusion of $5 \times 10^{11}$ vg/kg of AAV-FIX39-Padua vector results in sufficient and sustained Factor

IX production and activity to provide hemophilia B patients with meaningful and beneficial blood clotting activity to provide hemostasis.

**[0212]** As shown in Figures 19, 20A, 21A, 22A and 23A, Factor IX activity levels were at 28%, 41%, 26% and 33% of normal, for subjects 1-4 respectively, at 183, 102, 69 and 50 days after infusion. Subject #3 treated himself with an extended half-life product for a suspected ankle bleed 2 days after vector infusion; other than this there have been no factor infusions and no bleeds during the evaluation period.

**[0213]** Immunosuppressing agents (steroids) have not been administered to any of the subjects. In addition, in general there have been no sustained elevations of transaminases above the upper limit of normal, indicating no adverse effects of the treatment (Figures 20B, 21B, 22B and 23).

**[0214]** ELISPOTs were used to monitor T cell responses to AAV and to FIX in all four subjects and have shown no or very low responses. Of note, the time course of rise in Factor IX levels to a plateau level has been remarkably consistent to date (Figure 19). Modest fluctuations in antigen levels lead to greater shifts in activity levels, given the 8-fold increase in specific activity of the Factor IX Padua variant.

**[0215]** Published data (Nathwani et al., N Engl J Med. 371(21):1994-2004 (2014)) have shown long-term expression of Factor IX in men with hemophilia B infused with an AAV8 vector expressing wild-type Factor IX. However, levels of expression were low- ranging from 1.4%-2.2% normal at the lowest dose ($2 \times 10^{11}$ vector genomes [vg]/kg body weight) to 2.9-7.2% at the highest dose ($2 \times 10^{12}$ vg/kg). Moreover, 4/6 subjects infused at the highest dose required a course of immunosuppressant (prednisolone) to reduce rising transaminases associated with the highest dose (but not observed at lower doses of $2 \times 10^{11}$ or $6 \times 10^{11}$ vg/kg). Data from a natural history study of patients with hemophilia suggest that circulating levels of ~12% FIX are required to reduce the annual number of spontaneous joint bleeds to zero (den Uijl et al., Haemophilia 17(1):41-4 (2011)).

**[0216]** These are the first clinical results using a novel bioengineered AAV capsid expressing a high specific activity Factor IX transgene. The Factor IX activity levels seen in subjects 1-4 28%, 41%, 26% and 33% of normal are substantially greater circulating Factor IX levels than the those seen in prior studies, based on published data, and exceed the circulating Factor IX levels needed to reduce the annual number of spontaneous joint bleeds to zero.

**[0217]** Furthermore, the substantial Factor IX activity levels seen in this study were achieved with no recombinant Factor IX use since vector infusion, and without using immunosuppressing agents (steroids). These results show the development of an AAV-FIX vector that can direct high level clotting factor expression at low doses of AAV vector administration, so that immunosuppression is not required - an important goal for liver-directed gene therapy. Factor IX activity levels observed in this study have been sustained over the duration of the study period.

**Example 6. Reduced Immunogenicity of AAV-FIX39-Padua Vector**

**[0218]** For the current Phase I/II study, four subjects receiving 5e11 vg/kg of AAV-FIX39-Padua were monitored for potential immune responses against the AAV vector using a validated interferon-gamma (IFN-g) Enzyme Linked Immunospot (ELISPOT) assay. Purified PBMCs isolated from weekly blood draws were tested via interferon gamma ELISPOT assay. Six AAV capsid peptide pools, containing 24-25 peptides each were incubated with 2e5 cells in triplicate. T cell responses were detected using a biotinylated antibody against IFN-g, followed by colorimetric development and reported as spot-forming units (SFU) per million cells. The highest responding pool at each timepoint is shown as SFU/million cells. The historically used cutoff for positivity is >50 SFU and 3-fold media control (blue line). Subjects 840-003-001, 840-001-002, 840-001-004, and 840-001-005 (shown in black) have been followed as far out as weeks 26, 14, 11, and 8 respectively. ELISPOT results from a previous trial in which two subjects, CP-16 and PT17 received 1e12 vg/kg of the AAV8-FIX19 vector and one subject received 2e12 vg/kg of AAV8-FIX19, are shown in red.

**[0219]** Using the historically accepted value of >50 SFU and 3-fold the background (media) control as criteria for positive T cell response, there has been very little to no response in the three subjects as far out as 26-weeks post infusion (Figure 24A). This is in stark contrast to a previously unpublished study by our group using a codon optimized AAV8 vector to deliver the FIX transgene cassette to 3 subjects, in which robust IFN-g T cell responses were observed as early as the week 2 timepoint (Figure 24B). Other previously published studies using AAV-2 (Manno et al., 2006 Nat Med) and AAV-8 self-complementary vectors (Nathwani et al., 2011 NEJM) have also shown evidence of early T cell responses to the AAV capsid as well. Importantly, no responses against the transgene product have been observed in this trial.

**[0220]** It is hypothesized that the activation of a T-cell mediated immune response against transduced hepatocytes presenting AAV capsid T cell epitopes may play a role in subjects that show short-lived and eventual loss of transgene expression. Therefore, the reduced immunogenicity profile of the AAV-FIX39-Padua vector represents a promising improvement towards overall efficacy.

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form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

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# P A T E N T K R A V

1. Rekombinant adenoassocieret virus (rAAV)-vektor omfattende et genom og et kapsid, hvor genomet af nævnte rAAV-vektor omfatter en nukleinsyre omfattende en ikke-naturligt forekommende nukleotidsekvens som koder for humant Faktor IX-protein, hvor nævnte nukleotidsekvens koder for det samme humane Faktor IX-protein som nukleotidsekvensen i SEQ ID NO:10 koder for, og er mindst 85% identisk med nukleotidsekvensen i SEQ ID NO:10, og hvor kapsidet af nævnte rAAV-vektor omfatter et VP1-protein omfattende aminosyresekvensen i SEQ ID NO:4.

2. rAAV-vektor ifølge krav 1, hvor nævnte nukleotidsekvens som koder for humant Faktor IX-protein er mindst 90% identisk med nukleotidsekvensen i SEQ ID NO:10.

3. rAAV-vektor ifølge krav 1 eller 2, hvor nævnte ikke-naturligt forekommende nukleotidsekvens som koder for humant Faktor IX-protein, har et reduceret antal af CpG-dinukleotider sammenlignet med vildtypesekvensen som koder for humant Faktor IX.

4. rAAV-vektor ifølge et hvilket som helst af de foregående krav, hvor nævnte nukleinsyre yderligere omfatter mindst ét element valgt fra gruppen bestående af: en adenoassocieret virus (AAV) inverteret, terminal repeat (ITR), et ekspressionstyrende element operativt forbundet med nævnte nukleotidsekvens som koder for humant Faktor IX-protein, en polynukleotidfylder ("stuffer") og en transkriptionsterminator.

5. rAAV-vektor ifølge krav 4, hvor nævnte ekspressionsstyrende element bibringer ekspression i leveren og omfatter en promotor og eventuelt en enhancer.

6. rAAV-vektor ifølge krav 4 eller 5, hvor nævnte nukleinsyre omfatter en ITR fra AAV2-serotypen, en promotor og en enhancer som bibringer ekspression i leveren, operativt forbundet med nævnte nukleotidsekvens som koder for humant Faktor IX-protein, en polyadenyleringssekvens, en transkriptionsterminator og eventuelt en anden AAV2-ITR, hvor AVV2-ITR'en er placeret 5' for promotoren eller enhanceren eller 3' for transkriptionsterminatoren.

7. rAAV-vektor ifølge et hvilket som helst af krav 1-6, hvor nævnte nukleotidsekvens som koder for humant Faktor IX-protein er afbrudt af en intron.

8. rAAV-vektor ifølge et hvilket som helst af krav 1-7, hvor nævnte nukleinsyre yderligere omfatter en polynukleotidfylder ("stuffer").

9. rAAV-vektor ifølge krav 5 eller 6, hvor nævnte promotor er en human alfa1-antitrypsin (AAT)-promotor og nævnte enhancer er en apolipoprotein E (ApoE) HCR-1-eller -HCR-2 enhancer.

10. rAAV-vektor ifølge krav 5, 6 eller 9, hvor nævnte promoter omfatter nukleotidsekvensen i SEQ ID NO:15.

11. rAAV-vektor ifølge krav 5, 6, 9 eller 10, hvor nævnte enhancer omfatter nukleotidsekvensen i SEQ ID NO: 14.

12. rAAV-vektor ifølge et hvilket som helst af krav 6-11, hvor nævnte AAV2-ITR omfatter en af AAV2-ITR-sekvenserne som fundet i nukleotidsekvensen i SEQ ID NO:26.

13. rAAV-vektor ifølge et hvilket som helst af krav 7-12, hvor nævnte intron omfatter nukleotidsekvensen i SEQ ID NO:17.

14. rAAV-vektor ifølge et hvilket som helst af krav 7-12, hvor nukleotidsekvensen som koder for humant Faktor IX-protein og intronen omfatter nukleotidsekvensen i SEQ ID NO:25.

15. rAAV-vektor ifølge et hvilket som helst af krav 1-14 omfattende i rækkefølge en ApoE HCR-1-enhancer, en AAT-promoter, den ikke-naturligt forekommende nukleotidsekvens som koder for humant Faktor IX-protein, en polyadenyleringssekvens, en AAV2-ITR placeret 5' for enhanceren eller 3' for polyadenyleringssekvensen, og eventuelt en anden

AAV2-ITR i den modsatte position.

16. rAAV-vektor ifølge krav 15, hvor nukleotidsekvensen af nævnte ApoE-HCR-1-enhancer omfatter nukleotider 152-472 i SEQ ID NO:12, nukleotidsekvensen af nævnte AAT-promotor omfatter nukleotider 482-878 i SEQ ID NO:12, nukleotidsekvensen som koder for humant Faktor IX-protein omfatter nukleotider 908-995 og 2434-3731 i SEQ ID NO:12, nukleotidsekvensen af nævnte polyadenyleringssekvens omfatter nukleotider 3820-4047 af SEQ ID NO:12, og nukleotidsekvensen af nævnte AAV2-ITR omfatter en af AAV2-ITR-sekvenserne som fundet i nukleotidsekvensen i SEQ ID NO:26.

17. rAAV-vektor ifølge et hvilket som helst af krav 1 til 16 omfattende nukleotider 142-4096 af SEQ ID NO:12.

18. rAAV-vektor ifølge et hvilket som helst af krav 8-12 og 16, hvor nævnte nukleotidsekvens som koder for humant Faktor IX-protein, er afbrudt af en intron.

19. rAAV-vektor ifølge krav 18, hvor nævnte intron omfatter nukleotidsekvensen i SEQ ID NO:17.

20. rAAV-vektor ifølge krav 18, hvor nukleotidsekvensen som koder for humant Faktor IX-protein og intronen omfatter nukleotidsekvensen i SEQ ID NO:25.

21. rAAV-vektor ifølge et hvilket som helst af de foregående krav, hvor genomet af nævnte rAAV-vektor er enkeltstrenget.

22. rAAV-vektor ifølge et hvilket som helst af de foregående krav, hvor nævnte nukleotidsekvens som koder for humant Faktor IX-protein, er mindst 90% identisk med nukleotidsekvensen i SEQ ID NO:10.

23. Farmaceutisk sammensætning omfattende rAAV-vektoren ifølge et hvilket som helst af de foregående krav til anvendelse i behandlingen af hæmofili B.

# DRAWINGS

Drawing

Rh74 VP1 Amino Acid Sequence (SEQ ID NO:1)

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDNGRGLVLPGYKYLGPFNGLDKGEPV
NAADAAALEHDKAYDQQLQAGDNPYLRYNHADAEFQERLQEDTSFGGNLGRAVFQAKKRVLEPLGL
VESPVKTAPGKKRPVEPSPQRSPDSSTGIGKKGQQPAKKRLNFGQTGDSESVPDPQPIGEPPAGPSGLGS
GTMAAGGGAPMADNNEGADGVGSSSGNWHCDSTWLGDRVITTSTRTWALPTYNNHLYKQISNGTSG
GSTNDNTYFGYSTPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTQNEGTKTI
ANNLTSTIQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVGRSSFYCLEYFPSQ
MLRTGNNFEFSYNFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTQSTGGTAGTQQLLFSQAGPNN
MSAQAKNWLPGPCYRQQRVSTTLSQNNNSNFAWTGATKYHLNGRDSLVNPGVAMATHKDDEERFFP
SSGVLMFGKQGAGKDNVDYSSVMLTSEEEIKTTNPVATEQYGVVADNLQQQNAAPIVGAVNSQGALP
GMVWQNRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADPPTTFNQAKLASFIT
QYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSTNVDFAVNTEGTYSEPRPIGTRYLTRNL

Figure 1

Rh74 VP2 Amino Acid (SEQ ID NO:2):

TAPGKKRPVEPSPQRSPDSSTGIGKKGQQPAKKRLNFGQTGDSESVPDPQPIGEPPAGPSGLGSGTMAA
GGGAPMADNNEGADGVGSSSGNWHCDSTWLGDRVITTSTRTWALPTYNNHLYKQISNGTSGGSTND
NTYFGYSTPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTQNEGTKTIANNLT
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Figure 2

Rh74 VP3 Amino Acid (SEQ ID NO:3):

MAAGGGAPMADNNEGADGVGSSSGNWHCDSTWLGDRVITTSTRTWALPTYNNHLYKQISNGTSGGS
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MVWQNRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADPPTTFNQAKLASFITQ
YSTGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSTNVDFAVNTEGTYSEPRPIGTRYLTRNL

Figure 3

4-1 variant VP1 capsid amino acid sequence (SEQ ID NO:4)

1 MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDNGRGLVLPGYKYLGPFNGLD
61 KGEPVNAADAAALEHDKAYDQQLQAGDNPYLRYNHADAEFQERLQEDTSFGGNLGRAVFQ
121 AKKRVLEPLGLVESPVKTAPGKKRPVEPSPQRSPDSSTGIGKKGQQPAKKRLNFGQTGDS
181 ESVPDPQPIGEPPA**A**PSG**V**G**PN**TMAAGGGAPMADNNEGADGVGSSSGNWHCDSTWLGDRV
241 ITTSTRTWALPTYNNHLYKQISNGTSGGSTNDNTYFGYSTPWGYFDFNRFHCHFSPRDWQ
301 RLINNNWGFRPKRLNFKLFNIQVKEVTQNEGTKTIANNLTSTIQVFTDSEYQLPYVLGSA
361 HQGCLPPFPADVFMIPQYGYLTLNNGSQAV GRSSFYCLEYFPSQMLRTGNNFEFSYNFED
421 VPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTQSTGGTAGTQQLLFSQAGPNNMSAQAKNW
481 LPGPCYRQQRVSTTLSQNNNSNFAWTGATKYHLNGRDSLVNPGVAMATHKDDEERFFPSS
541 GVLMFGKQGAGKDNVDYSSVMLTSEEEIKTTNPVATEQYGVVADNLQQQNAAPIVGAVNS
601 QGALPGMVWQNRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADP
661 PTTFNQAKLASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSTNVDFAVNTE
721 GTYSEPRPIGTRYLTRNL

4-1 variant VP2 capsid amino acid sequence (SEQ ID NO:27)

TAPGKKRPVEPSPQRSPDSSTGIGKKGQQPAKKRLNFGQTGDSESVPDPQPIGEPPA**A**PSG**V**G**PN**T
MAAGGGAPMADNNEGADGVGSSSGNWHCDSTWLGDRVITTSTRTWALPTYNNHLYKQISNGTS
GGSTNDNTYFGYSTPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTQNEG
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TTFNQAKLASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSTNVDFAVNTEGTYSEPRPI
GTRYLTRNL

4-1 variant VP3 capsid amino acid sequence (SEQ ID NO:3)

