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(54) Title: COMPOSITION AND METHODS FOR HIGHLY EFFICIENT GENE TRANSFER USING AAV CAPSID VARIANTS

(57) Abstract: Improved compositions and methods for AAV mediated gene therapy are disclosed.

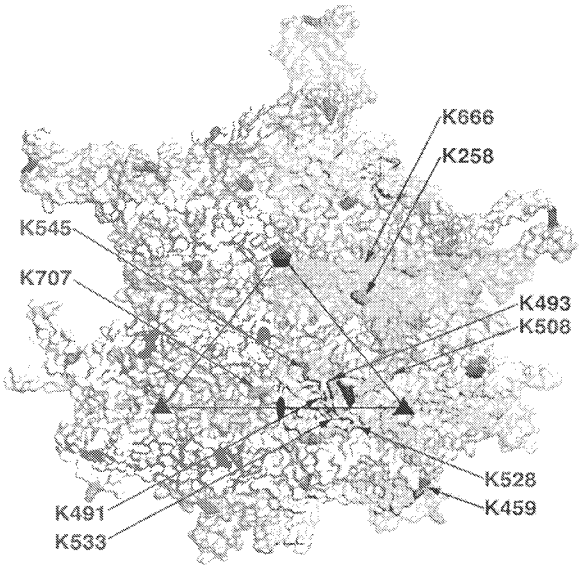

Figure 1A

WO 2013/158879 A1

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Composition and methods for highly efficient gene transfer using AAV capsid variants

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This application claims priority to US Provisional Application Nos. 61/635,273 and 61/794,995 filed April 18, 2012 and March 15, 2013 respectively, the entire contents of each being incorporated herein by reference as though set forth in full.

FIELD OF THE INVENTION

This application relates to the fields of gene therapy and molecular biology. More specifically, this invention provides adeno-associated viral vectors comprising protein capsid variants which improve the transduction efficiency of AAV vectors comprising therapeutically beneficial transgenes.

BACKGROUND OF THE INVENTION

Several publications and patent documents are cited throughout the specification in order to describe the state of the art to which this invention pertains. Each of these citations is incorporated herein by reference as though set forth in full.

Adeno-associated virus (AAV) is a small (20 nm), replication-defective, non-enveloped virus. Many distinct AAV serotypes have been characterized in human and nonhuman primates. The AAV genome is comprised of single-stranded DNA with 145 bp inverted terminal repeats (ITRs) at both ends. There are two open reading frames (ORFs), rep and cap. While the rep products are essential for AAV replication, 3 capsid proteins (VP1, VP2, and VP3) are expressed from the cap gene. VP1, VP2 and VP3 come together at 1:1:10 ratio to form an icosahedral capsid (Xie Q et al, 2002). During recombinant AAV (rAAV) vector production, an expression cassette flanked by ITRs is packaged into AAV capsid. The genes required for replication of AAV are not included in the cassette. Recombinant AAV is considered the safest and one of the most widely used viral vectors for in vivo gene transfer. The vectors can infect cells from multiple tissue types providing strong and persistent

transgene expression. They are also non-pathogenic and have a low immunogenicity profile (High KA, 2011).

One of the immediate goals for gene therapy trials is optimizing vectors to maximize tissue transduction while minimizing the vector dose. Upon entry into the cell, AAV capsid proteins are subject to proteasome mediated degradation. Phosphorylation of surface-exposed tyrosine residues of AAV capsid represents one of the first steps that leads to degradation of the virus via the ubiquitin-proteasome pathway (Zhong L *et al*, 2007). Most of the regulated proteolysis in the cell occurs through this pathway. Ubiquitin is a small protein (~8.5 kDa) that can be found in all eukaryotic cells. Ubiquitin is attached to the side-chain of amino-acids of a substrate protein. After additional ubiquitin proteins are attached to the substrate via the initially attached ubiquitin, a polyubiquitin chain is formed and the substrate is marked for degradation (Thrower JS *et al* 2000, Peng J *et al* 2003, Bedford L *et al* 2011). It has been shown that mutation of surface-exposed tyrosine residues leads to an increase in transduction efficiency of AAV2 vectors (Zhong L *et al*, 2008). More recently, several groups have shown that the strategy is effective also with other AAV serotypes in several tissues, including AAV serotype 6 and 8.

Clearly, a need exists in the art for compositions and methods which improve the transduction of AAV carrying clinically important transgenes in patients in need thereof.

SUMMARY OF THE INVENTION

In accordance with the present invention, novel AAV variants are provided which exhibit increased transduction efficiency when compared to AAV serotypes (e.g., AAV1, AAV2, AAV8, AAV-rh74), which lack the modifications disclosed herein. Such improved vectors are useful for transduction of a variety of tissues, including liver, muscle, brain, and retina.

In one embodiment, an adeno-associated virus (AAV) vector comprising an altered VP1 capsid protein is provided, the altered capsid protein comprising lysine residue substitutions, thereby reducing ubiquination of the capsid and increasing the transduction efficiency of variant AAV into target tissues and cells. In one embodiment, the vector further comprises a heterologous nucleic acid, (e.g., a minigene comprising AAV inverted terminal repeats and a heterologous nucleic acid sequence) operably linked to regulatory sequences which direct expression of a product from the heterologous nucleic acid sequence in a host cell. In a preferred embodiment, the AAV vector comprises one or more lysine

substitutions in VP1 as provided in the tables set forth herein. In another embodiment, the AAV vector is of the AAV8 serotype and contains an alteration provided in Table 3.

In a preferred embodiment, the AAV vectors of the invention comprising the variant capsid proteins are useful for expression of therapeutic peptides or therapeutic nucleic acids. Such peptides include, without limitation, an anti-viral RNAi molecule, Factor VIII, Factor IX or a functional fragment thereof. Additional expression products include for example, IgG, IgM, IgA, IgD, IgE, chimeric immunoglobulins, humanized antibodies, or single chain antibodies. In one aspect the expression product is an RNAi that is useful for inhibiting HCV infection and replication. In another embodiment, the expression product is an antisense nucleic acid useful for down modulating a target cell of interest.

In another embodiment of the invention, a pharmaceutical composition comprising the variant AAV vectors of the invention in a biologically compatible carrier is provided. Also encompassed by the present invention are cell cultures comprising the vectors disclosed herein.

The invention also encompasses a method of delivering a transgene to a cell in a subject, said method comprising the step of contacting the cell with an AAV vector as disclosed herein, wherein said AAV vector comprises the transgene, wherein the presence of lysine substitution in the VP1 capsid sequence in said vector is associated with reduced ubiquitination and increased transduction efficiency.

In a final aspect, the invention provides decoy viral variants that are inefficient at infecting cells but are effective to block antibody neutralization of viral variants carrying beneficial transgenes due to the structural similarities of the two viral variants. Exemplary capsid variants for this purpose include for example, K38R, K143R, K510R and K709R.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Lysines on the surface of AAV1, AAV2 and AAV8: PDB numbers of AAV serotypes used here are as follows: AAV1: 3NG9, AAV2: 1LP3, and AAV8: 2QA0. The arrows represent the respective Lysine residues. (A) There are 11 Lysines on the surface of AAV1 VP3. Residue colors are as follows: K258 red, K459 blue, K491 yellow, K493 magenta, K508 cyan, K528 dark salmon, K533 light green, K545 light blue (slate), K567 dark salmon, K666 light cyan, K707 gray. (B) There are 10 Lysines on the surface of AAV2 VP3. Residue colors are as follows: K258 red, K490 yellow, K507 cyan, K527 dark salmon, K532 light green, K544 light blue (slate), K549 light yellow, K556 light magenta,

K665 light cyan, K706 gray (C) There are 8 Lysines on the surface of AAV8 VP3. Residue colors are as follows : K259 red, K333 green, K510 cyan, K530 dark salmon, K547 light blue (slate), K569 dark salmon, K668 light cyan, K709 gray. Note that K528 and K567 of AAV1 and K530 and K569 of AAV8 are side by side in the strructure and showed with the same color.

Figure 2: Several Lysine residues of AAV8 capsid were mutated to Arginine. Blood from the animals was collected 8 weeks after virus injection via tail vein. hF.IX levels are detected by ELISA (A) The residues that are predicted to be ubiqutinated by the software with high and medium confidence levels were mutated to arginine. 2.5 x 10^{10} virus particles per mouse were injected via tail vein (B,C) The residues that are predicted with low confidence to be ubiqutinated by the software were mutated to arginine. 2.5 x 10^{9} virus particles per mouse were injected via tail vein. (B) K569R and K668R capsid mutations (C) Affect of K38R, K143R, K259R, K510R, K547R capsid mutations are compared to affect of K530R mutation.

Figure 3: Combination of the K to R capsid mutations (A) Combination of K137R, K33R and K530R mutations are still higher than the wild type but not statistically different from the K530R. (B) K709R mutation negatively affects the AAV8 transduction, Addition of K709R mutation to K(137/333/530)R mutant also decreases the transduction of the virus. C) Combination of multiple Lysine to arginine mutations does not increase the transduction rate. (D) Combination of three lysine to arginine residues with four or six tyrosine to phenylalanine residues decreases the transduction rate.

Figure 4: AAV1 transduction (A) CTL killing of HHL5-B7 hepatocytes transduced with AAV-1 lysine mutants at three different MOIs 5K, 50K and 500K. Peptide (IPQYGYLTL for AAV1; VPQYGYLTL for AAV2) was used as a positive control. LDH release correlates with the cell killing. (B) Total number of GFP positive cells and GFP expression was compared among different constructs at 50K and 500K MOIs.

Figure 5: AAV2 transduction: A) AAV2 K137R, K527R or K532R mutants were compared to WT AAV2 in terms of HHL5 cell line transduction rate. The cells were transfected at 10K, 50K, 100K and 500K MOIs and checked 24 hours later for GFP expression. B) A graph showing cytotoxicity as measured by LDL release of variants tested.

Figure 6: CTL assay in which target hepatocyte cells were transduced with AAV-2 vector at increasing concentrations and then incubated with HLA-matched effector cells. AAV vectors encoded wild-type AAV-2 capsid or single lysine mutations as indicated. Effectors were derived from PBMC expanded in vitro against AAV-2 MHC Class I epitope VPQYGYLTL and effector-to-target ratio was 10:1. Results are expressed as percentage of CTL activity (% cytotoxicity compared to cells treated with 10% SDS as a maximum cytotoxicity control after background subtraction) with respect to the wild type vector.