MAAGGGAPMADNNEGADGVGSSSGNWHCDSTWLGDRVITTSTRTWALPTYNNHLYKQISNGTS
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TKTIANNLTSTIQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVGRSSFYCL
EYFPSQMLRTGNNFEFSYNFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTQSTGGTAGTQQLLF
SQAGPNNMSAQAKNWLPGPCYRQQRVSTTLSQNNNSNFAWTGATKYHLNGRDSLVNPGVAMAT
HKDDEERFFPSSGVLMFGKQGAGKDNVDYSSVMLTSEEEIKTTNPVATEQYGVVADNLQQQNAAP
IVGAVNSQGALPGMVWQNRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADPP
TTFNQAKLASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSTNVDFAVNTEGTYSEPRPI
GTRYLTRNL

Figure 4

15-1 variant VP1 capsid amino acid sequence (SEQ ID NO:5)

1 MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQRQDNGRGLVLPGYRYLGPFNGLD
61 KGEPVNAADAAALEHDRAYDQQLQAGDNPYLRYNHADAEFQERLQEDTSFGGNLGRAVFQ
121 AKKRVLEPLGLVESPVRTAPGKKRPVEPSPQRSPDSSTGIGKKGQQPARKRLNFGQTGDS
181 ESVPDPQPIGEPPAAPSGVGPNTMAAGGGAPMADNNEGADGVGSSSGNWHCDSTWLGDRV
241 ITTSTRTWALPTYNNHLYRQISNGTSGGSTNDNTYFGYSTPWGYFDFNRFHCHFSPRDWQ
301 RLINNNWGFRPKRLNFKLFNIQVKEVTQNEGTRTIANNLTSTIQVFTDSEYQLPYVLGSA
361 HQGCLPPFPADVFMIPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFEFSYNFED
421 VPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTQSTGGTAGTQQLLFSQAGPNNMSAQAKNW
481 LPGPCYRQQRVSTTLSQNNNSNFAWTGATKYHLNGRDSLVNPGVAMATHRDDEERFFPSS
541 GVLMFGRQGAGRDNVDYSSVMLTSEEEIRTTNPVATEQYGVVADNLQQN AAPIVGAVNS
601 QGALPGMVWQNRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADP
661 PTTFNQAKLASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSTNVDFAVNTE
721 GTYSEPRPIGTRYLTRNL

Figure 5

15-2 variant VP1 capsid amino acid sequence (SEQ ID NO:6)

1 MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQRQD NGRGLVLPGY RYLGPFNGLD
61 KGEPVNAADAAALEHDRAYDQQLQAGDNPYLRYNHADAEF QERLQEDTSF GGNLGRAVFQ
121 AKKRVLEPLGLVESPVRTAPGKKRPVEPSPQRSPDSSTGI GKRGQQPARK RLNFGQTGDS
181 ESVPDPQPIGEPPAAPSGVGPNTMAAGGGAPMADNNEGAD GVGSSSGNWH CDSTWLGDRV
241 ITTSTRTWALPTYNNHLYRQISNGTSGGSTNDNTYFGYST PWGYFDFNRF HCHFSPRDWQ
301 RLINNNWGFRPKRLNFKLFNIQVKEVTQNEGTRTIANNLT STIQVFTDSE YQLPYVLGSA
361 HQGCLPPFPADVFMIPQYGYLTLNNGSQAVGRSSFYCLEY FPSQMLRTGN NFEFSYNFED
421 VPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTQSTGGTAGT QQLLFSQAGP NNMSAQAKNW
481 LPGPCYRQQRVSTTLSQNNNSNFAWTGATKYHLNGRDSLV NPGVAMATHR DDEERFFPSS
541 GVLMFGKQGAGRDNVDYSSVMLTSEEEIRTTNPVATEQYG VVADNLQQQN AAPIVGAVNS
601 QGALPGMVWQNRDVYLQGPIWAKIPHTDGNFHPSPLMGGF GLKHPPPQIL IKNTPVPADP
661 PTTFNQAKLASFITQYSTGQVSVEIEWELQKENSKRWNPE IQYTSNYYKS TNVDFAVNTE
721 GTYSEPRPIGTRYLTRNL

Figure 6

15-3/15-5 variant VP1 capsid amino acid sequence (SEQ ID NO:7)

1 MAADGYLPDWLEDNLSEGIR EWWDLKPGAPKPKANQQRQD NGRGLVLPGY RYLGPFNGLD
61 KGEPVNAADAAALEHDRAYDQQLQAGDNPYLRYNHADAEF QERLQEDTSF GGNLGRAVFQ
121 AKKRVLEPLGLVESPVRTAPGKKRPVEPSPQRSPDSSTGI GKRGQQPAKK RLNFGQTGDS
181 ESVPDPQPIGEPPAAPSGVGPNTMAAGGGAPMADNNEGAD GVGSSSGNWH CDSTWLGDRV
241 ITTSTRTWALPTYNNHLYRQISNGTSGGSTNDNTYFGYST PWGYFDFNRF HCHFSPRDWQ
301 RLINNNWGFRPKRLNFKLFNIQVKEVTQNEGTRTIANNLT STIQVFTDSE YQLPYVLGSA
361 HQGCLPPFPADVFMIPQYGYLTLNNGSQAVGRSSFYCLEY FPSQMLRTGN NFEFSYNFED
421 VPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTQSTGGTAGT QQLLFSQAGP NNMSAQAKNW
481 LPGPCYRQQRVSTTLSQNNNSNFAWTGATKYHLNGRDSLV NPGVAMATHR DDEERFFPSS
541 GVLMFGRQGAGRDNVDYSSVMLTSEEEIRTTNPVATEQYG VVADNLQQQN AAPIVGAVNS
601 QGALPGMVWQNRDVYLQGPIWAKIPHTDGNFHPSPLMGGF GLKHPPPQIL IKNTPVPADP
661 PTTFNQAKLASFITQYSTGQVSVEIEWELQKENSKRWNPE IQYTSNYYKS TNVDFAVNTE
721 GTYSEPRPIGTRYLTRNL

Figure 7

15-4 variant VP1 capsid amino acid sequence (SEQ ID NO:8)

1 MAADGYLPDWLEDNLSEGIREWWDLKPGAP KPKANQQRQD NGRGLVLPGY RYLGPFNGLD
61 KGEPVNAADAAALEHDRAYDQQLQAGDNPY LRYNHADAEF QERLQEDTSF GGNLGRAVFQ
121 AKKRVLEPLGLVESPVRTAPGKKRPVEPSP QRSPDSSTGI GKRGQQPAKK RLNFGQTGDS
181 ESVPDPQPIGEPPAAPSGVGPNTMAAGGGA PMADNNEGAD GVGSSSGNWH CDSTWLGDRV
241 ITTSTRTWALPTYNNHLYRQISNGTSGGST NDNTYFGYST PWGYFDFNRF HCHFSPRDWQ
301 RLINNNWGFRPKRLNFKLFNIQVKEVTQNE GTRTIANNLT STIQVFTDSE YQLPYVLGSA
361 HQGCLPPFPADVFMIPQYGYLTLNNGSQAV GRSSFYCLEY FPSQMLRTGN NFEFSYNFED
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601 QGALPGMVWQNRDVYLQGPIWAKIPHTDGN FHPSPLMGGF GLKHPPPQIL IKNTPVPADP
661 PTTFNQARLASFITQYSTGQVSVEIEWELQ KENSKRWNPE IQYTSNYYKS TNVDFAVNTE
721 GTYSEPRPIGTRYLTRNL

Figure 8

15-6 variant VP1 capsid amino acid sequence (SEQ ID NO:9)

1 MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQRQDNGRGLVLPGY RYLGPFNGLD
61 KGEPVNAADAAALEHDRAYDQQLQAGDNPYLRYNHADAEFQERLQEDTSF GGNLGRAVFQ
121 AKKRVLEPLGLVESPVRTAPGKKRPVEPSPQRSPDSSTGIGKKGQQPAKK RLNFGQTGDS
181 ESVPDPQPIGEPPAAPSGVGPNTMAAGGGAPMADNNEGADGVGSSSGNWH CDSTWLGDRV
241 ITTSTRTWALPTYNNHLYRQISNGTSGGSTNDNTYFGYSTPWGYFDFNRF HCHFSPRDWQ
301 RLINNNWGFRPKRLNFKLFNIQVKEVTQNEGTRTIANNLTSTIQVFTDSE YQLPYVLGSA
361 HQGCLPPFPADVFMIPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGN NFEFSYNFED
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601 QGALPGMVWQNRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQIL IKNTPVPADP
661 PTTFNQARLASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYYKS TNVDFAVNTE
721 GTYSEPRPIGTRYLTRNL

Figure 9

FIX39 nucleic acid sequence (SEQ ID NO:10)

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CCAAGCTGACTTGA

Figure 10

FIX19 nucleic acid sequence (SEQ ID NO:11)

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Figure 11

pAAV-ApoE_hAAT-FIX39 (SEQ ID NO:12)

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Figure 12A

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Figure 12A cont.

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Figure 12A cont.

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Figure 12A cont.

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Figure 12B

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Figure 12B cont.

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9301 ccgtcaagtc agcgtaatgc tctgccagtg ttacaaccaa ttaaccaatt ctgattagaa
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9541 tttcccctcg tcaaaaataa ggttatcaag tgagaaatca ccatgagtga cgactgaatc
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Figure 12B cont.

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 9781 ccggcgcagg aacactgcca gcgcatcaac aatattttca cctgaatcag gatattcttc
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 9961 gaccatctca tctgtaacat cattggcaac gctacctttg ccatgtttca gaaacaactc
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11041 gaaacgcctg gtatctttat agtcctgtcg ggtttcgcca cctctgactt gagcgtcgat
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Figure 12B cont.

pAAV-ApoE_hAAT-FIX39

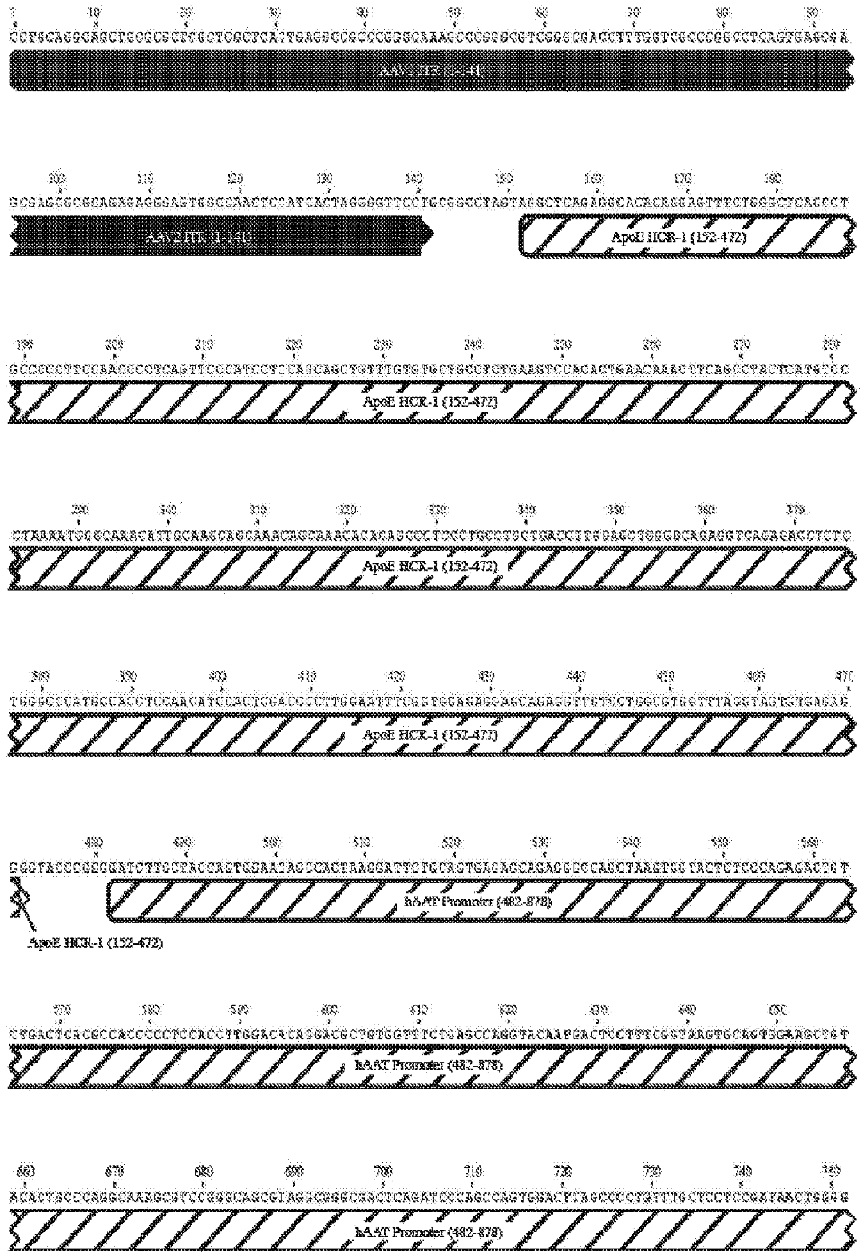


Figure 13

pAAV-ApoE_hAAT-FIX3

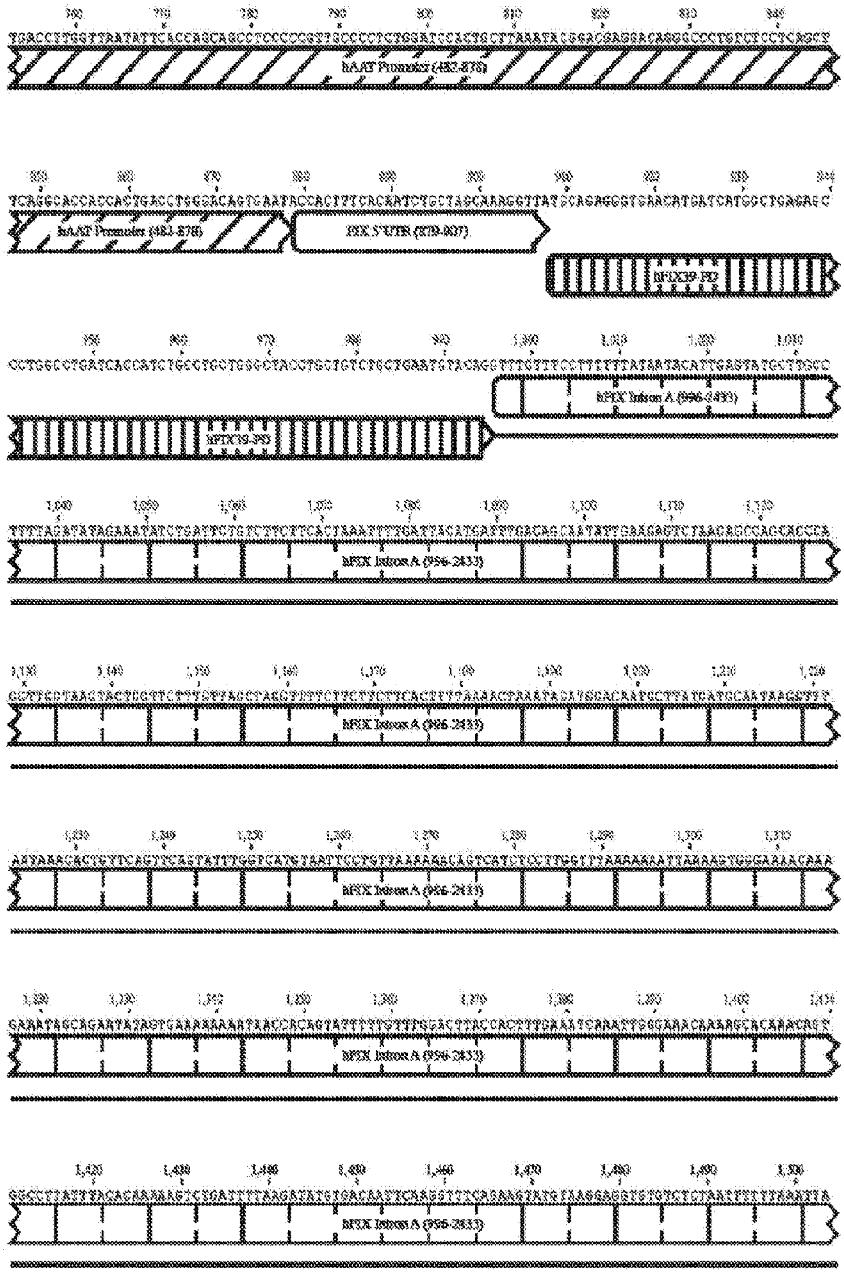

Figure 13 cont.

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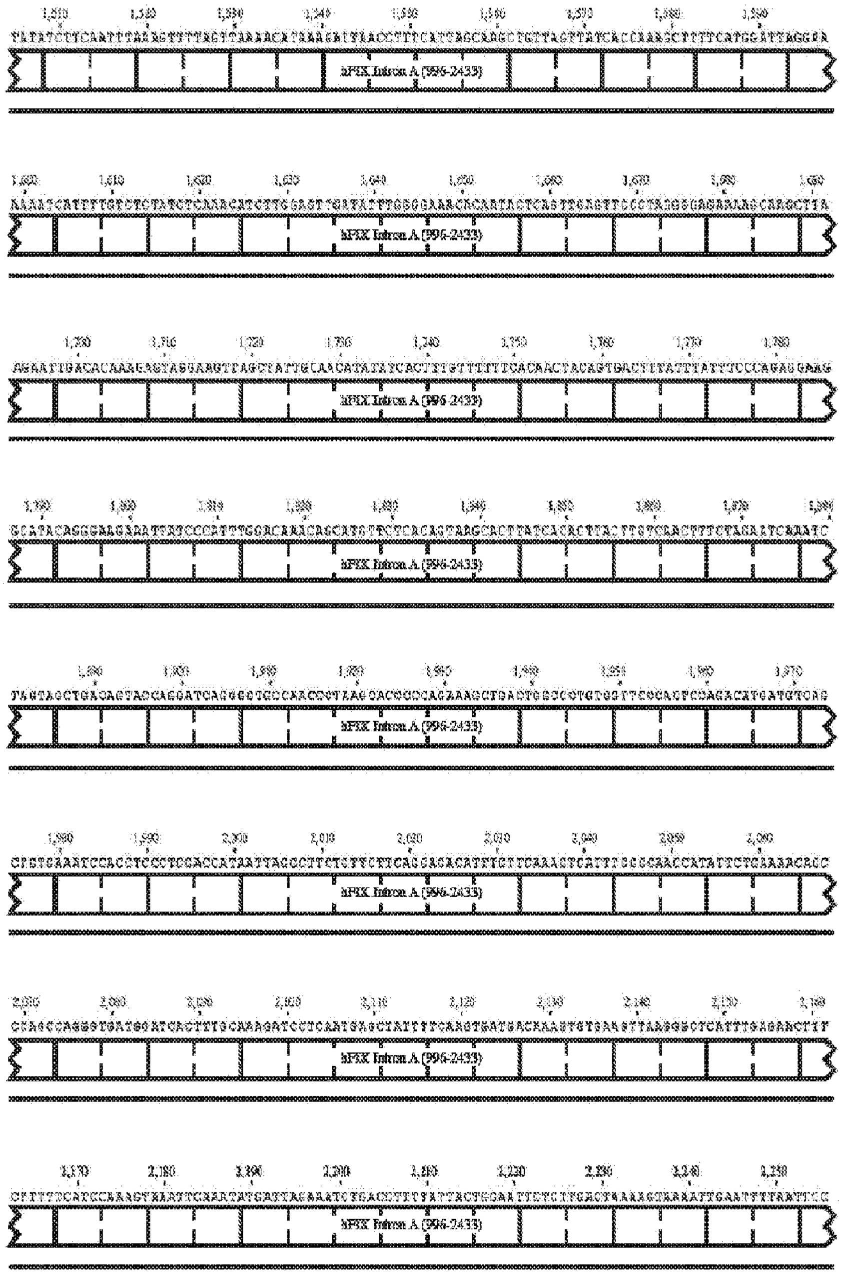


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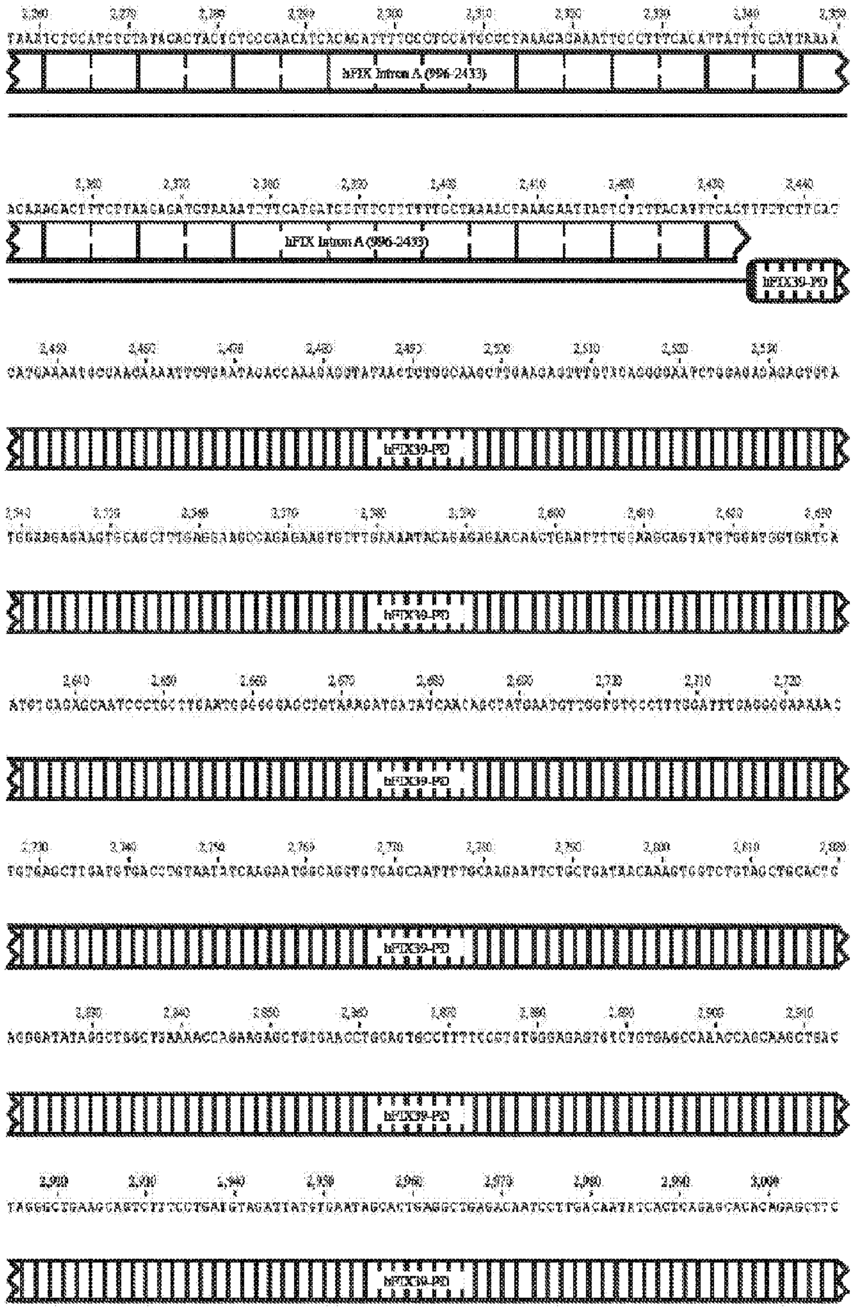


Figure 13 cont.

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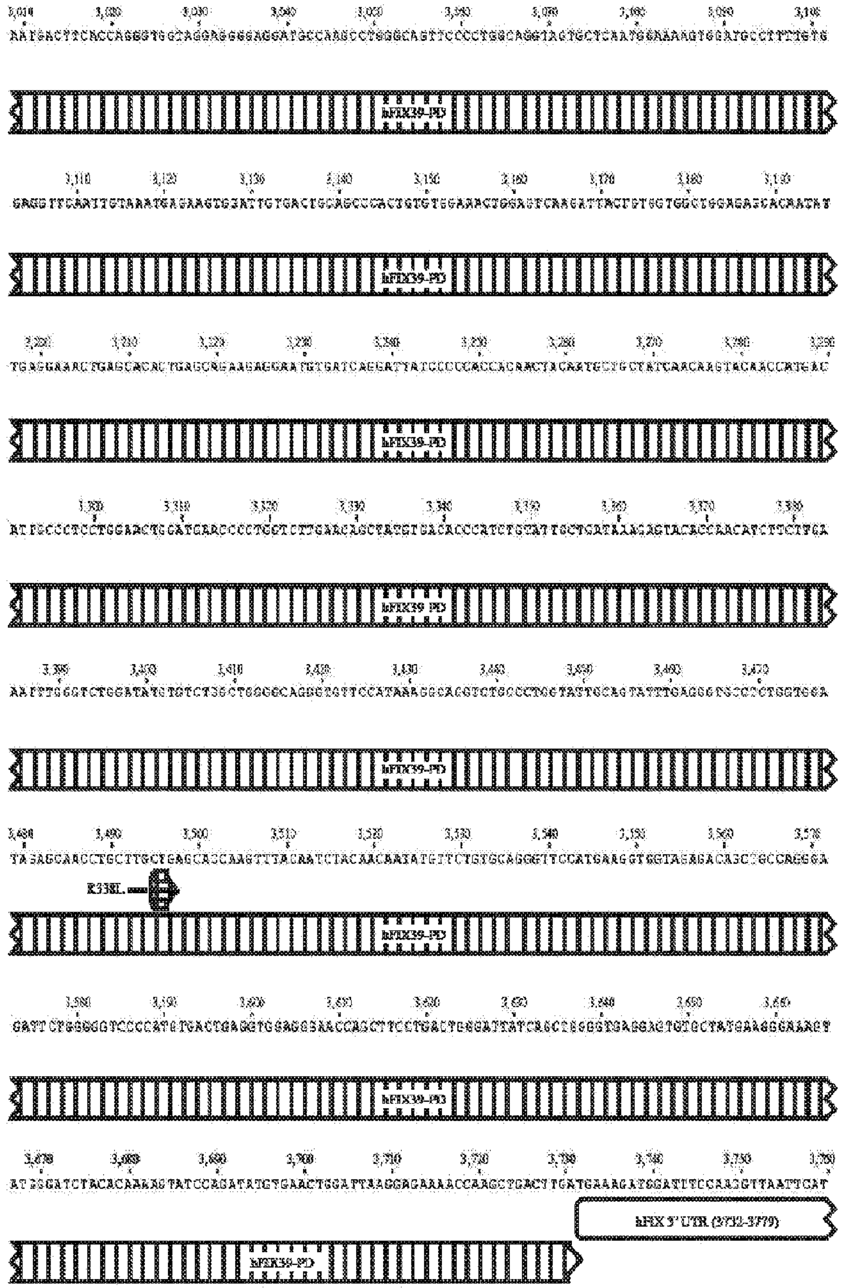


Figure 13 cont.

pAAV-ApoE_hAAT-FIX3

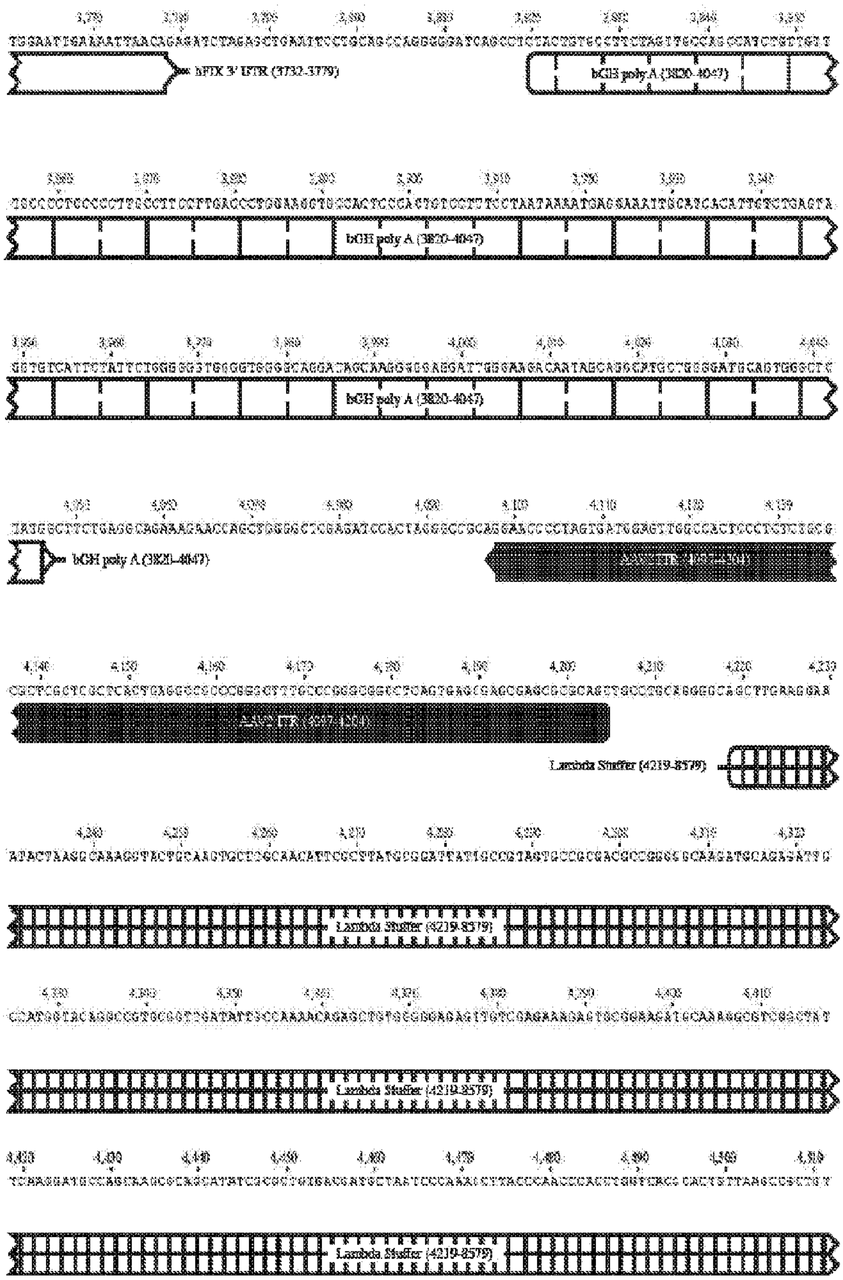


Figure 13 cont.