Figure 7: RH74 data: Human F.IX transgene expression levels in plasma measured by an ELISA specific for human F.IX.A) Definition of TMR=K(137/333/530)R, Definition of HMF= Y(253/275/447/703/707/733)F. B) Definition of HMR: K(137/259/333/530/552/569)R, Definition of 195/202: G195A+ L199V+ S201P+ G202N. While HMR+195/202, HMR+195/202+K(38)R, HMR+195/202+K(51)R, HMR+195/202+K(61)R , and HMR+195/202+K(77)R produced higher hFIX production upon injection to mice, HMR+195/202+K(122/123)R or HMR+195/202+K(142/413)R injection did not produce any detectable hFIX at all. C): RHM13_1 mutant produced similar hFIX levels compared to Rh74 WT whereas hFIX levels derived from RHM17_1-treated mice were barely above background levels. D) RHM14_2 mutant produced similar hFIX levels compared to Rh74 WT; RHM15_1 performance was in-between that of AAV8 and AAVrh74 WT.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, we have found that mutating the lysine residues on AAV capsids to arginine residues increases AAV transduction efficiency. Our initial experiments showed that a single substitution of a lysine residue predicted to be a target for ubiquitination resulted in higher levels of expression of the human factor IX (FIX) transgene in mice compared to animals receiving unmodified AAV vectors. The AAV lysine mutants described herein could be used to advantage to generate vectors that target the liver, CNS, muscle, and other organs with higher efficiency compared to the wild type AAV capsids. Thus, this discovery can be used to develop therapeutics to treat hemophilia A, B, Huntington's disease, and virtually any other disease that requires increased transduction levels of desirable transgenes into a target tissue of interest.

The following definitions are provided to facilitate an understanding of the present invention.

I. DEFINITIONS:

"Gene therapy" is the insertion of genes into an individual's cells and/or tissues to treat a disease, commonly hereditary diseases wherein a defective mutant allele is replaced or supplemented with a functional one.

"Adeno-associated viruses", from the parvovirus family, are small viruses with a genome of single stranded DNA. These viruses can insert genetic material at a specific site on chromosome 19 and are preferred because they are not associated with pathogenic disease in humans.

A "therapeutic" peptide or protein is 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 is one that otherwise confers a benefit to a subject, e.g., anti-cancer effects. Therapeutic peptides and proteins include, but are not limited to, CFTR (cystic fibrosis transmembrane regulator protein), dystrophin (including the protein product of dystrophin mini-genes, see, e.g, Vincent et al., (1993) Nature Genetics 5:130), utrophin (Tinsley et al., (1996) Nature 384:349), clotting factors (Factor XIII, Factor IX, Factor X, etc.), monoclonal antibodies (Lewis et al., 2002), erythropoietin, the LDL receptor, lipoprotein lipase, ornithine transcarbamylase, β-globin, α-globin, spectrin, α-antitrypsin, adenosine deaminase, hypoxanthine guanine phosphoribosyl transferase, β-glucocerebrosidase, sphingomyelinase, lysosomal hexosaminidase, branched-chain keto acid dehydrogenase, hormones, growth factors (e.g., insulin-like growth factors 1 and 2, platelet derived growth factor, epidermal growth factor, nerve growth factor, neurotrophic factor -3 and -4, brain-derived neurotrophic factor, glial derived growth factor, transforming growth factor α and β, and the like), cytokines (e.g., α-interferon, β-interferon, interferon-γ, interleukin-2, interleukin-4, interleukin 12, granulocyte-macrophage colony stimulating factor, lymphotoxin), suicide gene products (e.g., herpes simplex virus thymidine kinase, cytosine deaminase, diphtheria toxin, cytochrome P450, deoxycytidine kinase, and tumor necrosis factor), proteins conferring resistance to a drug used in cancer therapy, tumor suppressor gene products (e.g., p53, Rb, Wt-1, NF1, VHL, APC, and the like), and any other peptide or protein that has a therapeutic effect in a subject in need thereof.

Further exemplary therapeutic peptides or proteins include those that may be used in the treatment of a disease condition including, but not limited to, cystic fibrosis (and other

diseases of the lung), hemophilia A, hemophilia B, thalassemia, anemia and other blood disorders, AIDS, Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, epilepsy, and other neurological disorders, cancer, diabetes mellitus, muscular dystrophies (e.g., Duchenne, Becker), Gaucher's disease, Hurler's disease, adenosine deaminase deficiency, glycogen storage diseases and other metabolic defects, retinal degenerative diseases (and other diseases of the eye), and diseases of solid organs (e.g., brain, liver, kidney, heart).

The term "promoters" or "promoter" as used herein can refer to a DNA sequence that is located adjacent to a DNA sequence that encodes a recombinant product. A promoter is preferably linked operatively to an adjacent DNA sequence. A promoter typically increases an amount of recombinant product expressed from a DNA sequence as compared to an amount of the expressed recombinant product when no promoter exists. A promoter from one organism can be utilized to enhance recombinant product expression from a DNA sequence that originates from another organism. For example, a vertebrate promoter may be used for the expression of jellyfish GFP in vertebrates. In addition, one promoter element can increase an amount of recombinant products expressed for multiple DNA sequences attached in tandem. Hence, one promoter element can enhance the expression of one or more recombinant products. Multiple promoter elements are well-known to persons of ordinary skill in the art.

In one embodiment, high-level constitutive expression will be desired. Examples of such promoters include, without limitation, the retroviral Rous sarcoma virus (RSV) LTR promoter/enhancer, the cytomegalovirus (CMV) immediate early promoter/enhancer (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.

In another embodiment, inducible promoters may be desired. Inducible promoters are those which are regulated by exogenously supplied compounds, either in cis or in trans, including without limitation, the zinc-inducible sheep metallothionine (MT) promoter; the dexamethasone (Dex)-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 types of inducible promoters which may be useful in this context are those which are regulated by a specific physiological state, e.g., temperature, acute phase, or in replicating cells only.

In another embodiment, the native promoter for the transgene or nucleic acid sequence of interest will be used. The native promoter may be preferred when it is desired that expression of the transgene or the nucleic acid sequence should mimic the native expression. The native promoter may be used when expression of the transgene or other nucleic acid sequence must be regulated temporally or developmentally, or in a tissue-specific manner, or in response to specific transcriptional stimuli. In a further embodiment, other native expression control elements, such as enhancer elements, polyadenylation sites or Kozak consensus sequences may also be used to mimic the native expression.

In one embodiment, the recombinant viral genome comprises a transgene operably linked to a tissue-specific promoter. For instance, if expression in skeletal muscle is desired, a promoter active in muscle may be used. These include the 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 Li et al., Nat. Biotech., 17:241-245 (1999). Examples of promoters that are tissue-specific are known for liver 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.

The term "enhancers" or "enhancer" as used herein can refer to a DNA sequence that is located adjacent to the DNA sequence that encodes a recombinant product. Enhancer elements are typically located upstream of a promoter element or can be located downstream of or within a DNA coding sequence (e.g., a DNA sequence transcribed or translated into a recombinant product or products). Hence, an enhancer element can be located 100 base pairs, 200 base pairs, or 300 or more base pairs upstream or downstream of a DNA sequence that encodes recombinant product. Enhancer elements can increase an amount of recombinant product expressed from a DNA sequence above increased expression afforded by a promoter

element. Multiple enhancer elements are readily available to persons of ordinary skill in the art.

"Nucleic acid" or a "nucleic acid molecule" as used herein refers to any DNA or RNA molecule, either single or double stranded and, if single stranded, the molecule of its complementary sequence in either linear or circular form. In discussing nucleic acid molecules, a sequence or structure of a particular nucleic acid molecule may be described herein according to the normal convention of providing the sequence in the 5' to 3' direction. With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous in the naturally occurring genome of the organism in which it originated. For example, an "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a prokaryotic or eukaryotic cell or host organism.

A "vector" is a replicon, such as a plasmid, cosmid, bacmid, phage or virus, to which another genetic sequence or element (either DNA or RNA) may be attached so as to bring about the replication of the attached sequence or element.

An "expression operon" refers to a nucleic acid segment that may possess transcriptional and translational control sequences, such as promoters, enhancers, translational start signals (e.g., ATG or AUG codons), polyadenylation signals, terminators, and the like, and which facilitate the expression of a polypeptide coding sequence in a host cell or organism.

The terms "transform", "transfect", "transduce", shall refer to any method or means by which a nucleic acid is introduced into a cell or host organism and may be used interchangeably to convey the same meaning. Such methods include, but are not limited to, transfection, electroporation, microinjection, infection, PEG-fusion and the like.

The introduced nucleic acid may or may not be integrated (covalently linked) into nucleic acid of the recipient cell or organism. In bacterial, yeast, plant and mammalian cells, for example, the introduced nucleic acid may be maintained as an episomal element or independent replicon such as a plasmid. Alternatively, the introduced nucleic acid may become integrated into the nucleic acid of the recipient cell or organism and 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.

The term "selectable marker gene" refers to a gene that when expressed confers a selectable phenotype, such as antibiotic resistance, on a transformed cell or plant.

The term "operably linked" means that the regulatory sequences necessary for expression of the coding sequence are placed in the DNA molecule in the appropriate positions relative to the coding sequence so as to effect expression of the coding sequence. This same definition is sometimes applied to the arrangement of transcription units and other transcription control elements (e.g. enhancers) in an expression vector.

The term "oligonucleotide" as used herein refers to sequences, primers and probes of the present invention, and is defined as a nucleic acid molecule comprised of two or more ribo- or deoxyribonucleotides, preferably more than three. The exact size of the oligonucleotide will depend on various factors and on the particular application and use of the oligonucleotide.

The phrase "specifically hybridize" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule of the invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

The term "primer" as used herein refers to a DNA oligonucleotide, either single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to functionally act as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme, suitable cofactors and conditions such as a suitable temperature and pH, the primer may be extended at its 3' terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield a primer extension product. The primer may vary in length depending on the particular conditions and requirement of the application. For example, in diagnostic applications, the oligonucleotide primer is typically 15-25 or more nucleotides in length. The primer must be of sufficient complementarity to the desired template to prime the synthesis of the desired extension product, that is, to be able to anneal with the desired template strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use

in the initiation of synthesis by a polymerase or similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non-complementary nucleotide sequence may be attached to the 5' end of an otherwise complementary primer. Alternatively, non-complementary bases may be interspersed within the oligonucleotide primer sequence, provided that the primer sequence has sufficient complementarity with the sequence of the desired template strand to functionally provide a template-primer complex for the synthesis of the extension product.

Polymerase chain reaction (PCR) has been described in U.S. Patents Nos. 4,683,195, 4,800,195, and 4,965,188, the entire disclosures of which are incorporated by reference herein.

The term "isolated" may refer to a compound or complex that has been sufficiently separated from other compounds with which it would naturally be associated. "Isolated" is not meant to exclude artificial or synthetic mixtures with other compounds or materials, or the presence of impurities that do not interfere with fundamental activity or ensuing assays, and that may be present, for example, due to incomplete purification, or the addition of stabilizers.

The term "immune response" is meant to refer to any response to an antigen or antigenic determinant by the immune system of a vertebrate subject. Exemplary immune responses include humoral immune responses (e.g. production of antigen-specific antibodies) and cell-mediated immune responses (e.g. lymphocyte proliferation), as defined herein below.

II. METHODS OF USING AND METHODS OF ADMINISTRATION OF THE VARIANT ADENOASSOCIATED VIRAL VECTORS OF THE INVENTION

The methods of the present invention provide a means for delivering heterologous nucleic acid sequences into a broad range of host cells, including both dividing and non-dividing cells. The vectors and other reagents, methods and pharmaceutical formulations of the present invention are additionally useful in a method of administering a protein or peptide to a subject in need thereof, as a method of treatment or otherwise. In this manner, the protein or peptide may thus be produced in vivo in the subject. The subject may be in need of the protein or peptide because the subject has a deficiency of the protein or peptide, or because the production of the protein or peptide in the subject may impart some therapeutic effect, as a method of treatment or otherwise, and as explained further below.