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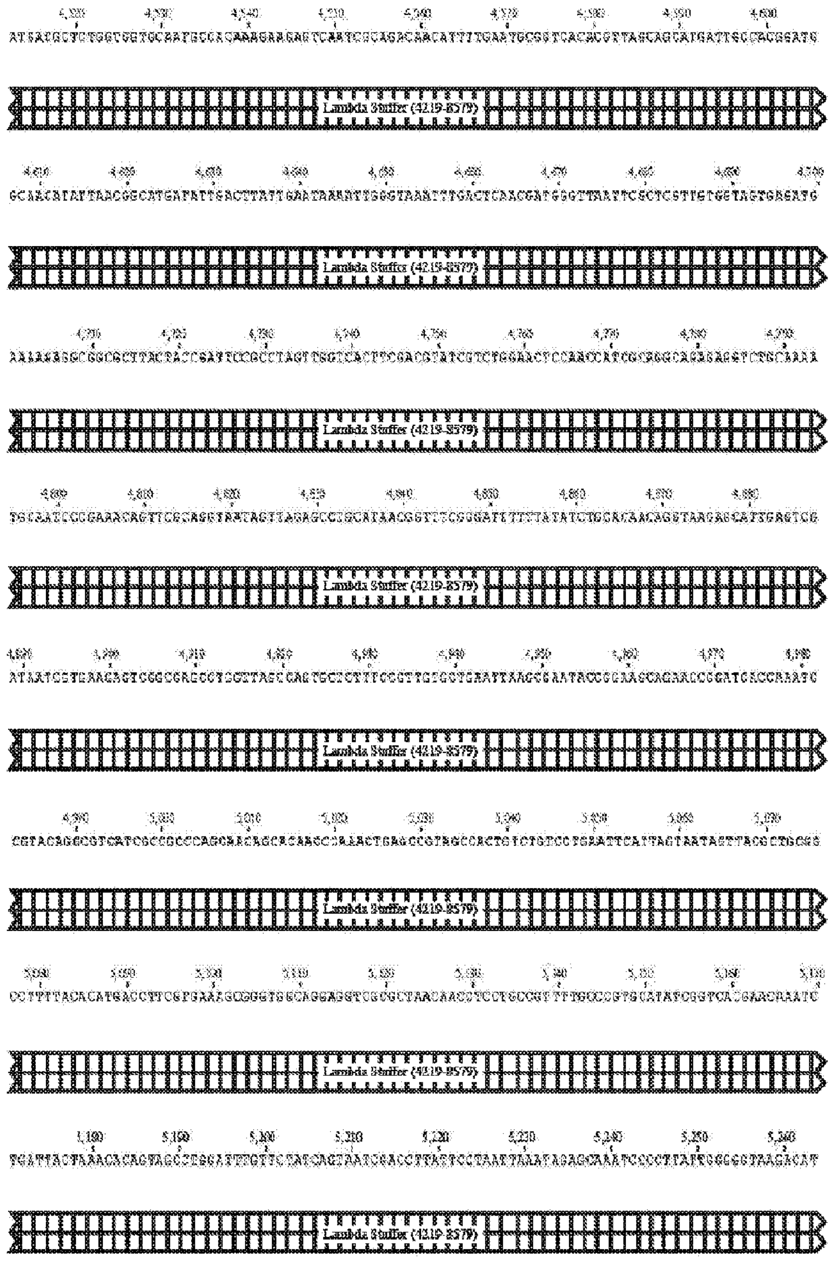


Figure 13 cont.

pAAV-ApoE_hAAT-FIX3

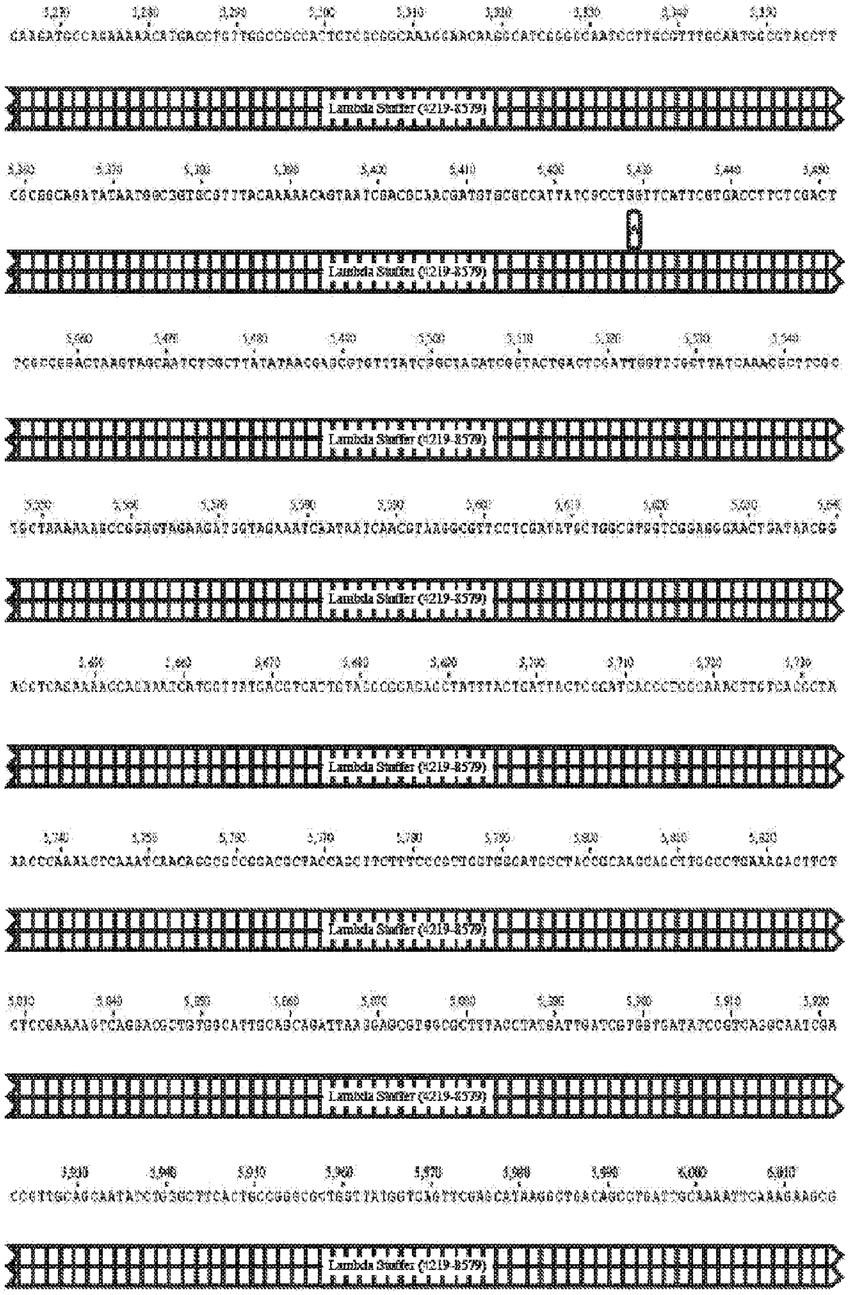


Figure 13 cont.

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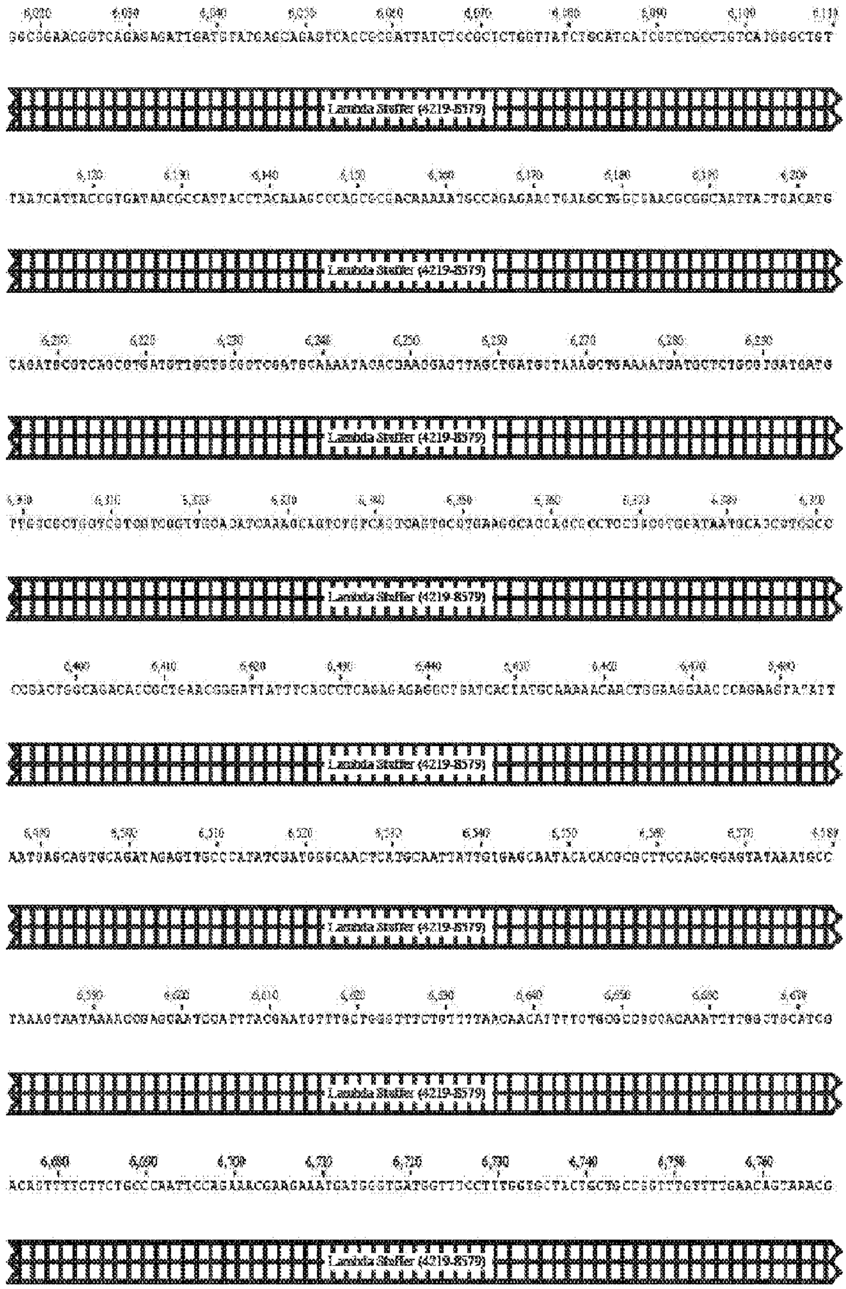


Figure 13 cont.

pAAV-ApoE_hAAT-FIX3

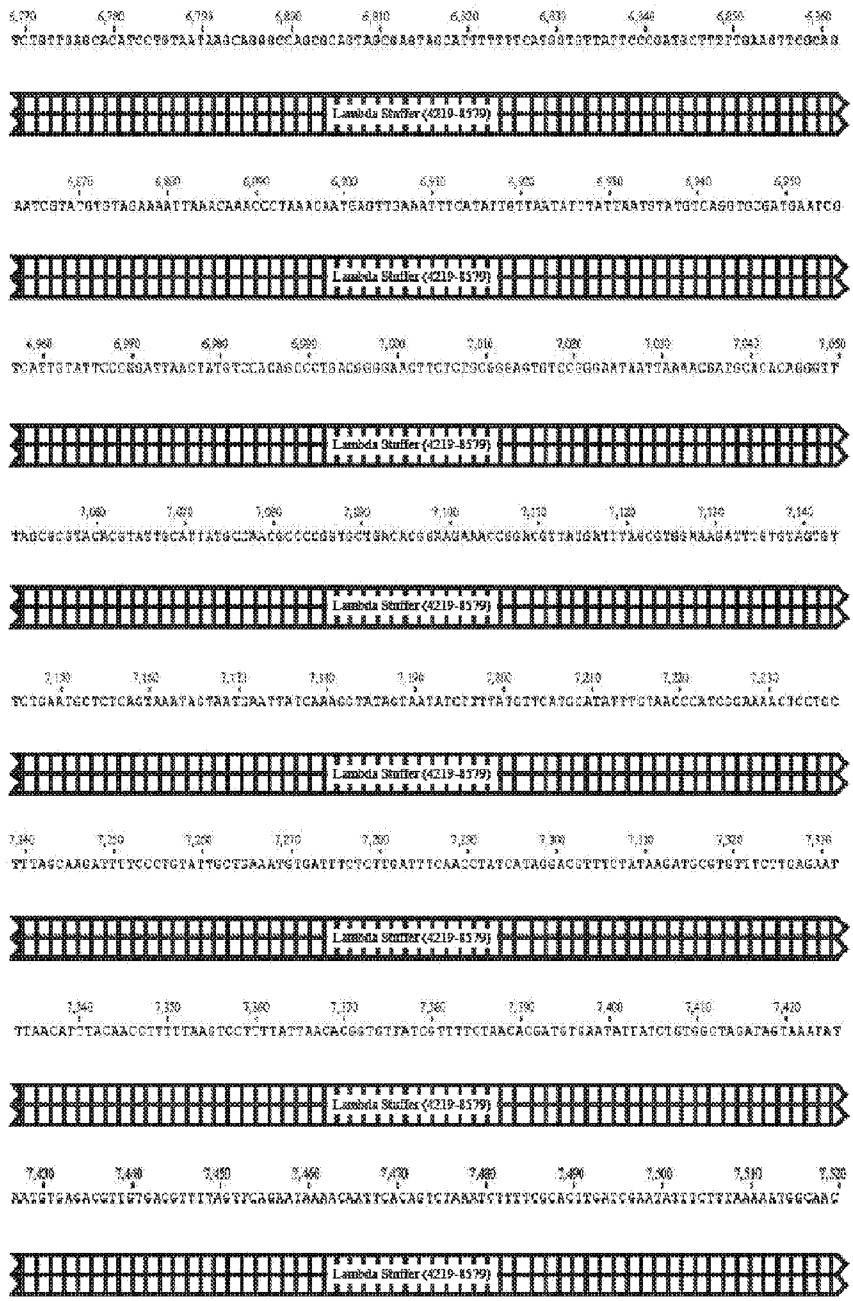


Figure 13 cont.

pAAV-ApoE_hAAT-FIX3

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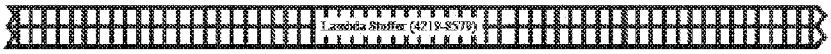


7,620 7,630 7,640 7,650 7,660 7,670 7,680 7,690 7,700
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7,710 7,720 7,730 7,740 7,750 7,760 7,770 7,780 7,790 7,800
ACAACCGACAGATGCATGTAAGGCCAACGTGCTCAAATCTTCATACAGAAAGATTTGAAGTAATATTTTAACCGCTAGATGAAGAGCAAGCGCA


7,810 7,820 7,830 7,840 7,850 7,860 7,870 7,880 7,890
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7,900 7,910 7,920 7,930 7,940 7,950 7,960 7,970 7,980 7,990
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8,000 8,010 8,020 8,030 8,040 8,050 8,060 8,070 8,080
TATGAAGGATTATTCCCTGGTGGTTGACTGATCACCATAACTGCTAATCATTCAAACTATTTAGTCTGTGACAGAGCCAACACGCAGTCTGTCA


8,090 8,100 8,110 8,120 8,130 8,140 8,150 8,160 8,170
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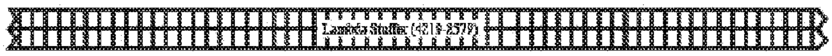


8,180 8,190 8,200 8,210 8,220 8,230 8,240 8,250 8,260 8,270
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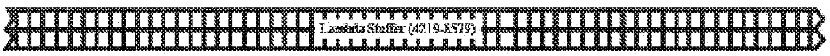


Figure 13 cont.

pAAV-ApoE_hAAT-FIX3

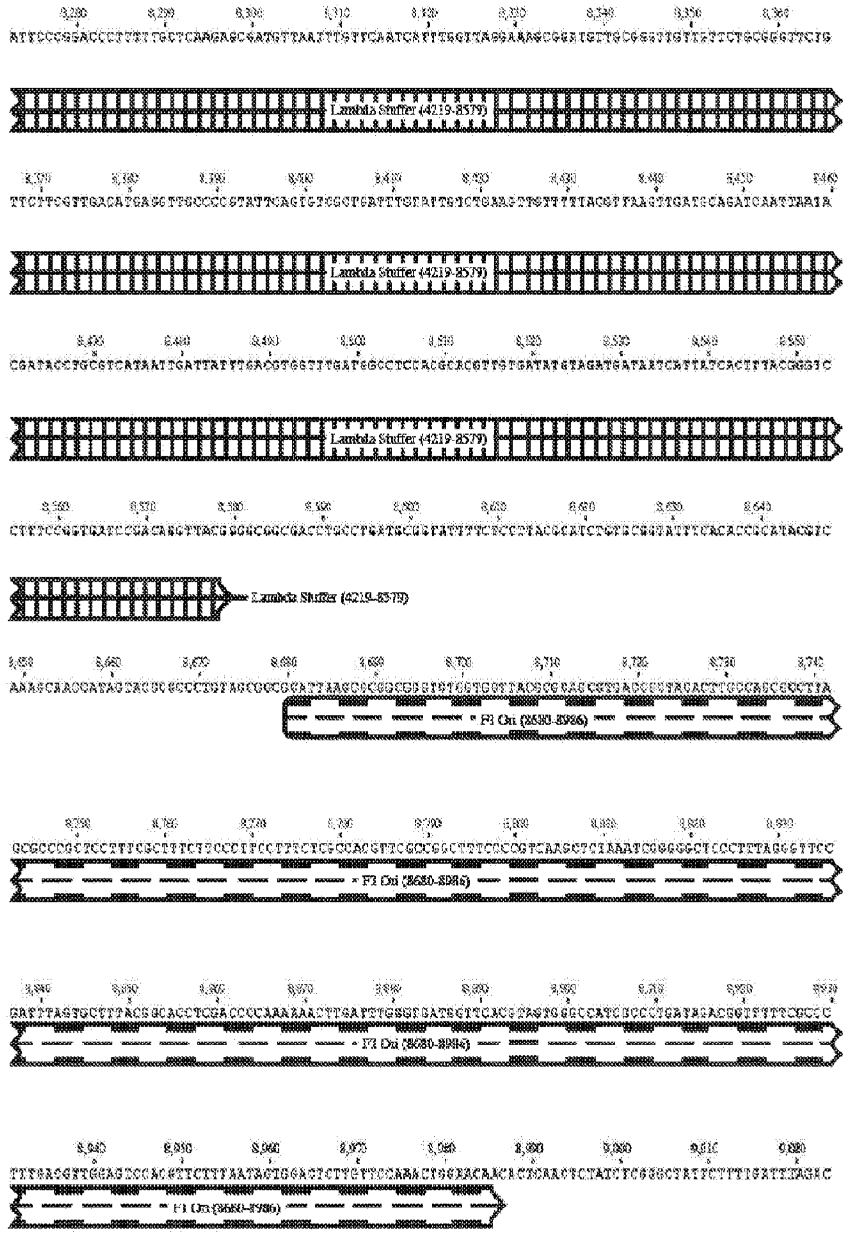


Figure 13 cont.