In general, the present invention may be employed to deliver any foreign nucleic acid with a biological effect to treat or ameliorate the symptoms associated with any disorder related to gene expression. Illustrative disease states include, but are not limited to: cystic fibrosis (and other diseases of the lung), hemophilia A, hemophilia B, thalassemia, anemia and other blood coagulation disorders, AIDs, Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, epilepsy, and other neurological disorders, cancer, diabetes mellitus, muscular dystrophies (e.g., Duchenne, Becker), Gaucher's disease, Hurler's disease, adenosine deaminase deficiency, glycogen storage diseases and other metabolic defects, retinal degenerative diseases (and other diseases of the eye), diseases of solid organs (e.g., brain, liver, kidney, heart), and the like.

In addition, the present invention may be employed to deliver nucleic acids encoding monoclonal antibodies or fragments thereof that are known to provide beneficial biological effects to treat or ameliorate the symptoms associated with cancers, infectious diseases, and autoimmune diseases such as rheumatoid arthritis. Other sequences may encode for example cytokines such as interferon-alpha that may modulate the course of a disease.

Gene transfer has substantial potential use in understanding and providing therapy for disease states. There are a number of inherited diseases in which defective genes are known and have been cloned. In some cases, the function of these cloned genes is known. In general, the above disease states fall into two classes: deficiency states, usually of enzymes, which are generally inherited in a recessive manner, and unbalanced states, 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. Thus the methods of the present invention permit the treatment of genetic diseases. As used herein, a disease state is treated by partially or wholly remedying the deficiency or imbalance that causes the disease or makes it more severe. The use of site-specific integration of nucleic sequences to cause mutations or to correct defects is also possible.

Finally, the instant invention finds further use in diagnostic and screening methods, whereby a gene of interest is transiently or stably expressed in a cell culture system, or alternatively, a transgenic animal model.

III. SUBJECTS, PHARMACEUTICAL FORMULATIONS, VACCINES, AND MODES OF ADMINISTRATION

The present invention finds use in both veterinary and medical applications. Suitable subjects include both avians and mammals, with mammals being preferred. The term "avian" as used herein includes, but is not limited to, chickens, ducks, geese, quail, turkeys and pheasants. The term "mammal" as used herein includes, but is not limited to, humans, bovines, ovines, caprines, equines, felines, canines, lagomorphs, etc. Human subjects are the most preferred. Human subjects include fetal, neonatal, infant, juvenile and adult subjects.

In particular embodiments, the present invention provides a pharmaceutical composition comprising a virus particle of the invention in a pharmaceutically-acceptable carrier or other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc. For injection, the carrier will typically be a liquid. For other methods of administration, the carrier may be either solid or liquid, such as sterile, pyrogen-free water or sterile pyrogen-free phosphate-buffered saline solution. For inhalation administration, the carrier will be respirable, and will preferably be in solid or liquid particulate form. As an injection medium, it is preferred to use water that contains the additives usual for injection solutions, such as stabilizing agents, salts or saline, and/or buffers.

In other embodiments, the present invention provides a pharmaceutical composition comprising a cell in which an AAV provirus is integrated into the genome in a pharmaceutically-acceptable carrier or other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc.

By "pharmaceutically acceptable" it is meant a material that is not biologically or otherwise undesirable, e.g., the material may be administered to a subject without causing any undesirable biological effects. Thus, such a pharmaceutical composition may be used, for example, in transfection of a cell ex vivo or in administering a viral particle or cell directly to a subject.

The present invention further provides a method of delivering a nucleic acid to a cell. For in vitro methods, the virus -may be administered to the cell by standard viral transduction methods, as are known in the art. Preferably, the virus particles are added to the cells at the appropriate multiplicity of infection according to standard transduction methods appropriate for the particular target cells. Titers of virus to administer can vary, depending upon the target cell type and the particular virus vector, and may be determined by those of skill in the art

without undue experimentation. Alternatively, administration of a parvovirus vector of the present invention can be accomplished by any other means known in the art.

Recombinant virus vectors are preferably administered to the cell in a biologically-effective amount. A "biologically-effective" amount of the virus vector is an amount that is sufficient to result in infection (or transduction) and expression of the heterologous nucleic acid sequence in the cell. If the virus is administered to a cell in vivo (e.g., the virus is administered to a subject as described below), a "biologically-effective" amount of the virus vector is an amount that is sufficient to result in transduction and expression of the heterologous nucleic acid sequence in a target cell.

The cell to be administered the inventive virus vector may be of any type, including but not limited to neural cells (including cells of the peripheral and central nervous systems, in particular, brain cells), lung cells, retinal cells, epithelial cells (e.g., gut and respiratory epithelial cells), muscle cells, pancreatic cells (including islet cells), hepatic cells, myocardial cells, bone cells (e.g., bone marrow stem cells), hematopoietic stem cells, spleen cells, keratinocytes, fibroblasts, endothelial cells, prostate cells, germ cells, and the like. Alternatively, the cell may be any progenitor cell. As a further alternative, the cell can be a stem cell (e.g., neural stem cell, liver stem cell). Moreover, the cells can be from any species of origin, as indicated above.

In particular embodiments of the invention, cells are removed from a subject, the parvovirus vector is introduced therein, and the cells are then replaced back into the subject. Methods of removing cells from subject for treatment ex vivo, followed by introduction back into the subject are known in the art. Alternatively, the rAAV vector is introduced into cells from another subject, into cultured cells, or into cells from any other suitable source, and the cells are administered to a subject in need thereof.

Suitable cells for ex vivo gene therapy include, but are not limited to, liver cells, neural cells (including cells of the central and peripheral nervous systems, in particular, brain cells), pancreas cells, spleen cells, fibroblasts (e.g., skin fibroblasts), keratinocytes, endothelial cells, epithelial cells, myoblasts, hematopoietic cells, bone marrow stromal cells, progenitor cells, and stem cells.

Dosages of the cells to administer to a subject will vary upon the age, condition and species of the subject, the type of cell, the nucleic acid being expressed by the cell, the mode of administration, and the like. Typically, at least about 10^2 to about 10^8, preferably about 10^3 to about 10^6 cells, will be administered per dose. Preferably, the cells will be administered in a "therapeutically-effective amount".

A "therapeutically-effective" amount as used herein is an amount that is sufficient to alleviate (e.g., mitigate, decrease, reduce) at least one of the symptoms associated with a disease state. Alternatively stated, a "therapeutically-effective" amount is an amount that is sufficient to provide some improvement in the condition of the subject.

A further aspect of the invention is a method of treating subjects in vivo with the inventive virus particles. Administration of the parvovirus particles of the present invention to a human subject or an animal in need thereof can be by any means known in the art for administering virus vectors.

Exemplary modes of administration include oral, rectal, transmucosal, topical, transdermal, inhalation, parenteral (e.g., intravenous, subcutaneous, intradermal, intramuscular, and intraarticular) administration, and the like, as well as direct tissue or organ injection, alternatively, intrathecal, direct intramuscular, intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspenions in liquid prior to injection, or as emulsions. Alternatively, one may administer the virus in a local rather than systemic manner, for example in a depot or sustained-release formulation.

In particularly preformed embodiments of the invention, the nucleotide sequence of interest is delivered to the liver of the subject. Administration to the liver may be achieved by any method known in art, including, but not limited to intravenous administration, intraportal administration, intrabilary administration, intra-arterial administration, and direct injection into the liver parenchyma.

Preferably, the cells (e.g., liver cells) are infected by a recombinant parvovirus vector encoding a peptide or protein, the cells express the encoded peptide or protein and secrete it into the circulatory system in a therapeutically-effective amount (as defined above). Alternatively, the vector is delivered to and expressed by another cell or tissue, including but not limited to, brain, pancreas, spleen or muscle.

In other preferred embodiments, the inventive parovirus particles are administered intramuscularly, more preferably by intramuscular injection or by local administration (as defined above). In other preferred embodiments, the parovirus particles of the present invention are administered to the lungs.

The parovirus vector disclosed herein may be administered to the lungs of a subject by any suitable means, but are preferably administered by administering an aerosol suspension of respirable particles comprised of the inventive parvovirus vectors, which the

subject inhales. The respirable particles may be liquid or solid. Aerosols of liquid particles comprising the inventive parvovirus vectors may be produced by any suitable means, such as with a pressure-driven aerosol nebulizer or an ultrasonic nebulizer, as is known to those of skill in art. See, e.g. U.S. Pat. No. 4,501,729. Aerosols of solid particles comprising the inventive virus vectors may likewise be produced with any solid particulate medicament aerosol generator, by techniques known in the pharmaceutical art.

Dosages of the inventive parvovirus particles will depend upon the mode of administration, the disease or condition to be treated, the individual subject's condition, the particular virus vector, and the gene to be delivered and can be determined in a routine manner. Exemplary doses for achieving therapeutic effects are virus titers of at least about 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13}, 10^{14}, 10^{15} ,10^{16} transducting units or more, preferably about 10^8 to 10^{13} transducting units, yet more preferably 10^{12} transducing units.

In summary, the parvovirus vectors, reagents, and methods of the present invention can be used to direct a nucleic acid to either dividing or non-dividing cells, and to stably express the heterologous nucleic acid therein. Using this vector system, it is now possible to introduce into cells, in vitro or in vivo, genes that encode proteins that affect cell physiology. The vectors of the present invention can thus be useful in gene therapy for disease states or for experimental modification of cell physiology.

The following example is provided to illustrate certain embodiments of the invention. It is not intended to limit the invention in anyway.

EXAMPLE I

Lysine to Arginine mutations affect AAV transduction rate and MHC delivery

Identification of lysine residues to be targeted in AAV1 and AAV8 vectors

We used the UbPred software to predict the possible ubiquitination sites on AAV1, AAV2, AAV8 and Rh74 capsid proteins (Radivojac P *et al*, 2010). UbPred software is available online at http://www.ubpred.org/index.html. The output of the analysis is the prediction of the lysine residues important for ubiquitination within the indicated AAV serotype capsid sequence. See Figure 1 and Table 1. These are the following:

AAV1 Capsid Protein VP1 Sequence:
MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLGPFNGL
DKGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADAEFQERLQEDTSFGGNLGRAVF

QAKKRVLEPLGLVEEGAKTAPGKKRPVEQSPQEPDSSSGIGKTGQQPAKKRLNFGQTGDSES
VPDPQPLGEPPATPAAVGPTTMASGGGAPMADNNEGADGVGNASGNWHCDSTWLGDRVIT
TSTRTWALPTYNNHLYKQISSASTGASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLINN
NWGFRPKRLNFKLFNIQVKEVTTNDGVTTIANNLTSTVQVFSDSEYQLPYVLGSAHQGCLPP
FPADVFMIPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFTFSYTFEEVPFHSSYAHS
QSLDRLMNPLIDQYLYYLNRTQNQSGSAQNKDLLFSRGSPAGMSVQPKNWLPGPCYRQQRV
SKTKTDNNNSNFTWTGASKYNLNGRESIINPGTAMASHKDDEDKFFPMSGVMIFGKESAGAS
NTALDNVMITDEEEIKATNPVATERFGTVAVNFQSSSTDPATGDVHAMGALPGMVWQDRD
VYLQGPIWAKIPHTDGHFHPSPLMGGFGLKNPPPQILIKNTPVPANPPAEFSATKFASFITQYST
GQVSVEIEWELQKENSKRWNPEVQYTSNYAKSANVDFTVDNNGLYTEPRPIGTRYLTRP

Lysine

position	Score	Ubiquitinated	
26	0.59	No	
31	0.55	No	
33	0.47	No	
38	0.61	No	
51	0.54	No	
61	0.72	Yes	Medium confidence
77	0.63	Yes	Low confidence
84	0.72	Yes	Medium confidence
122	0.17	No	
123	0.26	No	
137	0.90	Yes	High confidence
142	0.62	Yes	Low confidence
143	0.81	Yes	Medium confidence
161	0.70	Yes	Medium confidence
168	0.25	No	
169	0.26	No	
258	0.49	No	
310	0.14	No	
315	0.15	No	
322	0.48	No	
459	0.81	Yes	Medium confidence
476	0.43	No	
491	0.20	No	
493	0.28	No	
508	0.62	Yes	Low confidence
528	0.67	Yes	Low confidence
533	0.70	Yes	Medium confidence
545	0.65	Yes	Low confidence
567	0.66	Yes	Low confidence
621	0.50	No	
641	0.50	No	
650	0.45	No	
666	0.58	No	
689	0.65	Yes	Low confidence
693	0.65	Yes	Low confidence
707	0.78	Yes	Medium confidence

Legend:

Label	Score range	Sensitivity	Specificity
Low confidence	0.62 < s < 0.69	0.464	0.903
Medium confidence	0.69 < s < 0.84	0.346	0.950
High confidence	0.84 < s < 1.00	0.197	0.989

Desirable mutants in AAV-1

ID	*AAV-1 mutant*
MUT1-1	**K61R**
MUT1-2	**K84R**
MUT1-3	**K137R**
MUT1-4	**K143R**
MUT1-5	**K161R**
MUT1-6	**K459R**
MUT1-7	**K528R**
MUT1-8	**K533R**
MUT1-9	**K707R**

AAV8 capsid protein VP1 sequence:

MAADGYLPDWLEDNLSEGIREWWALKPGAPKPKANQQKQDDGRGLVLPGYKYLGPFNGL
DKGEPVNAADAAALEHDKAYDQQLQAGDNPYLRYNHADAEFQERLQEDTSFGGNLGRAVF
QAKKRVLEPLGLVEEGAKTAPGKKRPVEPSPQRSPDSSTGIGKKGQQPARKRLNFGQTGDSE
SVPDPQPLGEPPAAPSGVGPNTMAAGGGAPMADNNEGADGVGSSSGNWHCDSTWLGDRVI
TTSTRTWALPTYNNHLYKQISNGTSGGATNDNTYFGYSTPWGYFDFNRFHCHFSPRDWQRLI
NNNWGFRPKRLSFKLFNIQVKEVTQNEGTKTIANNLTSTIQVFTDSEYQLPYVLGSAHQGCLP
PFPADVFMIPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFQFTYTFEDVPFHSSYAH
SQSLDRLMNPLIDQYLYYLSRTQTTGGTANTQTLGFSQGGPNTMANQAKNWLPGPCYRQQR
VSTTTGQNNNSNFAWTAGTKYHLNGRNSLANPGIAMATHKDDEERFFPSNGILIFGKQNAAR
DNADYSDVMLTSEEEIKTTNPVATEEYGIVADNLQQQNTAPQIGTVNSQGALPGMVWQNRD
VYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADPPTTFNQSKLNSFITQYST
GQVSVEIEWELQKENSKRWNPEIQYTSNYYKSTSVDFAVNTEGVYSEPRPIGTRYLTRNL

Lysine position	Score	Ubiquitinated	
26	0.52	No	
31	0.51	No	
33	0.16	No	
38	0.62	Yes	Low confidence
51	0.56	No	
61	0.68	Yes	Low confidence
77	0.65	Yes	Low confidence
122	0.17	No	

123	0.32	No	
137	0.92	Yes	High confidence
142	0.53	No	
143	0.65	Yes	Low confidence
162	0.28	No	
163	0.29	No	
170	0.24	No	
259	0.49	No	
312	0.09	No	
317	0.12	No	
324	0.43	No	
333	0.75	Yes	Medium confidence
478	0.59	No	
510	0.50	No	
530	0.71	Yes	Medium confidence
547	0.40	No	
569	0.66	Yes	Low confidence
623	0.47	No	
643	0.44	No	
652	0.51	No	
668	0.64	Yes	Low confidence
691	0.66	Yes	Low confidence
695	0.67	Yes	Low confidence
709	0.68	Yes	Low confidence

Legend:

Label	Score range	Sensitivity	Specificity
Low confidence	0.62 < s < 0.69	0.464	0.903
Medium confidence	0.69 < s < 0.84	0.346	0.950
High confidence	0.84 < s < 1.00	0.197	0.989

Table 3 appended hereto provides the yield of vector obtained using the different capsid mutants, including AAV8 of the invention. The table also indicates whether the mutation resulted in increased, decreased or comparable transduction efficiencies when compared to wild type vectors of the same serotype.

AAV2 capsid protein VP1 sequence:

MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGYKYLGPFNGLDK
GEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADAEFQERLKEDTSFGGNLGRAVFQA
KKRVLEPLGLVEEPVKTAPGKKRPVEHSPVEPDSSSGTGKAGQQPARKRLNFGQTGDADSVP
DPQPLGQPPAAPSGLGTNTMATGSGAPMADNNEGADGVGNSSGNWHCDSTWMGDRVITTS
TRTWALPTYNNHLYKQISSQSGASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLINNNW
GFRPKRLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPA
DVFMVPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFTFSYTFEDVPFHSSYAHSQS
LDRLMNPLIDQYLYYLSRTNTPSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYRQQRVSKT
SADNNNSEYSWTGATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGVLIFGKQGSEKTNV

DIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGVLPGMVWQDRDVYL
QGPIWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKNTPVPANPSTTFSAAKFASFITQYSTGQ
VSVEIEWELQKENSKRWNPEIQYTSNYNKSVNVDFTVDTNGVYSEPRPIGTRYLTRNL

Lysine position	Score	Ubiquitinated	
24	0.20	No	
26	0.49	No	
33	0.38	No	
39	0.89	Yes	High confidence
51	0.53	No	
61	0.67	Yes	Low confidence
77	0.68	Yes	Low confidence
92	0.56	No	
105	0.58	No	
122	0.14	No	
123	0.18	No	
137	0.87	Yes	High confidence
142	0.58	No	
143	0.77	Yes	Medium confidence
161	0.70	Yes	Medium confidence
169	0.31	No	
258	0.51	No	
309	0.14	No	
314	0.17	No	
321	0.46	No	
490	0.73	Yes	Medium confidence
507	0.61	No	
527	0.78	Yes	Medium confidence
532	0.75	Yes	Medium confidence
544	0.61	No	
549	0.68	Yes	Low confidence
556	0.62	Yes	Low confidence
620	0.47	No	
640	0.45	No	
649	0.42	No	
665	0.54	No	
688	0.68	Yes	Low confidence
692	0.68	Yes	Low confidence
706	0.66	Yes	Low confidence

Legend:

Label	Score range	Sensitivity	Specificity
Low confidence	$0.62 \leq s \leq 0.69$	0.464	0.903
Medium confidence	$0.69 \leq s \leq 0.84$	0.346	0.950
High confidence	$0.84 \leq s \leq 1.00$	0.197	0.989

AAV-Rh74 capsid protein VP1 sequence

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDNGRGLVLPGYKYLGPFNGL

DKGEPVNAADAAALEHDKAYDQQLQAGDNPYLRYNHADAEFQERLQEDTSFGGNLGRAVF
QAKKRVLEPLGLVESPVKTAPGKKRPVEPSPQRSPDSSTGIGKKGQQPAKKRLNFGQTGDSES
VPDPQPIGEPPAGPSGLGSGTMAAGGGAPMADNNEGADGVGSSSGNWHCDSTWLGDRVITT
STRTWALPTYNNHLYKQISNGTSGGSTNDNTYFGYSTPWGYFDFNRFHCHFSPRDWQRLINN
NWGFRPKRLNFKLFNIQVKEVTQNEGTKTIANNLTSTIQVFTDSEYQLPYVLGSAHQGCLPPF
PADVFMIPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFEFSYNFEDVPFHSSYAHS
QSLDRLMNPLIDQYLYYLSRTQSTGGTAGTQQLLFSQAGPNNMSAQAKNWLPGPCYRQQRV
STTLSQNNNSNFAWTGATKYHLNGRDSLVNPGVAMATHKDDEERFFPSSGVLMFGKQGAG
KDNVDYSSVMLTSEEEIKTTNPVATEQYGVVADNLQQQNAAPIVGAVNSQGALPGMVWQN
RDVYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADPPTTFNQAKLASFITQ
YSTGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSTNVDFAVNTEGTYSEPRPIGTRYLTRNL

Lysine position	Score	Ubiquitinated	
26	0.60	No	
31	0.53	No	
33	0.27	No	
38	0.23	No	
51	0.38	No	
61	0.68	Yes	Low confidence
77	0.65	Yes	Low confidence
122	0.17	No	
123	0.30	No	
137	0.90	Yes	High confidence
142	0.35	No	
143	0.35	No	
162	0.36	No	
163	0.33	No	
169	0.27	No	
170	0.25	No	
259	0.46	No	
312	0.13	No	
317	0.16	No	
324	0.46	No	
333	0.77	Yes	Medium confidence
478	0.51	No	
510	0.49	No	
530	0.67	Yes	Low confidence
547	0.52	No	
552	0.69	Yes	Medium confidence
569	0.67	Yes	Low confidence
623	0.49	No	
643	0.44	No	
652	0.54	No	
668	0.56	No	
691	0.68	Yes	Low confidence
695	0.67	Yes	Low confidence
709	0.69	Yes	Medium confidence

Legend:

Label	Score range	Sensitivity	Specificity

Low confidence	0.62 < s < 0.69	0.464	0.903
Medium confidence	0.69 < s < 0.84	0.346	0.950
High confidence	0.84 < s < 1.00	0.197	0.989

Desirable mutants in AAV-rh74 (also see Table 3)

ID	*AAV-rh74 mutant*
Mut74-1	**K26R**
Mut74-2	**K31R**
Mut74-3	**K33R**
Mut74-4	**K38R**
Mut74-5	**K51R**
Mut74-6	**K77R**
Mut74-7	**K137R**
Mut74-8	**K163R**
Mut74-9	**K169R**
Mut74-10	**K259R**
Mut74-11	**K333R**
Mut74-12	**K530R**
Mut74-13	**K547R**
Mut74-14	**K552R**
Mut74-15	**K569R**
Mut74-16	**K668R**
Mut74-17	**K691R**
Mut74-18	**K695R**
Mut74-19	**K709R**

The following primer sets were utilized to create the lysine containing capsid variants of the invention:

Primers used for mutagenesis:

- AAV1 primers:

AAV1 K61R
Sense: 5'- CTT CAA CGG ACT CGA CAG GGG GGA GCC -3'
Antisense: 5'- GGC TCC CCC CTG TCG AGT CCG TTG AAG -3'