pAAV-ApoE_hAAT-FIX3

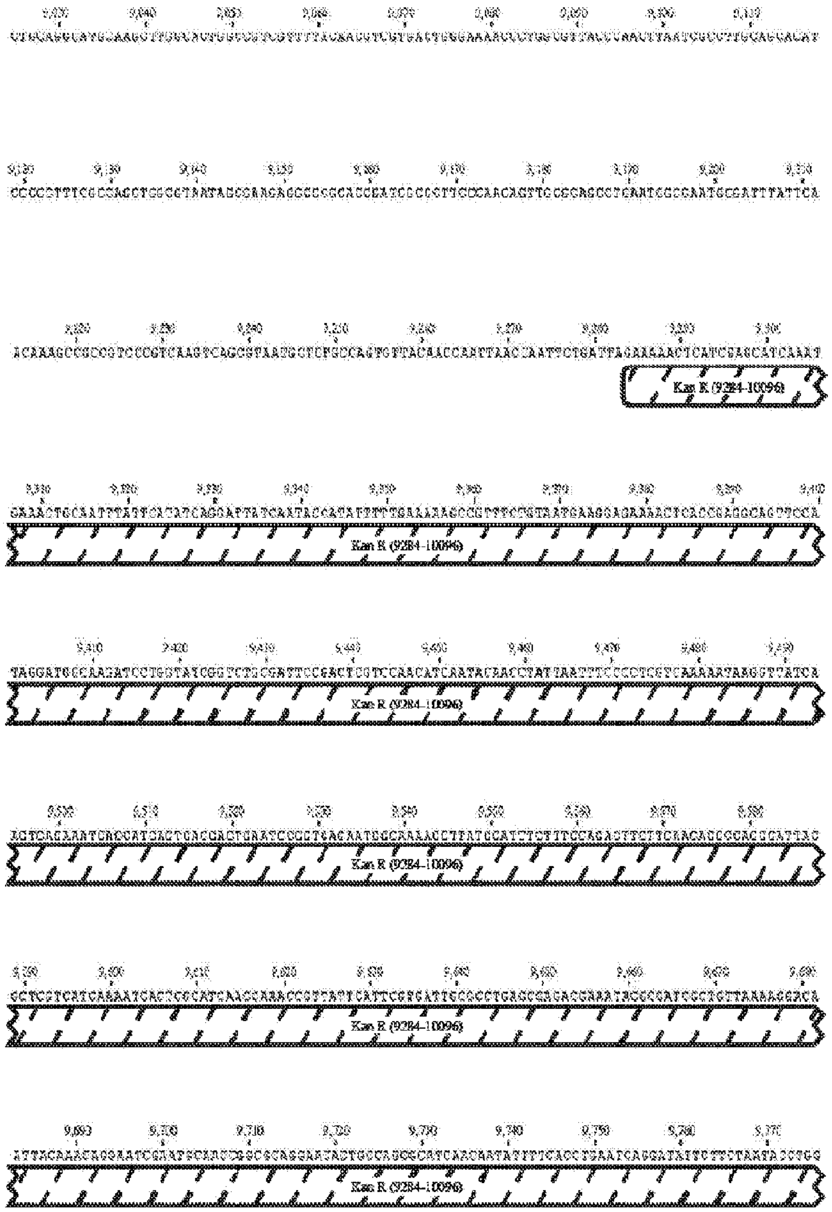


Figure 13 cont.

pAAV-ApoE_hAAT-FIX3

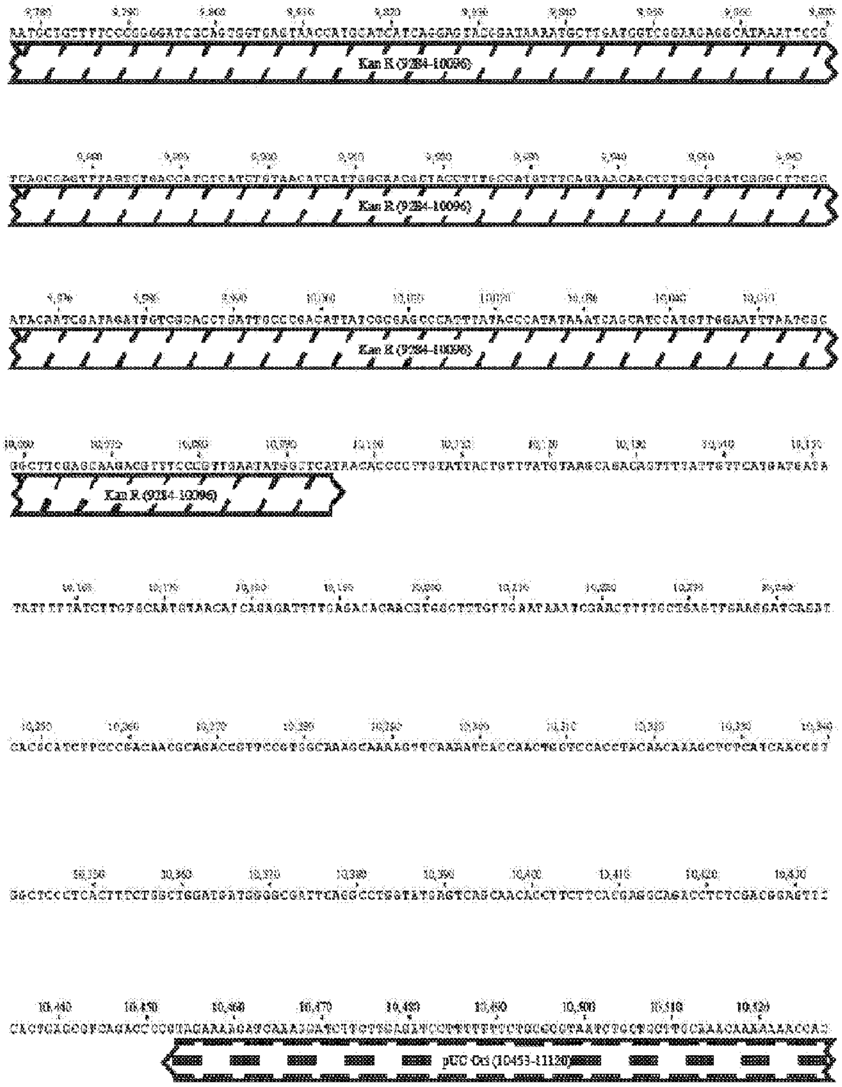


Figure 13 cont.

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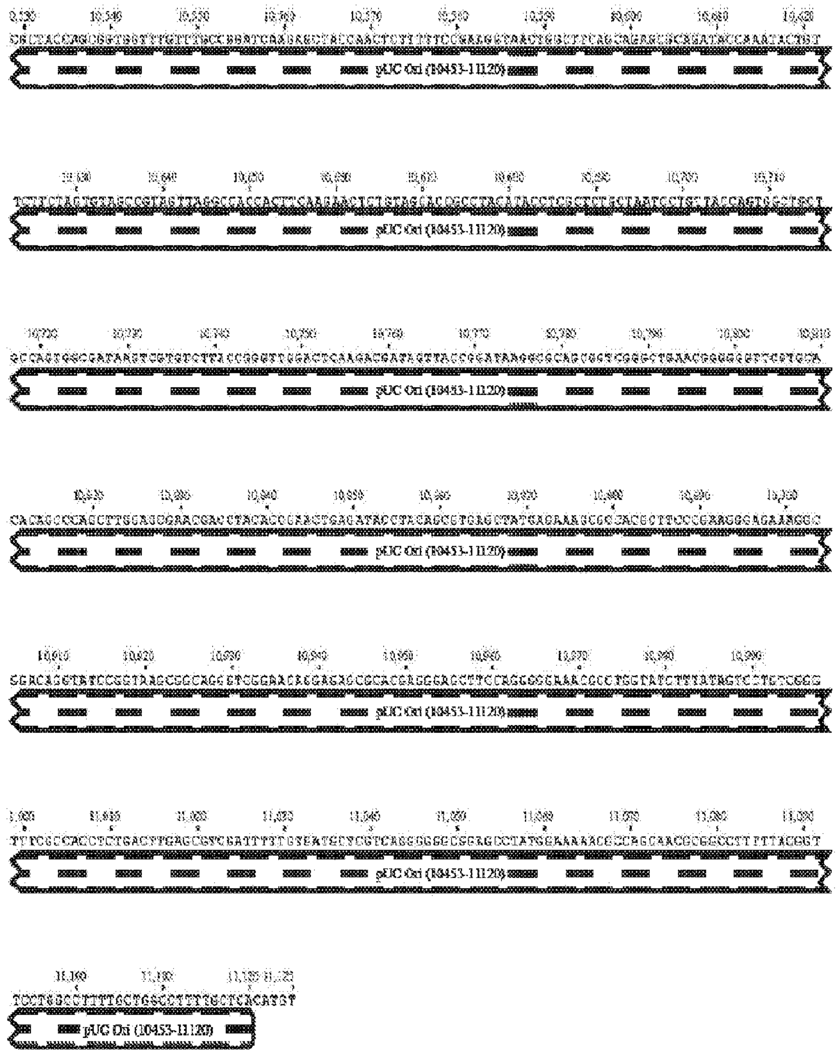


Figure 13 cont.

Intron A nucleic acid sequence (SEQ ID NO:17):

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CCTAAATCTCCATGTGTATACAGTACTGTGGGAACATCACAGATTTTGGCTCCATGCCCTAAAGAG
AAATTGGCTTTCAGATTATTTGGATTAAAAACAAAGACTTTCTTAAGAGATGTAAAATTTTCATGA
TGTTTTCTTTTTTGCTAAAACTAAAGAATTATTCTTTTACATTTCAG

Figure 14

FIX39 nucleic acid sequence including intron A (intron A is underlined) (SEQ ID NO:25)

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CTGCTGTCTGCTGAATGTACAGGTTTGTTTCCTTTTTTATAATACATTGAGTATGCTTGCCTTTTAGA
TATAGAAATATCTGATTCTGTCTTCTTCACTAAATTTTGATTACATGATTTGACAGCAATATTGAAG
AGTCTAACAGCCAGCACCCAGGTTGGTAAGTACTGGTTCTTTGTTAGCTAGGTTTTCTTCTTCTTCA
CTTTTAAAACTAAATAGATGGACAATGCTTATGATGCAATAAGGTTTAATAAACACTGTTCAGTTC
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GAAAACAAAGAAATAGCAGAATATAGTGAAAAAAAATAACCACAGTATTTTTGTTTGGACTTACC
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ATGATGTCAGCTGTGAAATCCACCTCCCTGGACCATAATTAGGCTTCTGTTCTTCAGGAGACATTT
GTTCAAAGTCATTTGGGCAACCATATTCTGAAAACAGCCCAGCCAGGGTGATGGATCACTTTGCAA
AGATCCTCAATGAGCTATTTTCAAGTGATGACAAAGTGTGAAGTTAAGGGCTCATTTGAGAACTTT
CTTTTTCATCCAAAGTAAATTCAAATATGATTAGAAATCTGACCTTTTATTACTGGAATTCTCTTGA
CTAAAAGTAAAATTGAATTTTAATTCCTAAATCTCCATGTGTATACAGTACTGTGGGAACATCACA
GATTTTGGCTCCATGCCCTAAAGAGAAATTGGCTTTCAGATTATTTGGATTAAAAACAAAGACTTT
CTTAAGAGATGTAAAATTTTCATGATGTTTTCTTTTTTGCTAAAACTAAAGAATTATTCTTTTACAT
TTCAGTTTTTCTTGATCATGAAAATGCCAACAAAATTCTGAATAGACCAAAGAGGTATAACTCTGG
CAAGCTTGAAGAGTTTGTACAGGGGAATCTGGAGAGAGAGTGTATGGAAGAGAAGTGCAGCTTTG
AGGAAGCCAGAGAAGTGTTTGAAAATACAGAGAGAACAACTGAATTTTGGAAGCAGTATGTGGAT
GGTGATCAATGTGAGAGCAATCCCTGCTTGAATGGGGGGAGCTGTAAAGATGATATCAACAGCTA
TGAATGTTGGTGTCCCTTTGGATTTGAGGGGAAAAACTGTGAGCTTGATGTGACCTGTAATATCAA
GAATGGCAGGTGTGAGCAATTTTGCAAGAATTCTGCTGATAACAAAGTGGTCTGTAGCTGCACTGA
GGGATATAGGCTGGCTGAAAACCAGAAGAGCTGTGAACCTGCAGTGCCTTTTCCCTGTGGGAGAG
TGTCTGTGAGCCAAACCAGCAAGCTGACTAGGGCTGAAGCAGTCTTTCCTGATGTAGATTATGTGA
ATAGCACTGAGGCTGAGACAATCCTTGACAATATCACTCAGAGCACACAGAGCTTCAATGACTTC
ACCAGGGTGGTAGGAGGGGAGGATGCCAAGCCTGGGCAGTTCCCCTGGCAGGTAGTGCTCAATGG
AAAAGTGGATGCCTTTTGTGGAGGTTCAATTGTAAATGAGAAGTGGATTGTGACTGCAGCCCACTG
TGTGGAAACTGGAGTCAAGATTACTGTGGTGGCTGGAGAGCACAATATTGAGGAAACTGAGCACA
CTGAGCAGAAGAGGAATGTGATCAGGATTATCCCCCACCACAACTACAATGCTGCTATCAACAAG
TACAACCATGACATTGCCCTCCTGGAACTGGATGAACCCCTGGTCTTGAACAGCTATGTGACACCC
ATCTGTATTGCTGATAAAGAGTACACCAACATCTTCTTGAAATTTGGGTCTGGATATGTGTCTGGCT
GGGGCAGGGTGTTCCATAAAGGCAGGTCTGCCCTGGTATTGCAGTATTTGAGGGTGCCTCTGGTGG
ATAGAGCAACCTGCTTGCTGAGCACCAAGTTTACAATCTACAACAATATGTTCTGTGCAGGGTTCC
ATGAAGGTGGTAGAGACAGCTGCCAGGGAGATTCTGGGGGTCCCCATGTGACTGAGGTGGAGGGA
ACCAGCTTCCTGACTGGGATTATCAGCTGGGGTGAGGAGTGTGCTATGAAGGGAAAGTATGGGAT
CTACACAAAAGTATCCAGATATGTGAACTGGATTAAGGAGAAAACCAAGCTGACTTGA

Figure 15

Transduction efficiency of the AAV-4-1 variant capsid (SEQ ID NO:4) analyzed in an *in vitro* setting