AAV1 K84R
Sense: 5'- GCC TAC GAC CAG CAG CTC AGA GCG GGT GAC -3'
Antisense: 5'- GTC ACC CGC TCT GAG CTG CTG GTC GTA GGC -3'

AAV1 K137R
Sense: 5'- TGG TTG AGG AAG GCG CTA GGA CGG CTC CT -3'
Antisense: 5'- AGG AGC CGT CCT AGC GCC TTC CTC AAC CA -3'

AAV1 K143R
Sense: 5'- CTA AGA CGG CTC CTG GAA AGA GAC GTC CGG TAG -3'
Antisense:5'- CTA CCG GAC GTC TCT TTC CAG GAG CCG TCT TAG -3'

AAV1 K161R
Sense: 5'- CGG GCA TCG GCA GGA CAG GCC AGC A -3'
Antisense:5'- TGC TGG CCT GTC CTG CCG ATG CCC G -3'

AAV1 K459R
Sense: 5'- AGT CCG GAA GTG CCC AAA ACA GGG ACT TGC TGT -3'
Antisense:5'- ACA GCA AGT CCC TGT TTT GGG CAC TTC CGG ACT -3'

AAV1 K528R
Sense: 5'- GCA CTG CTA TGG CCT CAC ACA GAG ACG ACG AAG -3'
Antisense:5'- CTT CGT CGT CTC TGT GTG AGG CCA TAG CAG TGC -3'

AAV1 K533R
Sense: 5'- CAA AGA CGA CGA AGA CAG GTT CTT TCC CAT GAG CG -3'
Antisense:5'-CGC TCA TGG GAA AGA ACC TGT CTT CGT CGT CTT TG -3'

AAV1 K707R
Sense: 5'- TGCAGTACACATCCAATTATGCAAGATCTGCCAACG TTG -3'
Antisense:5'- CAACGTTGGCAGATCTTGCATAATTGGATGTGTACTGCA -3'

- AAV8 Primers

AAV8 K137R
Sense: 5'- GGT TGA GGA AGG CGC TAG GAC GGC TCC TGG -3'
Antisense:5'- CCA GGA GCC GTC CTA GCG CCT TCC TCA ACC -3'

AAV8 K333R
Sense: 5'- GCA GAA TGA AGG CAC CAG GAC CAT CGC CAA TAA CC -3'
Antisense:5'- GGT TAT TGG CGA TGG TCC TGG TGC CTT CAT TCT GC -3'

AAV8 K530R
Sense: 5'- GCA TCG CTA TGG CAA CAC ACA GAG ACG ACG AGG -3'
Antisense:5'- CCT CGT CGT CTC TGT GTG TTG CCA TAG CGA TGC -3'

AAV8 K709R
Sense: 5'- GTACACCTCCAACTACTACAGATCTACAAGTGTGGACTTTG -3'
Antisense:5'- CAAAGTCCACACTTGTAGATCTGTAGTAGTTGGAGGTGTAC -3'

- AAV2 Primers

AAV2 K39R
Sense: 5'-GCCCGCAGAGCGGCATAGGGACGACAG-3'
Antisense: 5'-CTGTCGTCCCTATGCCGCTCTGCGGGC-3'

AAV2 K137R
Sense: 5'-CCTGGTTGAGGAACCTGTTAGGACGGCTCCGG-3'
Antisense: 5'-CCGGAGCCGTCCTAACAGGTTCCTCAACCAGG-3'

AAV2 K143R
Sense: 5'-AGACGGCTCCGGGAAAAAGGAGGCCGGTA-3'

Antisense: 5'-TACCGGCCTCCTTTTTCCCGGAGCCGTCT-3'

AAV2 K161R
Sense: 5'-CCTCGGGAACCGGAAGGGCGGGCC-3'
Antisense: 5'-GGCCCGCCCTTCCGGTTCCCGAGG-3'

AAV2 K490R
Sense: 5'-CCGCCAGCAGCGAGTATCAAGGACATCTGCGG-3'
Antisense: 5'-CCGCAGATGTCCTTGATACTCGCTGCTGGCGG-3'

AAV2 K527R
Sense: 5'-CGGCCATGGCAAGCCACAGGGACGATGAA-3'
Antisense: 5'-TTCATCGTCCCTGTGGCTTGCCATGGCCG-3'

AAV2 K532R
Sense: 5'-ACAAGGACGATGAAGAAAGGTTTTTTCCTCAGAGCGG-3'
Antisense: 5'-CCGCTCTGAGGAAAAAACCTTTCTTCATCGTCCTTGT-3'

- AAV-rh74 Primers

AAV-rh74 K137R
Sense: 5'-CTGGTTGAATCGCCGGTTAGGACGGCTCCTG-3'
Antisense: 5'-GACCAACTTAGCGGCCAATCCTGCCGAGGAC-3'

AAV-rh74 K333R
Sense: 5'-GCAGAATGAAGGCACCAGGACCATCGCCAATAACC-3'
Antisense: 5'-GGTTATTGGCGATGGTCCTGGTGCCTTCATTCTGC-3'

AAV-rh74 K530R
Sense: 5'-GTTGCCATGGCTACCCACAGGGACGACGAA-3'
Antisense: 5'-TTCGTCGTCCCTGTGGGTAGCCATGGCAAC-3'

AAV-rh74 K552R
Sense: 5'-GGAAACAGGGAGCTGGAAGAGACAACGTGGACTAT-3'
Antisense: 5'-ATAGTCCACGTTGTCTCTTCCAGCTCCCTGTTTCC-3'

AAV-rh74 K569R
Sense: 5'-CTAACCAGCGAGGAAGAAATAAGGACCACCAACCC-3'
Antisense: 5'-GGGTTGGTGGTCCTTATTTCTTCCTCGCTGGTTAG-3'

AAV-rh74 K691R
Sense: 5'-CGAGTGGGAGCTGCAGAGGGAGAACAGCAA-3'
Antisense: 5'-TTGCTGTTCTCCCTCTGCAGCTCCCACTCG-3'

AAV-rh74 K695R
Sense: 5'-GCTGCAGAAGGAGAACAGCAGACGCTGGAACC-3'
Antisense: 5'-GGTTCCAGCGTCTGCTGTTCTCCTTCTGCAGC-3'

AAV-rh74 K709R
Sense: 5'-AGTACACTTCCAACTACTACAGATCTACAAATGTGGACTTTGC-3'
Antisense: 5'-GCAAAGTCCACATTTGTAGATCTGTAGTAGTTGGAAGTGTACT-3'

Table 5 provides a series of AAV vectors derived from mutagenesis that were used to package liver-specific AAV transgene expression cassettes for FIX. Packaging efficiency was indistinguishable from that observed with wild type, unmodified AAV8 vectors.

Lot number	Lysine mutant	Preparation size (Roller Bottles)	Yield per roller bottle (vector genomes)
KA712	AAV8 K137R	10	1.53E13
KA713	AAV8 K333R	10	9.46E12
KA714	AAV8 K530R	10	1.61E13
KA535	AAV8 (wild type)	10	6.63E12

Capsid mutants which contain 2, 3, 4, 5, 6, 7 or more altered lysine residues in any of the capsid proteins described herein to further increase transduction efficiency are also within the scope of the invention. The data also reveals that certain mutations result in variants which exhibited significantly reduced transduction efficiency. Such variants could be used in combination with variants which exhibit increased transduction efficiencies, to act as decoys to neutralize or saturate an antibody directed immune response to the incoming vectors, thereby enabling vectors carrying desirable transgenes to more efficiently enter cells.

Figure 1 shows schematic diagrams of the capsid surface. The data presented in Figures 2A, 2B and 2C demonstrate that altering lysine residues in the VP1 capsid on AAV8 alters the level of transgene produced due to altered transduction levels. Figure 3A, 3B and 3C show the effects of single and multiple mutations on HF.IX production in transduced cells. Figure 3D demonstrates that a combination mutations, e.g., three lysine to arginine residues with four or six tyrosine to phenylalanine residues decreases transduction rates.

CTL Killing of HHL5-B7 Hepatocytes using AAV lysine mutants

We assessed CTL killing of hepatocytes transduced with certain of the AAV lysine mutants disclosed here. The following materials and methods were employed to assess CTL killing of transduced hepatocytes.

Vector generation

AAV vectors were produced in HEK-293 cells using a triple transfection approach as previously described (Matsushita, 1998) and purified with cesium chloride gradient

centrifugation methods (Ayuso, 2010). AAV epitope peptides were synthesized by Genemed Synthesis and resuspended at a concentration of 5mg/ml in 100% DMSO.

***In vitro* expansion of T cells**

Human PBMCs (Cellular Technology LTD) were thawed, washed, counted, and resuspended at a concentration of 2 x 10^6 cells/ml in AIM-V lymphocyte media (Gibco) containing 3% human serum (Bioreclamation), 1% L-glutamine (Gibco), and 1% penicillin/streptomycin (Gibco). For each expansion condition, 1x10^6 (500 µl) cells were added per well in a 24 well plate (BD Falcon) in a volume of 500 µl. An additional 1x10^6 (500 µl) of autologous irradiated splenocytes (3000 rad) were also added to each well as feeder cells, along with 2.5 µg/ml of human β-2-microglobulin (Lee Biosolutions), and 10 ng/ml of human recombinant IL-7 (R&D Systems). Cells were expanded in the presence of AAV peptide at a final concentration of 10 µg/ml at 37°C in 5% CO_2. Human IL-2 (Roche) at a concentration of 10 ng/ml was added to the cell culture after the first 24 hours and replenished every 48 hours thereafter. Cells were divided into new wells as necessary and antigenic stimulation (antigen and feeder cells) was repeated every 7-10 days for up to 3 rounds of restimulation.

CTL ASSAY

CTL assay was performed as previously described (Pien, 2009). Briefly, lactate dehydrogenase (LDH) release following CTL-mediated target lysis was measured with the CytoTox 96 Non Radioactive Cytotoxicity Assay (Promega). Four thousand HHL5 hepatocyte target cells were plated in each well of a Microtest Primaria flat-bottom 96-well plate (BD Falcon) in DMEM containing no serum. Target cells were transduced at an MOI of 5000, 50,000, and 500,000 of AAV capsid and incubated for 18 hours at 37°C 5% CO_2. Following treatment and incubation, plated target cells were washed once with media prior to the addition of epitope-specific cytotoxic T lymphocytes, expanded as described above. CTLs were added at an effector-target cell ratio of 10:1 for 4 hours at 37°C, 5% CO_2 and LDH was measured after a 30 minute incubation at room temperature with enzymatic substrate read at 490nm using a spectrophotometer (Spectramax).

Flow cytometry

GFP expression following AAV transduction was measured by flow cytometry. Human hepatocytes from cell lines HHL5 or Huh7 were plated in DMEM containing 10%

fetal bovine serum, 1% L-glutamine (Gibco), and 1% penicillin/streptomycin (Gibco) at a density of 250,000 cells/well in a Primaria Multiwell 24-well plate (BD Falcon). Cells were transduced with 5000, 50000, or 5000000 MOI of AAV vector and incubated for 18 hours at 37°C 5% CO_2. Following incubation, cells were trypsinized, washed twice with PBS 2% FBS, and fixed with 2% paraformaldehyde. Samples were aquired on a FACS Canto II flow cytometer using the FACSDiva® (BD Biosciences) and further analysis was performed using Flowjo® software (Treestar).

CTL ASSAY RESULTS

In order to further test effect of the lysine mutations on viral transduction, we utilized an in vitro CTL-mediated cytotoxicity assay previously developed by our lab to test the functionality of AAV vectors (Pien et al.). AAV1 and AAV2 transduction results are shown in Figures 4 and 5 and 6. In Figure 4, all lysine mutations in the AAV-1 capsid resulted in a decrease in CTL-mediated killing of target cells, suggesting that the lysine mutations led to less efficient processing and presentation of surface antigen upon transduction. Furthermore, the effect of lysine mutations appears to be additive, with the triple and quadruple lysine mutants showing the greatest decrease of CTL-mediated killing (Figure 4A, B). Mutations to the AAV-2 capsid showed a similar effect. See Figures 5 and 6. Figure 7 shows the transduction results obtained when Rh74 variants were tested.

In summary, we have found that mutating the lysine residues on AAV capsids to arginine residues increases AAV transduction efficiency. Our experiments identified several variants that upon transduction, resulted in higher levels of expression of the human factor IX (FIX) transgene in mice compared to animals receiving unmodified AAV vectors.

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While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

Table 1

Comparison of AAV1, AAV2 and AAV8 lysine positions and their Ubiqutination predictions

Color Label: Green: Low confidence, Blue: Medium confidence, Red: High confidence, Black: No ubiquitination prediction

```
AAV1      MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLGPFNGLD 60
AAV8      MAADGYLPDWLEDNLSEGIREWWALKPGAPKPKANQQKQDDGRGLVLPGYKYLGPFNGLD 60
AAV2      MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGYKYLGPFNGLD 60
AAV-rh74  MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDNGRGLVLPGYKYLGPFNGLD 60

AAV1      KGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADAEFQERLQEDTSFGGNLGRAVFQ 120
AAV8      KGEPVNAADAAALEHDKAYDQQLQAGDNPYLRYNHADAEFQERLQEDTSFGGNLGRAVFQ 120
AAV2      KGEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADAEFQERLKEDTSFGGNLGRAVFQ 120
AAV-rh74  KGEPVNAADAAALEHDKAYDQQLQAGDNPYLRYNHADAEFQERLQEDTSFGGNLGRAVFQ 120

AAV1      AKKRVLEPLGLVEEGAKTAPGKKRPVEQSPQ-EPDSSSGIGKTGQQPAKKRLNFGQTGDS 179
AAV8      AKKRVLEPLGLVEEGAKTAPGKKRPVEPSPQRSPDSSTGIGKKGQQPARKRLNFGQTGDS 180
AAV2      AKKRVLEPLGLVEEPVKTAPGKKRPVEHSPV-EPDSSSGTGKAGQQPARKRLNFGQTGDA 179
AAV-rh74  AKKRVLEPLGLVESPVKTAPGKKRPVEPSPQRSPDSSTGIGKKGQQPAKKRLNFGQTGDS 180

AAV1      ESVPDPQPLGEPPATPAAVGPTTMASGGGAPMADNNEGADGVGNASGNWHCDSTWLGDRV 239
AAV8      ESVPDPQPLGEPPAAPSGVGPNTMAAGGGAPMADNNEGADGVGSSSGNWHCDSTWLGDRV 240
AAV2      DSVPDPQPLGQPPAAPSGLGTNTMATGSGAPMADNNEGADGVGNSSGNWHCDSTWMGDRV 239
AAV-rh74  ESVPDPQPIGEPPAGPSGLGSGTMAAGGGAPMADNNEGADGVGSSSGNWHCDSTWLGDRV 240

AAV1      ITTSTRTWALPTYNNHLYKQISSASTG-ASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQ 298
AAV8      ITTSTRTWALPTYNNHLYKQISNGTSGGATNDNTYFGYSTPWGYFDFNRFHCHFSPRDWQ 300
AAV2      ITTSTRTWALPTYNNHLYKQIS--SQSGASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQ 297
AAV-rh74  ITTSTRTWALPTYNNHLYKQISNGTSGGSTNDNTYFGYSTPWGYFDFNRFHCHFSPRDWQ 300

AAV1      RLINNNWGFRPKRLNFKLFNIQVKEVTTNDGVTTIANNLTSTVQVFSDSEYQLPYVLGSA 358
AAV8      RLINNNWGFRPKRLSFKLFNIQVKEVTQNEGTKTIANNLTSTIQVFTDSEYQLPYVLGSA 360
AAV2      RLINNNWGFRPKRLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSA 357
AAV-rh74  RLINNNWGFRPKRLNFKLFNIQVKEVTQNEGTKTIANNLTSTIQVFTDSEYQLPYVLGSA 360

AAV1      HQGCLPPFPADVFMIPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFTFSYTFEE 418
AAV8      HQGCLPPFPADVFMIPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFQFTYTFED 420
AAV2      HQGCLPPFPADVFMVPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFTFSYTFED 417
AAV-rh74  HQGCLPPFPADVFMIPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFEFSYNFED 420

AAV1      VPFHSSYAHSQSLDRLMNPLIDQYLYYLNRTQNQSGSAQNKDLLFSRGSPAGMSVQPKNW 478
AAV8      VPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTQTTGGTANTQTLGFSQGGPNTMANQAKNW 480
AAV2      VPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTNTPSGTTTQSRLQFSQAGASDIRDQSRNW 477
AAV-rh74  VPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTQSTGGTAGTQQLLFSQAGPNNMSAQAKNW 480
```

AAV1 LPGPCYRQQRVSKTKTDNNNSNFTWTGASKYNLNGRESIINPGTAMASHKDDEDKFFPMS 538
AAV8 LPGPCYRQQRVSTTTGQNNNSNFAWTAGTKYHLNGRNSLANPGIAMATHKDDEERFFPSN 540
AAV2 LPGPCYRQQRVSKTSADNNNSEYSWTGATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQS 537
AAV-rh74 LPGPCYRQQRVSTTLSQNNNSNFAWTGATKYHLNGRDSLVNPGVAMATHKDDEERFFPSS 540

AAV1 GVMIFGKESAGASNTALDNVMITDEEEIKATNPVATERFGTVAVNFQSSSTDPATGDVHA 598
AAV8 GILIFGKQNAARDNADYSDVMLTSEEEIKTTNPVATEEYGIVADNLQQQNTAPQIGTVNS 600
AAV2 GVLIFGKQGSEKTNVDIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNT 597
AAV-rh74 GVLMFGKQGAGKDNVDYSSVMLTSEEEIKTTNPVATEQYGVVADNLQQQNAAPIVGAVNS 600

AAV1 MGALPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKNPPPQILIKNTPVPANP 658
AAV8 QGALPGMVWQNRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADP 660
AAV2 QGVLPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKNTPVPANP 657
AAV-rh74 QGALPGMVWQNRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADP 660

AAV1 PAEFSATKFASFITQYSTGQVSVEIEWELQKENSKRWNPEVQYTSNYAKSANVDFTVDNN 718
AAV8 PTTFNQSKLNSFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSTSVDFAVNTE 720
AAV2 STTFSAAKFASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYNKSVNVDFTVDTN 717
AAV-rh74 PTTFNQAKLASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSTNVDFAVNTE 720

AAV1 GLYTEPRPIGTRYLTRP- 735
AAV8 GVYSEPRPIGTRYLTRNL 738
AAV2 GVYSEPRPIGTRYLTRNL 735
AAV-rh74 GTYSEPRPIGTRYLTRNL 738

Table 2
Ubiquitination prediction of AAV8 capsid

Lysine	surface	prediction by ubipred	VECTOR PRODUCED
26	NA(not in VP3)		
31	NA(not in VP3)		
33	NA(not in VP3)		
38	NA(not in VP3)	Low confidence	Yes
51	NA(not in VP3)		
61	NA(not in VP3)	Low confidence	
77	NA(not in VP3)	Low confidence	
122	NA(not in VP3)		
123	NA(not in VP3)		
137	NA(not in VP3)	High confidence	YES
142	NA(not in VP3)		
143	NA(not in VP3)	Low confidence	Yes
162	NA(not in VP3)		
163	NA(not in VP3)		
170	NA(not in VP3)		
259	YES		YES
312			
317			
324			
333	YES	Medium confidence	YES
478			
510	YES		YES
530		Medium Confidence	YES
547	YES		YES
569	YES	Low confidence	YES
623			
643			
652			NOT PACKED
668	YES	Low confidence	YES
691		Low confidence	
695		Low confidence	
709	YES	Low confidence	YES

UbPred software (http://www.ubpred.org/):

Radivojac P., Vacic, V., Haynes, C., Cocklin, R. R., Mohan, A., Heyen, J. W., Goebl, M. G., and Iakoucheva, L. M. Identification, Analysis and Prediction of Protein Ubiquitination Sites. *Proteins: Structure, Function, and Bioinformatics. 78(2):365-380. (2010)*

Table 3

AAV8 mutation	Vector	YIELD (vector concentration vg/RB)	Comparison to WT
WT	hAAT-hFIX16	1.24 E+13	NA
K38R	hAAT-hFIX16	2.32 E+13	decreased
K137R	hAAT-hFIX16	1.53 E+13	increased
K143R	hAAT-hFIX16	2.21 E+13	decreased
K259R	hAAT-hFIX16	1.03 E+13	slight increase
K333R	hAAT-hFIX16	9.46 E+12	increased
K510R	hAAT-hFIX16	1.04 E+13	decreased
K530R	hAAT-hFIX16	1.61 E+13	increased
K547R	hAAT-hFIX16	1.17 E+13	same
K569R	hAAT-hFIX16	1.38 E+13	increased
K668R	hAAT-hFIX16	1.41 E+13	same
K709R	hAAT-hFIX16	1.38 E+13	decreased
K530R/K569R	hAAT-hFIX16	1.34 E+13	same
K(137/333/530) R	hAAT-hFIX16	7.00 E+12	increased
K(137/333/530/709)R	hAAT-hFIX16	6.64 E+12	decreased
K(137/333/530/569)R	hAAT-hFIX16	9.03 E+12	same
K(137/259/333/530/547/569)R	hAAT-hFIX16	7.62 E+12	same
Y(253/275/447/703/707/733)Y	hAAT-hFIX16	9.87 E+12	slight increase
Y(253/275/447/703/707/733)Y + K (137/333/530) R	hAAT-hFIX16	3.24 E+12	decreased
Y (253/275/447/733) F	hAAT-hFIX16	8.58 E+12	slight increase
Y(253/275/447/733)F + K(137/333/530)R	hAAT-hFIX16	3.30 E+12	decreased
K(137/333/530/547)R	hAAT-hFIX16	5.85 E+12	Not tested

K(137/259/333/530/547)R	hAAT-hFIX16	7.68 E+12	Not tested
Y733F	hAAT-hFIX16	8.30 E+12	increased
Y(733/447) F	hAAT-hFIX16	1.19 E+13	increased
Y(703/707)F	hAAT-hFIX16	1.05 E+13	increased
Y(733/447/253)F	hAAT-hFIX16	1.35 E+13	increased
Y(733/447/275) F	hAAT-hFIX16	7.26 E+12	increased