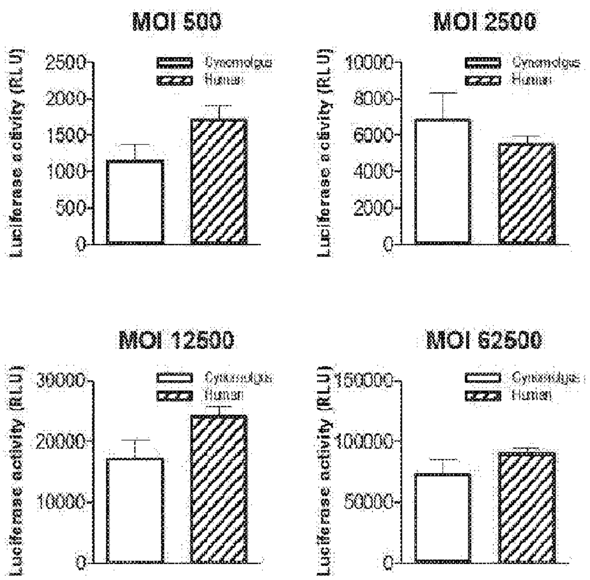
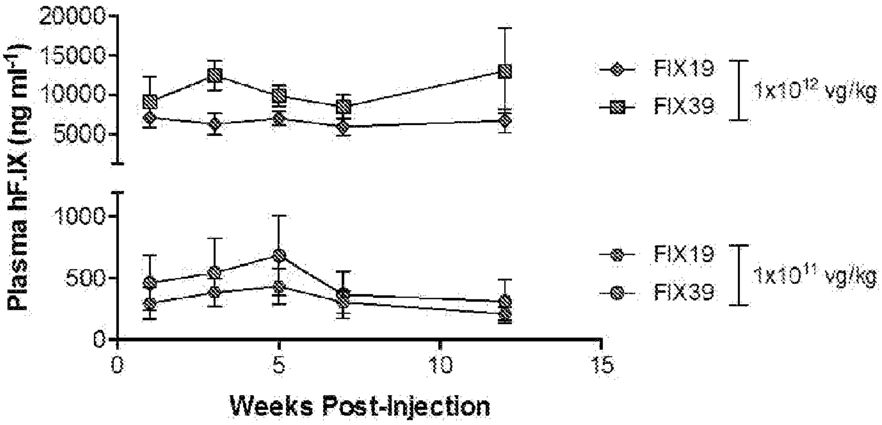



Figure 16



Plasma hF.IX (ng ml-1)
20000
15000
10000
5000
1000
500
0
0
5
10
15
Weeks Post-Injection
FIX19
FIX39
1x10^12 vg/kg
FIX19
FIX39
1x10^11 vg/kg


Figure 17

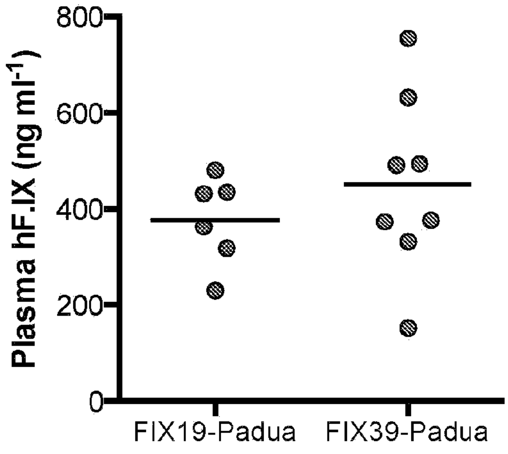



Figure 18

Comparison of early FIX:C activity levels for first 4 Subjects

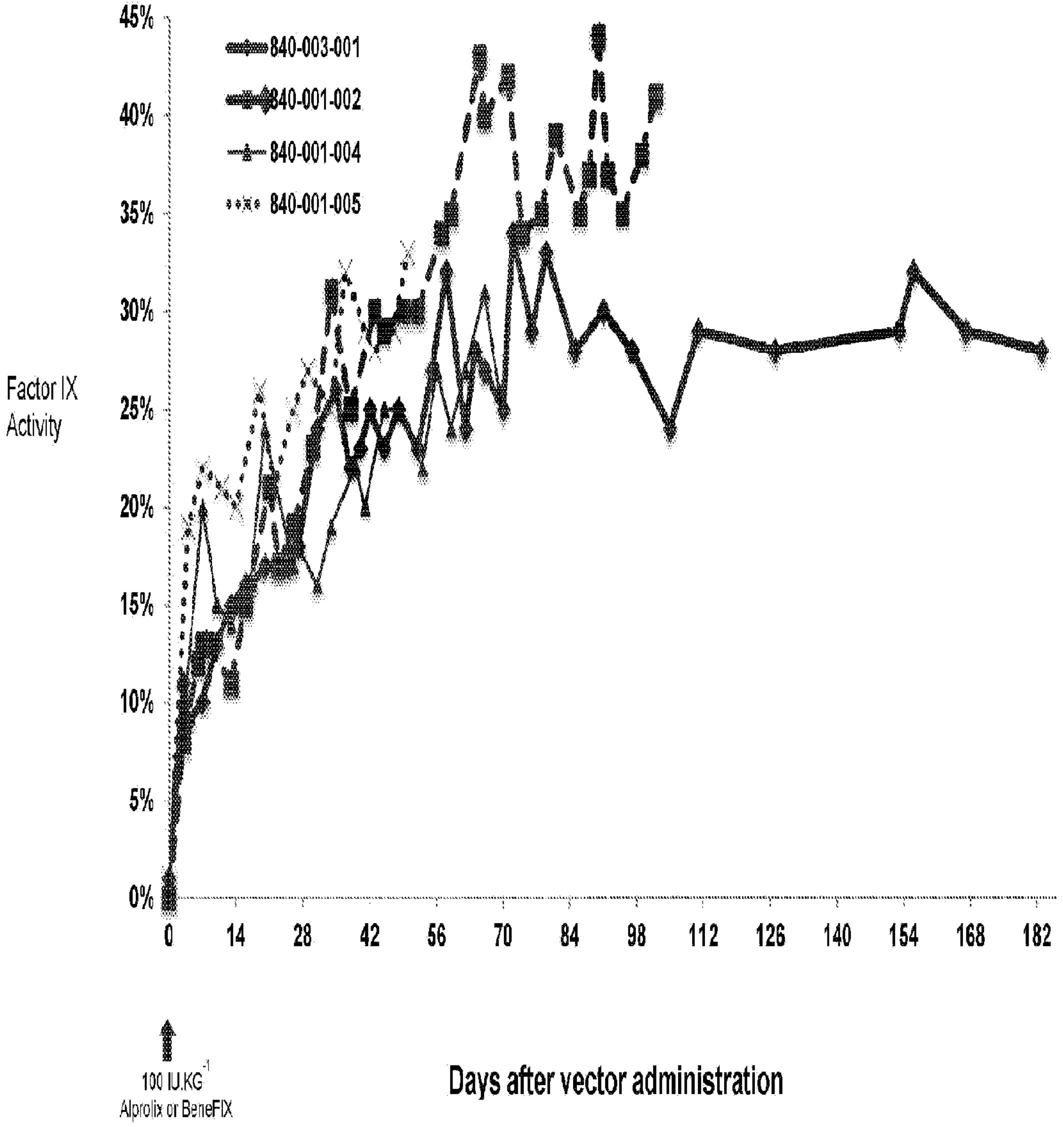



Figure 19

AAV-FIX39-Padua: 1st subject day 183 FIX:C activity level at 28%
Subject 1 [$5x10^{11}$ vg/kg] at 183 days since last Alprolix infusion
Dose: $4.08x10^{13}$ vg of AAV-FIX39-Padua + $2.01x10^{14}$ cp of Empty Capsids [1:5]

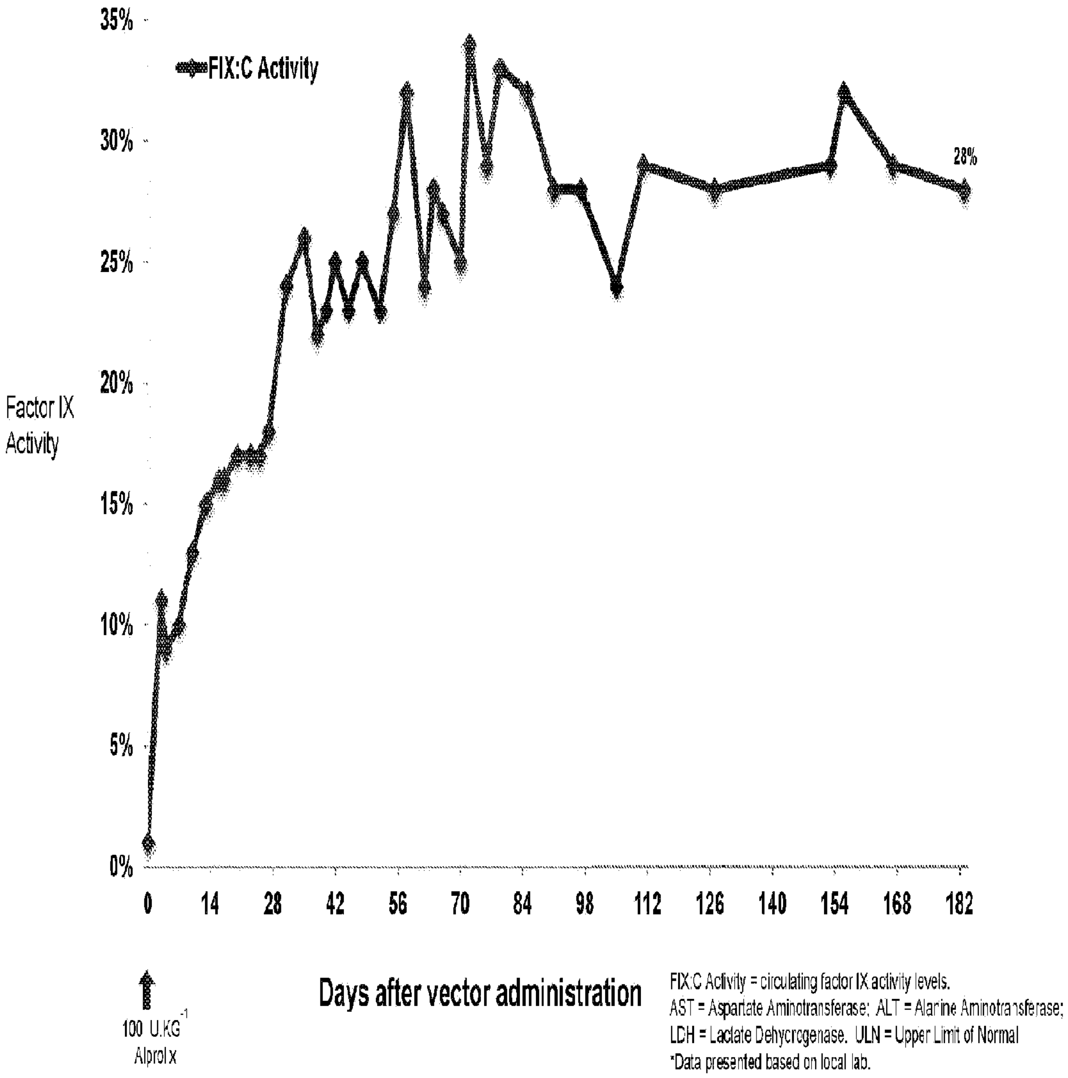



Figure 20A

AAV-FIX39-Padua: 1st subject day 183 Liver Functions
Subject 1 [5x10^11 vg/kg] at 183 days since last Alprolix infusion
4.08x10^13 vg of AAV-FIX39-Padua + 2.01x10^14 cp of Empty Capsids [1:5]

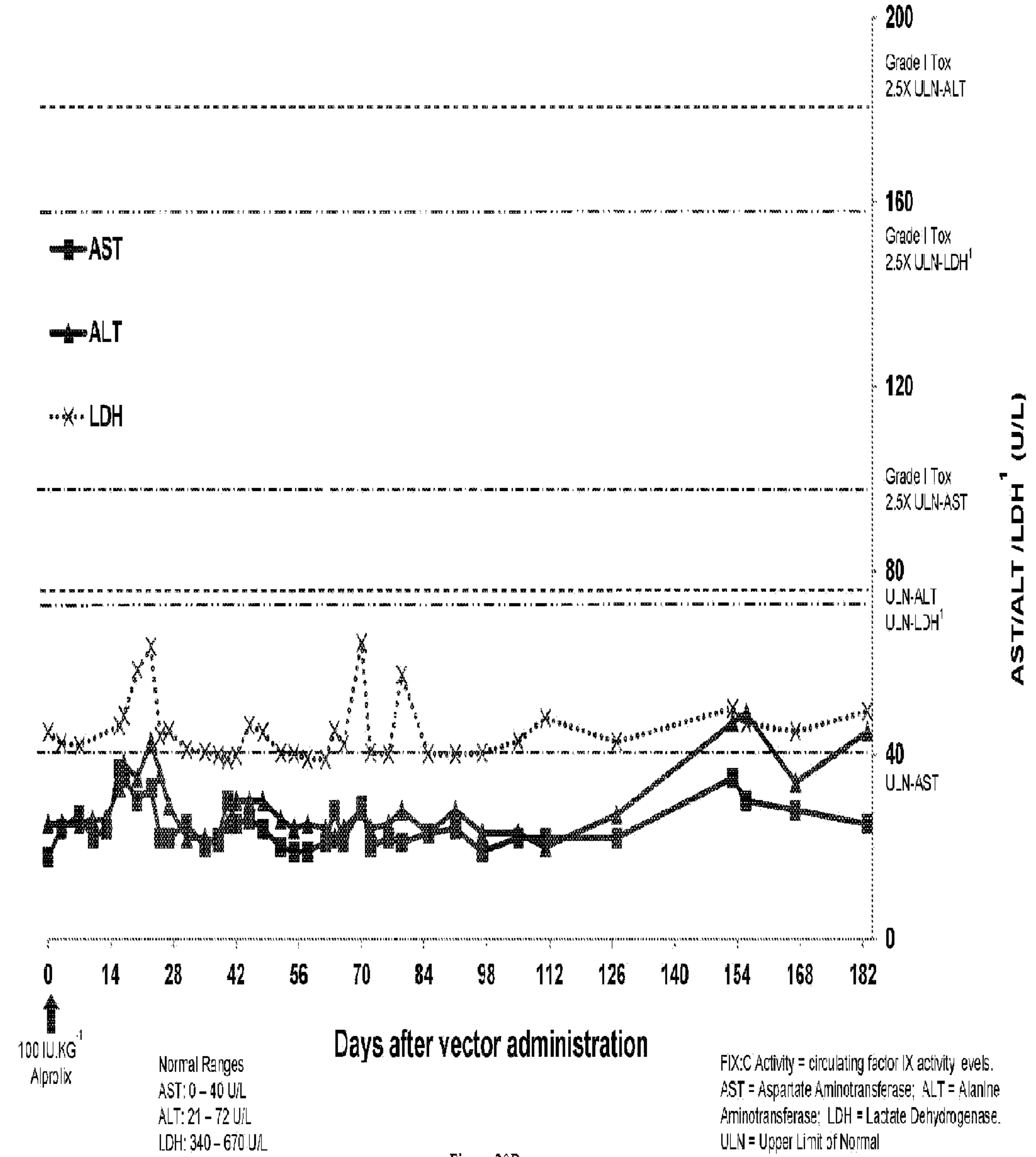



Figure 20B

AAV-FIX39-Padua: 2nd subject day 102 FIX:C Activity Level at 41%
Subject 2 [5x10$^{11}$ vg/kg] at 102 days since last BeneFIX infusion
Dose: 2.84x10$^{13}$ vg of AAV-FIX39-Padua + 1.39x10$^{14}$ cp of Empty Capsids [1:5]