AAV1 mutation	**Vector**	**YIELD (vector concentration vg/RB)**
AAV1 WT	CMV-GFP	6.71E+12
AAV1 K137R/K459R/K533R	CMV-GFP	8.35E+12
AAV1 K(137/459/533/707)R	CMV-GFP	6.97E+12
AAV1 K137R	CMV-GFP	7.96E+12
AAV1 K459R	CMV-GFP	6.57E+12
AAV1 K533R	CMV-GFP	5.00E+12
AAV1 K707R	CMV-GFP	5.66E+12
AAV1 K528R	CMV-GFP	7.28E+12
AAV1 Y50A	CMV-GFP	7.93E+12

MHC delivery

AAV2 mutation	**Vector**	**YIELD (vector concentration vg/RB)**
AAV2	CMV GFP	1.15e12
AAV2 K137R	CMV-GFP	6.24E+12
AAV2 K532R	CMV-GFP	5.73E+12
AAV2 K527R	CMV-GFP	4.92E+12
AAV2 Y50A	CMV-GFP	5.59E+12

RH74 mutation	Vector	YIELD (vector concentration vg/RB)
RH74 WT	hAAT-hFIX19	1.51E+13
Rh74 TMR	hAAT-hFIX16	7.62E+12
Rh74 TMR+552R	hAAT-hFIX16	6.91E+12
Rh74 TMR+709R	hAAT-hFIX16	4.18E+12
Rh74 HMF	hAAT-hFIX16	6.83E+12
Rh74 HMR+195/202	hAAT-hFIX19	3.79E+12
Rh74 HMR+195/202+K38R	hAAT-hFIX19	3.00E+12
Rh74 HMR+195/202+K51R	hAAT-hFIX19	3.54E+12
Rh74 HMR+195/202+K61R	hAAT-hFIX19	5.14E+12
Rh74 HMR+195/202+K77R	hAAT-hFIX19	5.86E+12
Rh74 HMR+195/202+K(122/123)R	hAAT-hFIX19	5.51E+12
Rh74 HMR+195/202+K(142/143)R	hAAT-hFIX19	5.75E+12
RHM13_1	hAAT-hFIX19	7.3132E+12
RHM 17_1	hAAT-hFIX19	5.928E+12
RHM14_2	hAAT-hFIX19	6.084E+12
RHM15_1	hAAT-hFIX19	5.0396E+12

Definition of TMR= K(137/333/530)R
Definition of HMF= Y(253/275/447/703/707/733)F
Definition of HMR : K(137/259/333/530/552/569)R
Definition of 195/202 : G195A+ L199V+ S201P+ G202N

5

WHAT IS CLAIMED IS:

1. An improved adeno-associated virus (AAV) vector comprising a VP1 capsid protein comprising one or more lysine substitutions, said vector further comprising a minigene comprising AAV inverted terminal repeats and a heterologous nucleic acid sequence operably linked to regulatory sequences which direct expression of a product from the heterologous nucleic acid sequence in a host cell, said lysine substitution being effective to inhibit ubiquitination of said capsid protein, thereby increasing transduction of said AAV vector into a target cell.

2. The AAV vector of claim 1 having a serotype selected from the group consisting of AAV1, AAV2, AAV3, AAV4, AAV-rh74, AAV5, AAV6, AAV7, AAV8 and AAV9.

3. The AAV vector of claim 1, comprising a VP1 capsid protein having at least one lysine substitution at a lysine residue shown in the tables, wherein said at least one substitution increases transduction efficiency.

4. The AAV vector of claim 2 comprising an altered AAV8 VP1capsid protein selected from the group consisting of AAV8 K137R, AAV8 K333R and AAV8 K530R.

5. The AAV vector according to any one of the previous claims wherein the expression product of the heterologous nucleic acid sequence is a therapeutic peptide or nucleic acid.

6. The AAV vector according to claim 5, wherein the therapeutic peptide is a coagulation factor selected from the group consisting of Factor VIII, Factor IX or a functional fragment thereof.

7. The AAV8 vector according to claim 4, wherein the expression product of the heterologous nucleic acid sequence is an IgG, IgM, IgA, IgD, IgE, chimeric immunoglobulin, humanized antibody, or a single chain antibody.

8. The AAV8 vector according to claim 7, wherein the expression product of the heterologous nucleic acid sequence is a chimeric immunoglobulin.

9. The AAV8 vector according to claim 4, wherein the expression product of the heterologous nucleic acid sequence is a single chain antibody.

10. The AAV vector of claim 5 wherein the expression product is an antiviral RNAi.

11. The AAV vector of claim 10, wherein said inhibitory RNA is effective to inhibit HCV infection and replication.

12. The AAV vector of claim 10, wherein said inhibitory RNA is effective to inhibit expression of a eukaryotic target gene.

13. The AAV vector of claim 4, wherein the transgene encodes a disease-modifying cytokine.

14. The AAV vector of claim 4, wherein the transgene encodes a pair of zinc finger nucleases.

15. The AAV vector of claim 1 comprising 2, 3, or 4 lysine substitutions.

16. The AAV vector of claim 3 further comprising a second AAV vector comprising a VP1 capsid protein having at least one lysine substitution at a lysine residue shown in the tables, wherein said substitution reduces transduction efficiency, delivery of said second AAV vector being effective to neutralize an antibody response to a first AAV vector.

17. A pharmaceutical composition comprising the AAV vector according to claim 3, and a physiological compatible carrier therefor.

18. A cell culture comprising the AAV vector according to claim 1.

19. A method of delivering a transgene to a cell in a subject, said method comprising the step of contacting the cell with the pharmaceutical composition of according to claim 17 wherein said AAV vector comprises the transgene, wherein the presence of said lysine substitution in said VP1 capsid protein is associated a reduction in ubiquitination of said capsid, thereby increasing transduction efficiency of target cells with said vector.

20. The method of claim 19, wherein said transgene is Factor IX.

21. A pharmaceutical composition comprising the AAV vectors according to claim 16 , and a physiological compatible carrier therefor.

22. A method of delivering a transgene to a cell in a subject, said method comprising the step of contacting the cell with the pharmaceutical composition of according to claim 21 wherein said first AAV vector comprises the transgene, wherein the presence of said lysine substitution in said VP1 capsid protein is associated a reduction in ubiquitination of said capsid, thereby increasing transduction efficiency of target cells with said vector and said second vector exhibits reduced transduction efficiency relative to wild type and is effective to neutralize an undesired antibody response to said first AAV vector.