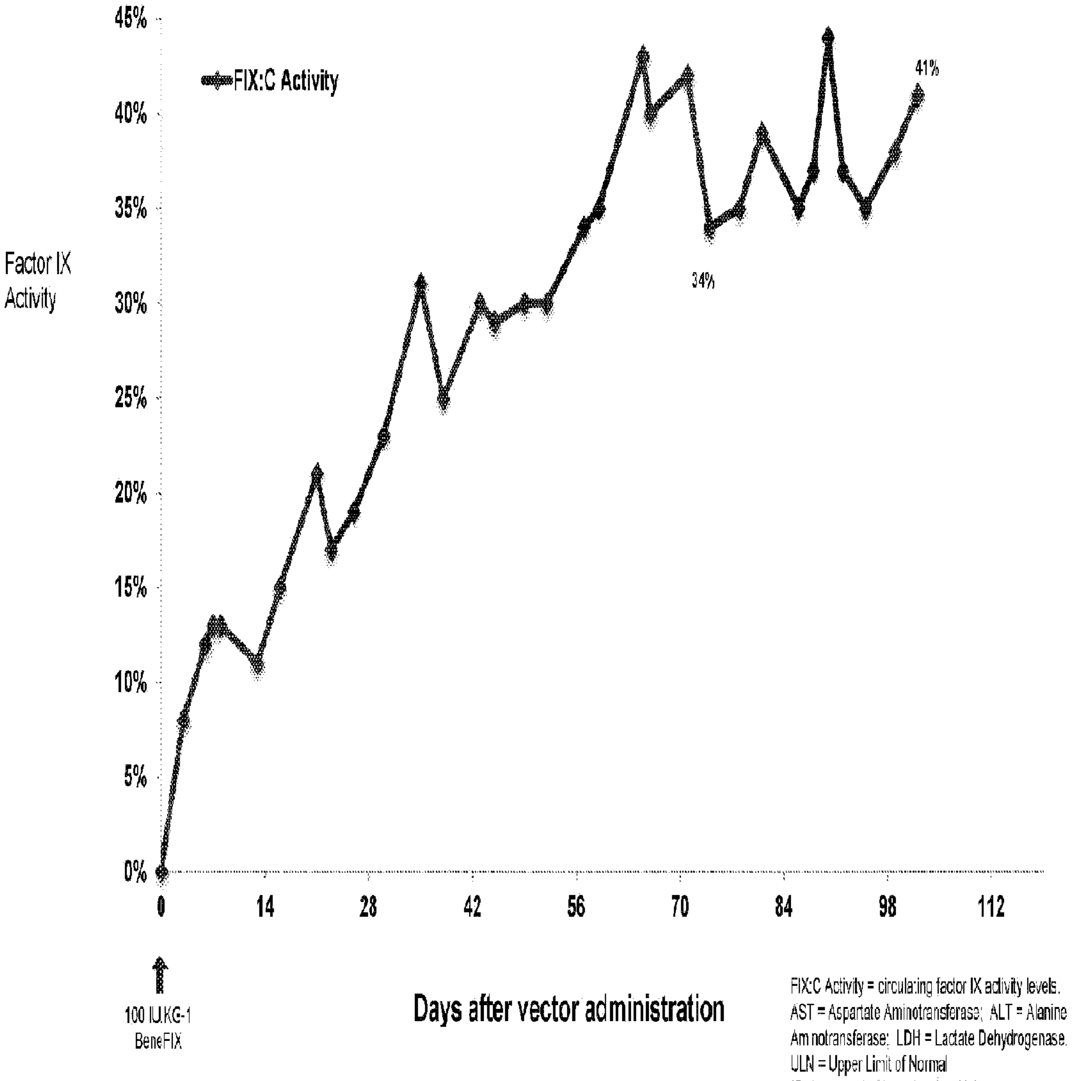



Figure 21A

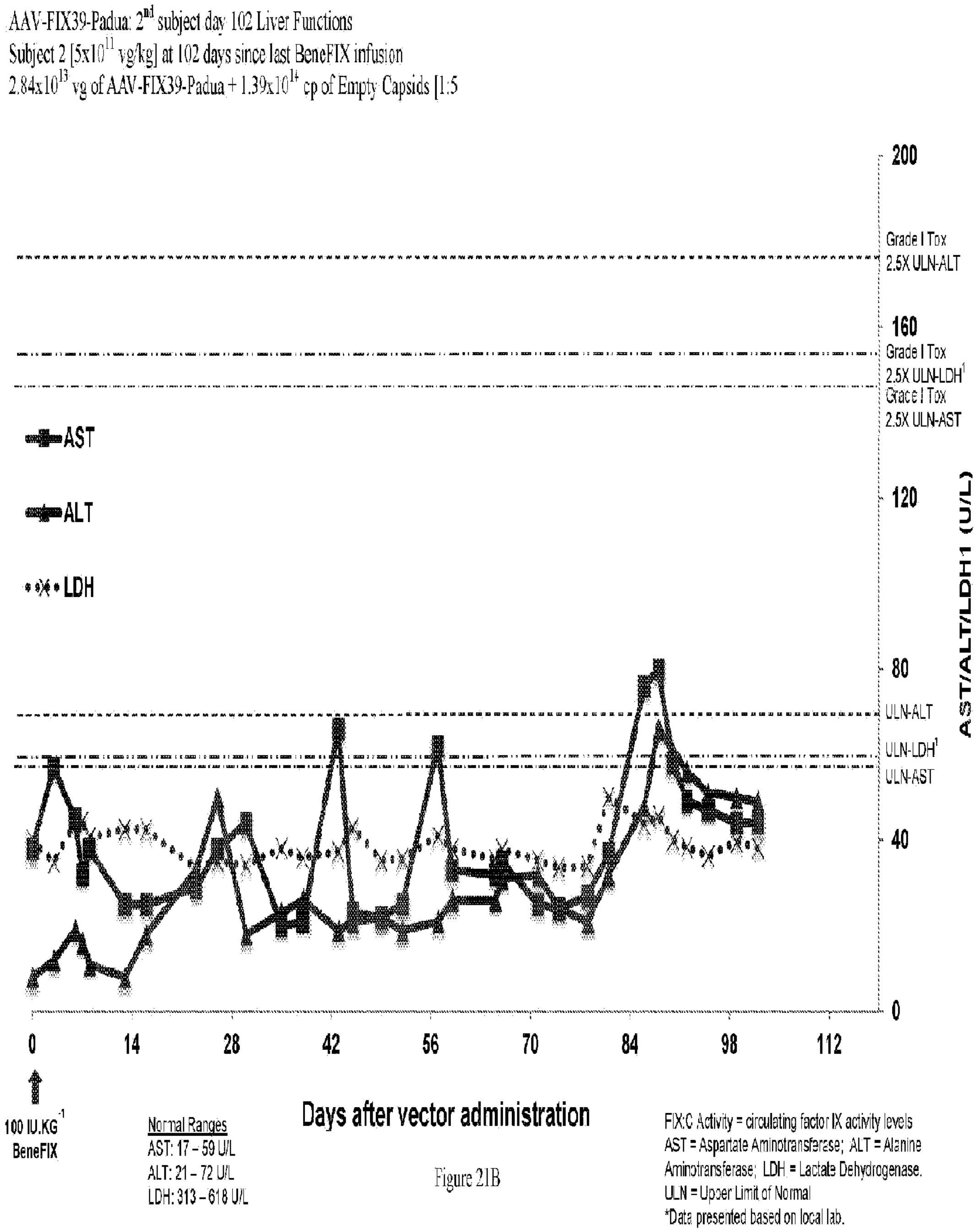

AAV-FIX39-Padua: 2nd subject day 102 Liver Functions
Subject 2 [5x10^11 vg/kg] at 102 days since last BeneFIX infusion
2.84x10^13 vg of AAV-FIX39-Padua + 1.39x10^14 cp of Empty Capsids [1:5
AST
ALT
LDH
Grade I Tox 2.5X ULN-ALT
Grade I Tox 2.5X ULN-LDH1
Grade I Tox 2.5X ULN-AST
ULN-ALT
ULN-LDH1
ULN-AST
AST/ALT/LDH1 (U/L)
0
40
80
120
160
200
0 14 28 42 56 70 84 98 112
100 IU.KG-1 BeneFIX
Days after vector administration
Normal Ranges
AST: 17 – 59 U/L
ALT: 21 – 72 U/L
LDH: 313 – 618 U/L
FIX:C Activity = circulating factor IX activity levels
AST = Aspartate Aminotransferase; ALT = Alanine Aminotransferase; LDH = Lactate Dehydrogenase.
ULN = Upper Limit of Normal
*Data presented based on local lab.

Figure 21B

AAV-FIX39-Padua: 3rd subject day 69 FIX:C Activity Level at 26%
Subject 3 [5x10$^{11}$ vg/kg] at 67 days since last Alprolix infusion
5.38x10$^{13}$ vg of AAV-FIX39-Padua + 1.93x10$^{14}$ cp of Empty Capsids [1:4]

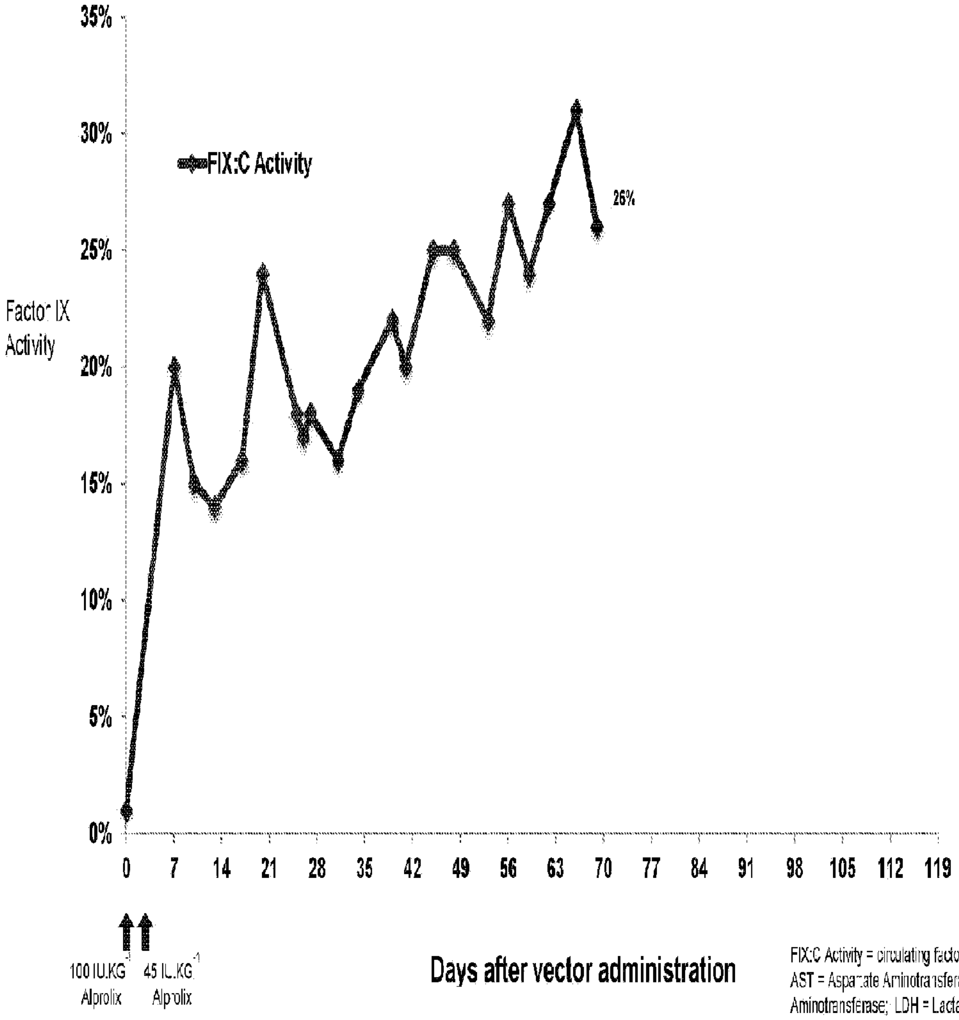
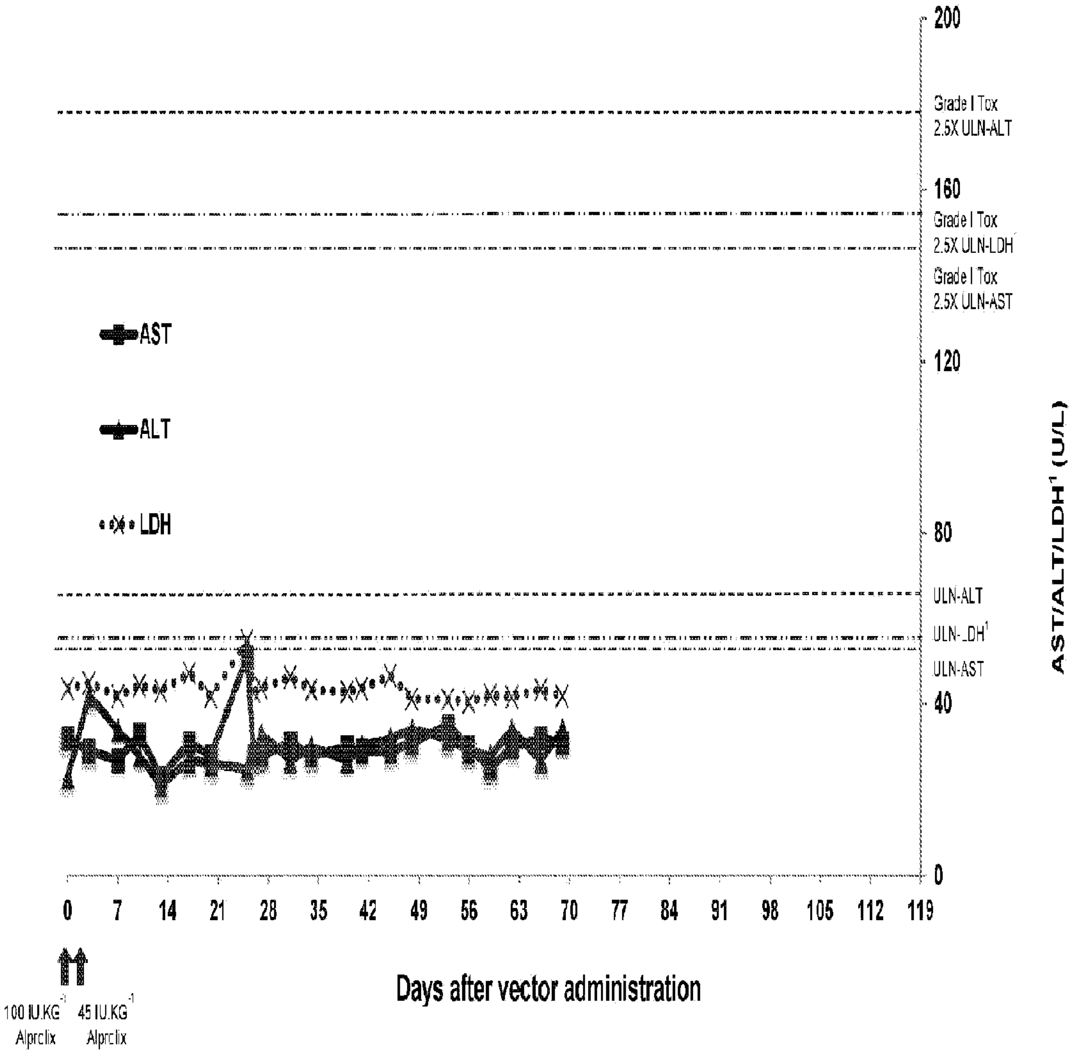



Figure 22A

FIX:C Activity = circulating factor IX activity levels.
AST = Aspartate Aminotransferase; ALT = Alanine Aminotransferase; LDH = Lactate Dehydrogenase.
ULN = Upper Limit of Normal
*Data presented based on local lab.

AAV-FIX39-Padua: 3rd subject day 69 Liver Functions
Subject 3 [5x10^11 vg/kg] at 67 days since last Alprolix infusion
5.38x10^13 vg of AAV-FIX39-Padua + 1.93x10^14 cp of Empty Capsids [ :4]




Normal Ranges
AST: 17 – 59 U/L
ALT: 21 – 72 U/L
LDH: 313 – 618 U/L

Figure 22B

FIX:C Activity = circulating factor IX activity levels.
AST = Aspartate Aminotransferase; ALT = Alanine Aminotransferase; LDH = Lactate Dehydrogenase.
ULN = Upper Limit of Normal
*Data presented based on local lab.

AAV-FIX39-Padua: 4th subject day 50 FIX:C Activity Level at 33%
Subject 4 [$5x10^{-1}$ vg/kg] at 50 days since last BeneFIX infusion
$5.57x10^{13}$ vg of AAV-FIX39-Padua + $2.22x10^{14}$ cp of Empty Capsids [1:4]

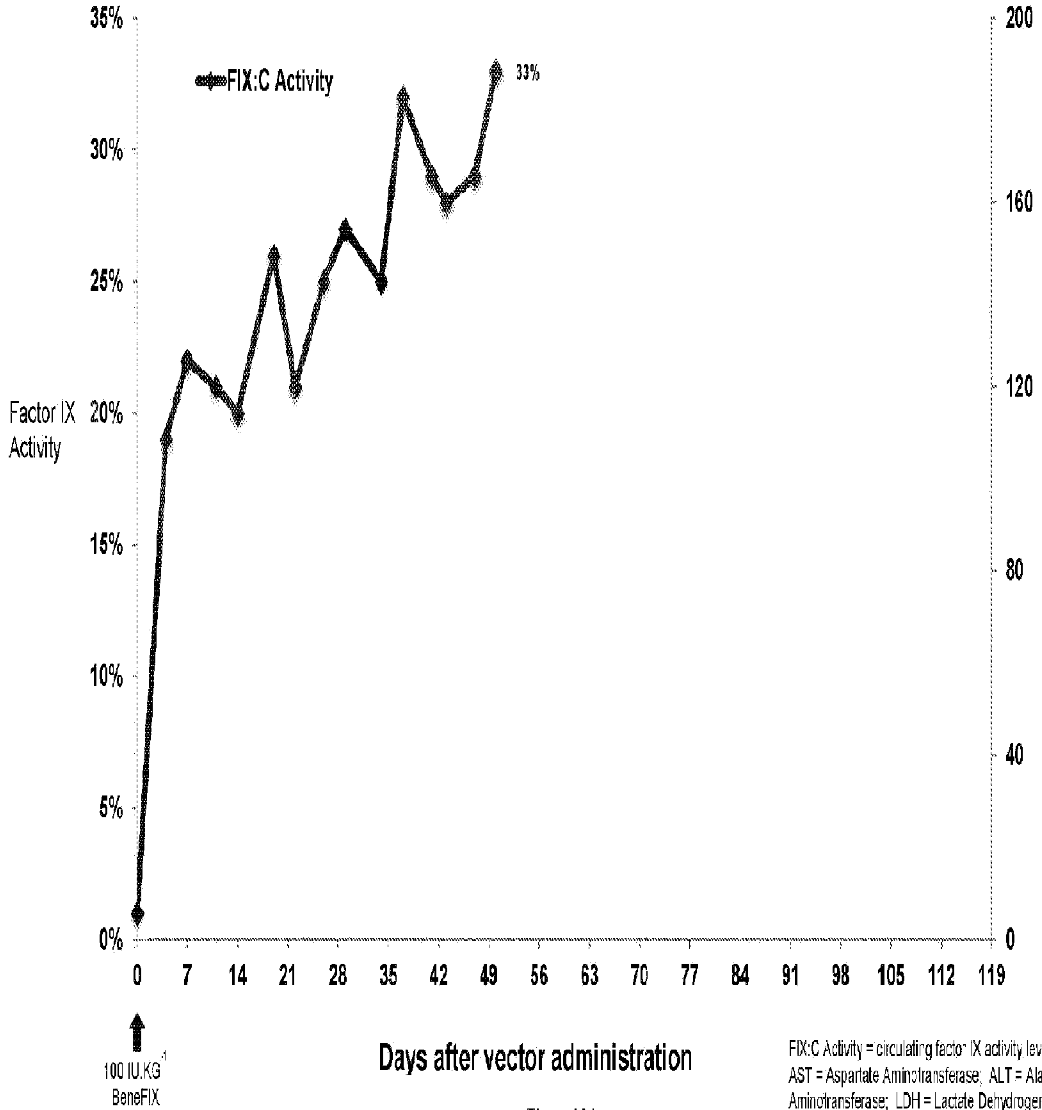



Figure 23A

FIX:C Activity = circulating factor IX activity levels.
AST = Aspartate Aminotransferase; ALT = Alanine Aminotransferase; LDH = Lactate Dehydrogenase.
ULN = Upper Limit of Normal
*Data presented based on local lab.

AAV-FIX39-Padua: 4th subject day 50 Liver Functions
Subject 4 [5x10^11 vg/kg] at 50 days since last BeneFIX infusion
5.57x10^13 vg of AAV-FIX39-Padua + 2.22x10^14 cp of Empty Capsids [1:4]

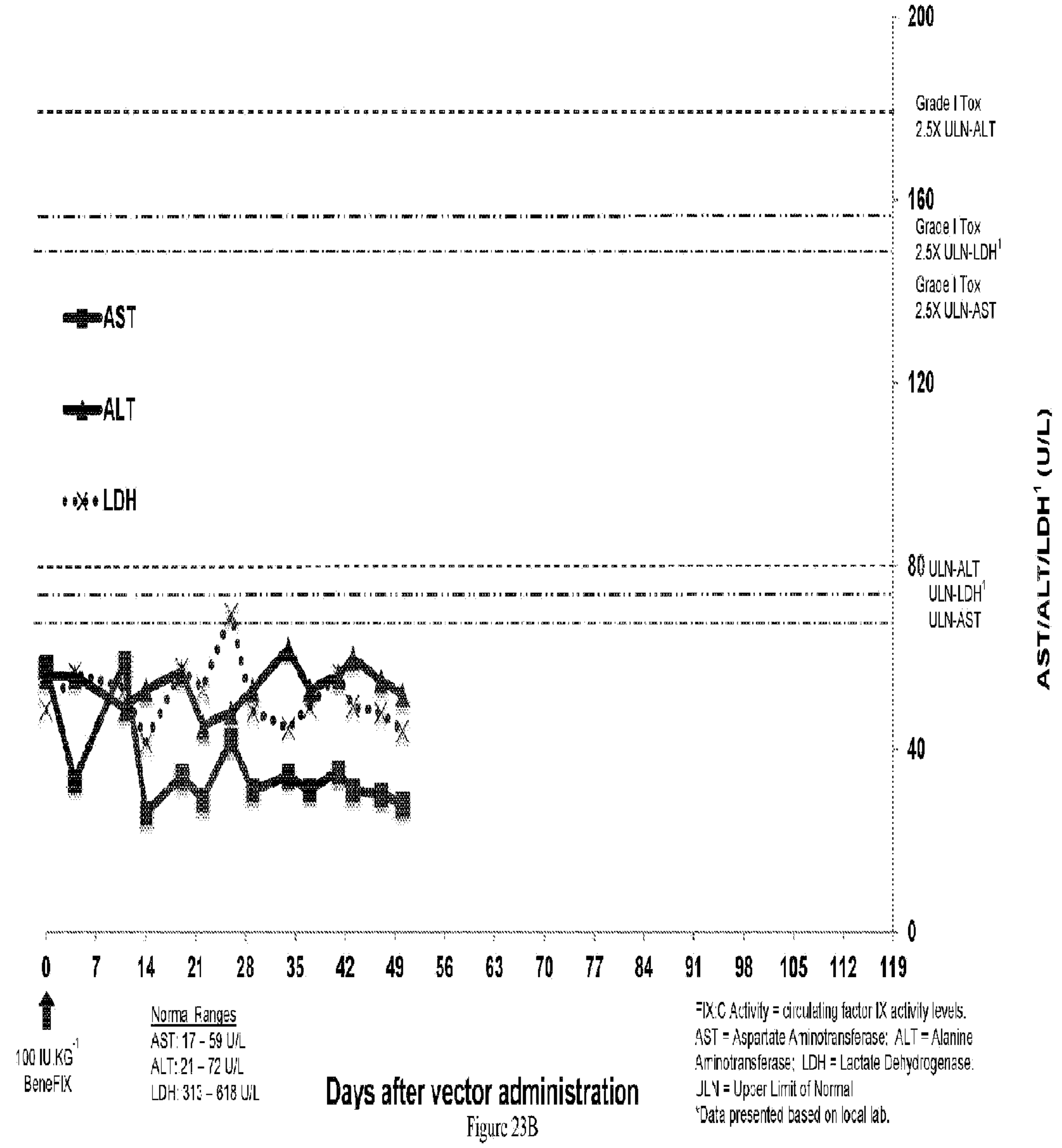



Figure 23B

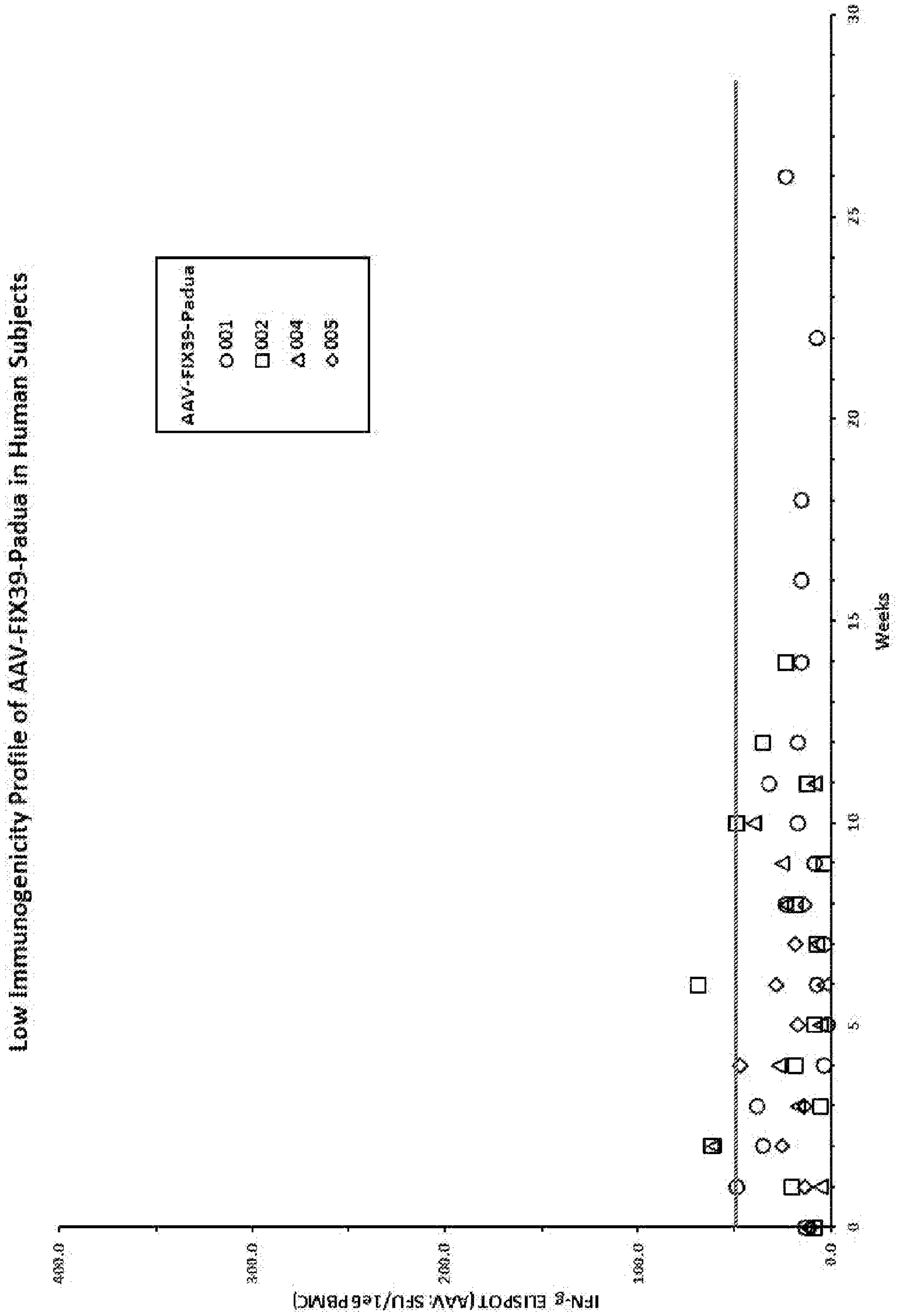
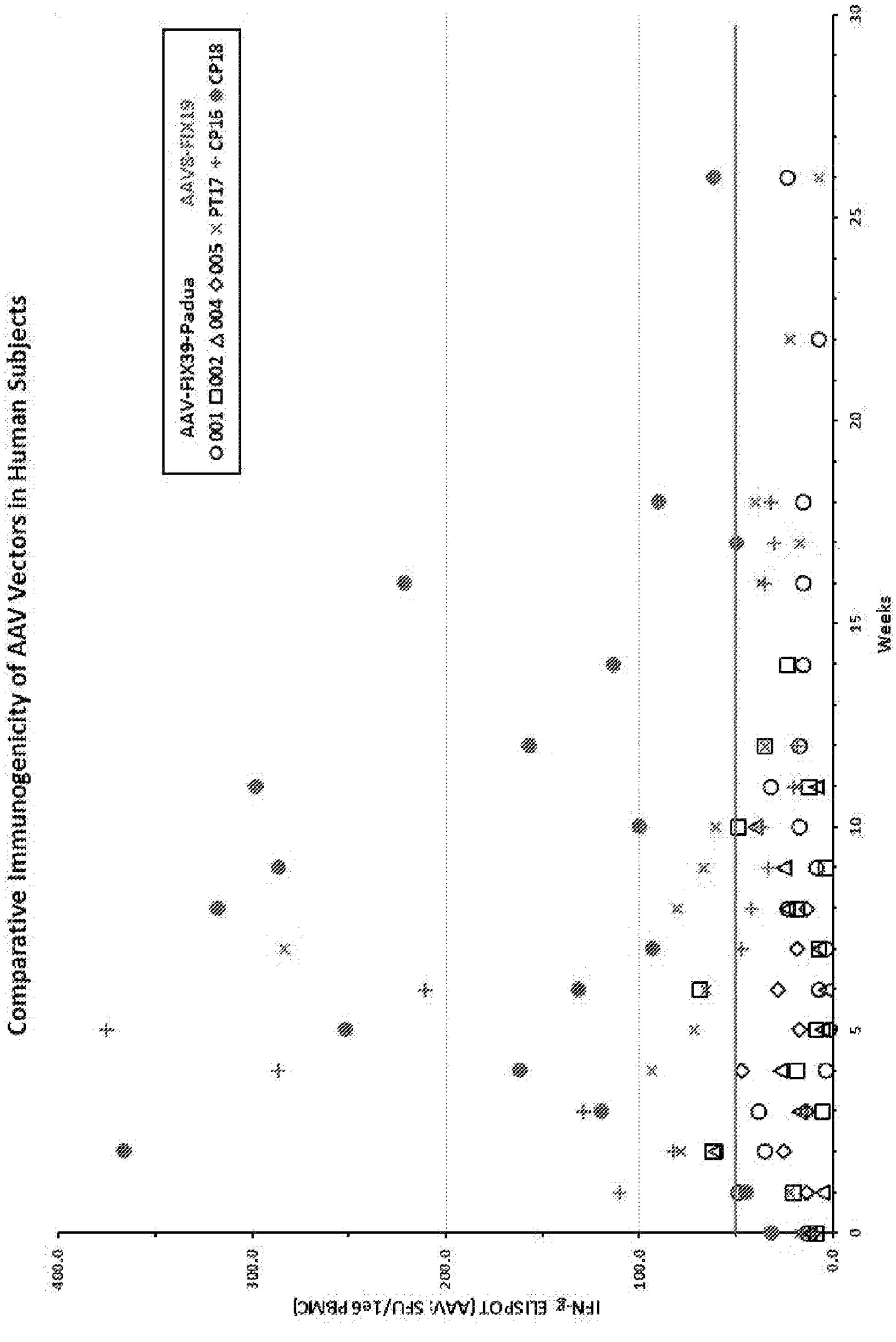

Low Immunogenicity Profile of AAV-FIX39-Padua in Human Subjects
AAV-FIX39-Padua
001
002
004
005
IFN-g ELISPOT (AAV: SFU/1e6 PBMC)
400.0
300.0
200.0
100.0
0.0
0
5
10
15
20
25
30
Weeks

Figure 24A



Comparative Immunogenicity of AAV Vectors in Human Subjects
AAV-FIX39-Padua
AAV8-FIX19
○ 001 □ 002 △ 004 ◇ 005 × PT17 + CP16 ● CP18
IFN-g ELISPOT (AAV: SFU/1e6 PBMC)
400.0
300.0
200.0
100.0
0.0
0
5
10
15
20
25
30
Weeks


Figure 24B

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.