23. The method of claim 22, wherein said transgene is Factor IX.

AAV1

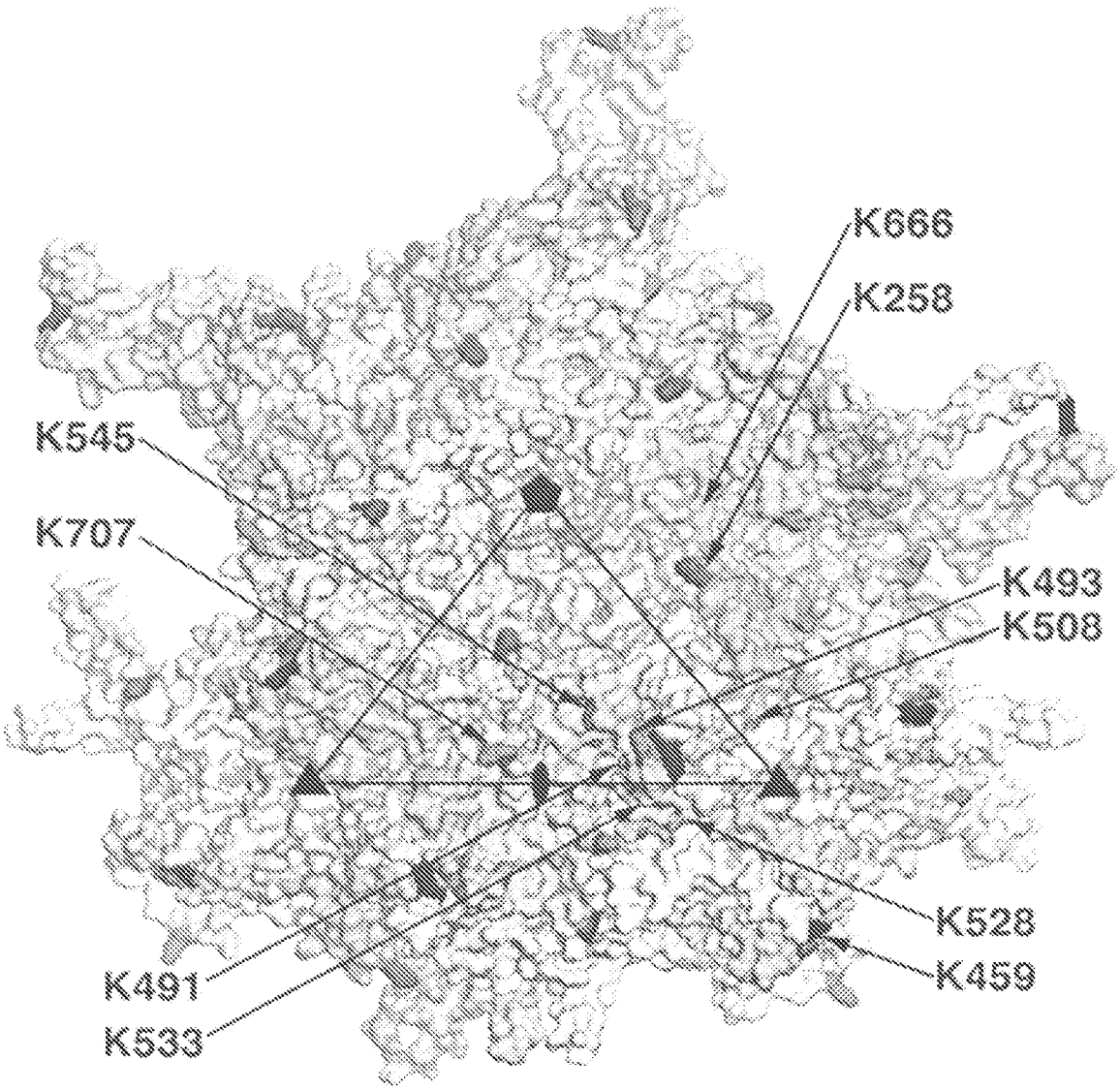

Figure 1A

AAV2

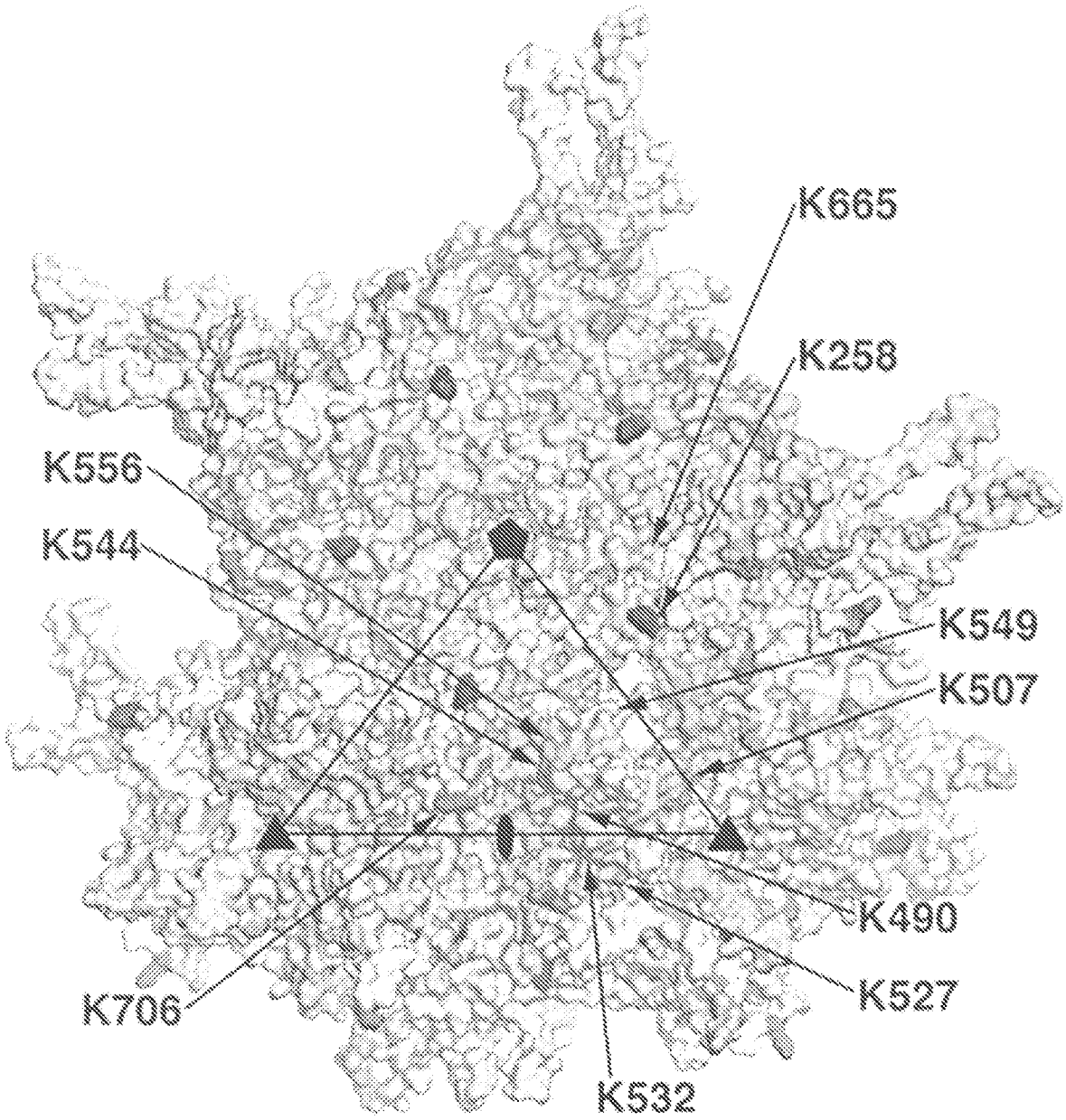

Figure 1B

AAV8

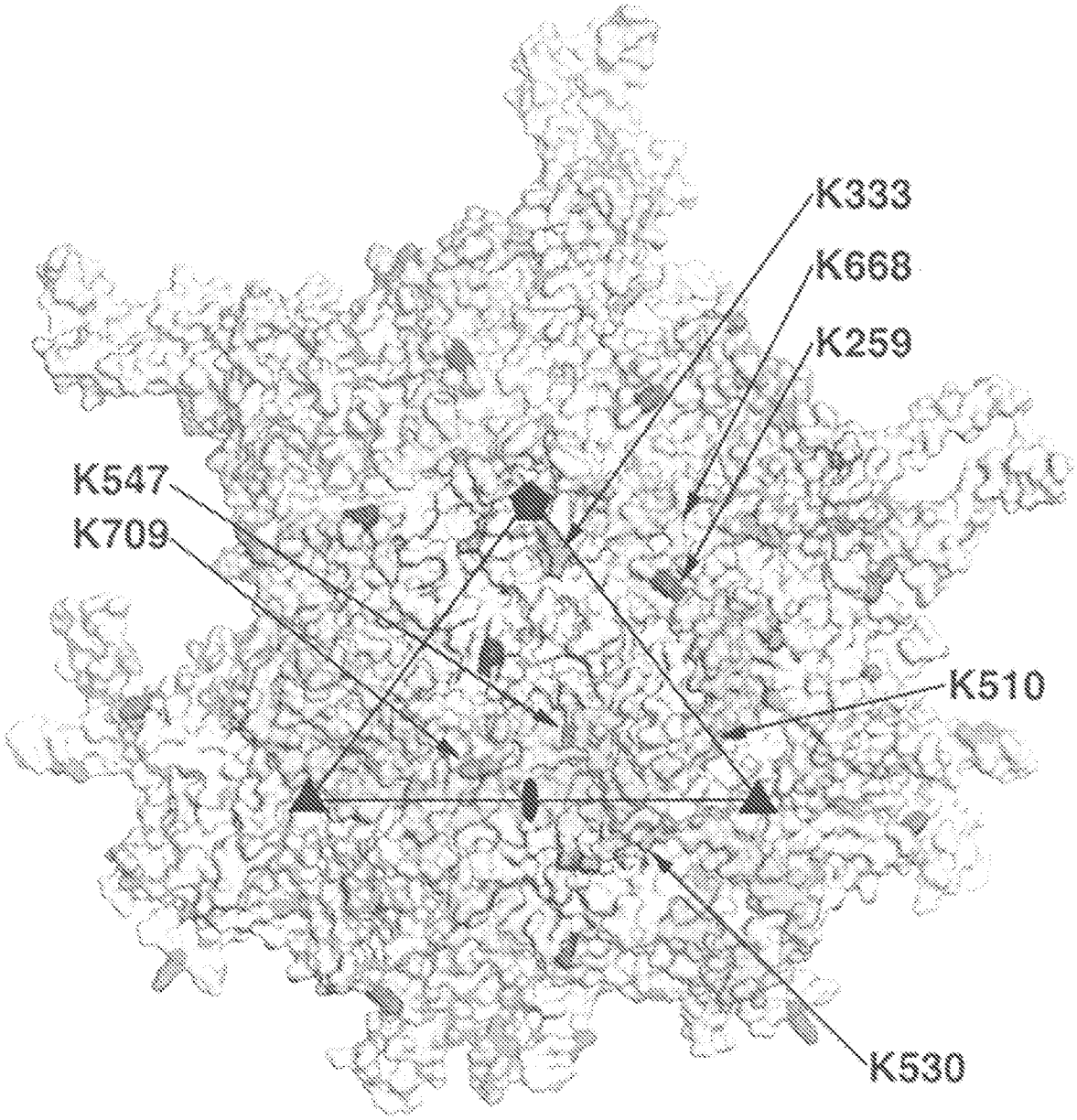

Figure 1C

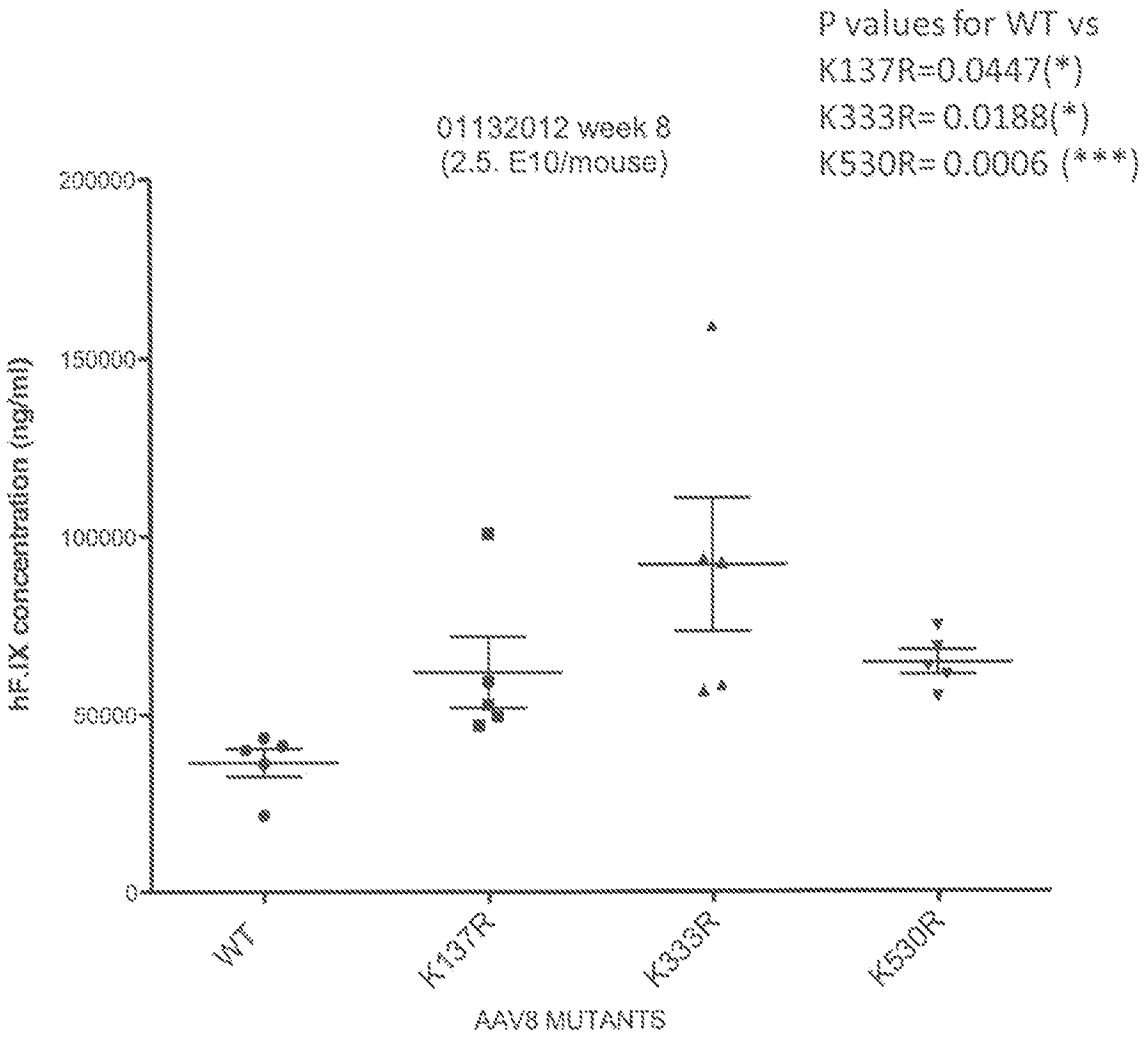
P values for WT vs
K137R=0.0447(*)
K333R= 0.0188(*)
K530R= 0.0006 (***)
01132012 week 8
(2.5. E10/mouse)
hF.IX concentration (ng/ml)
200000
150000
100000
50000
0
WT
K137R
K333R
K530R
AAV8 MUTANTS

Figure 2A

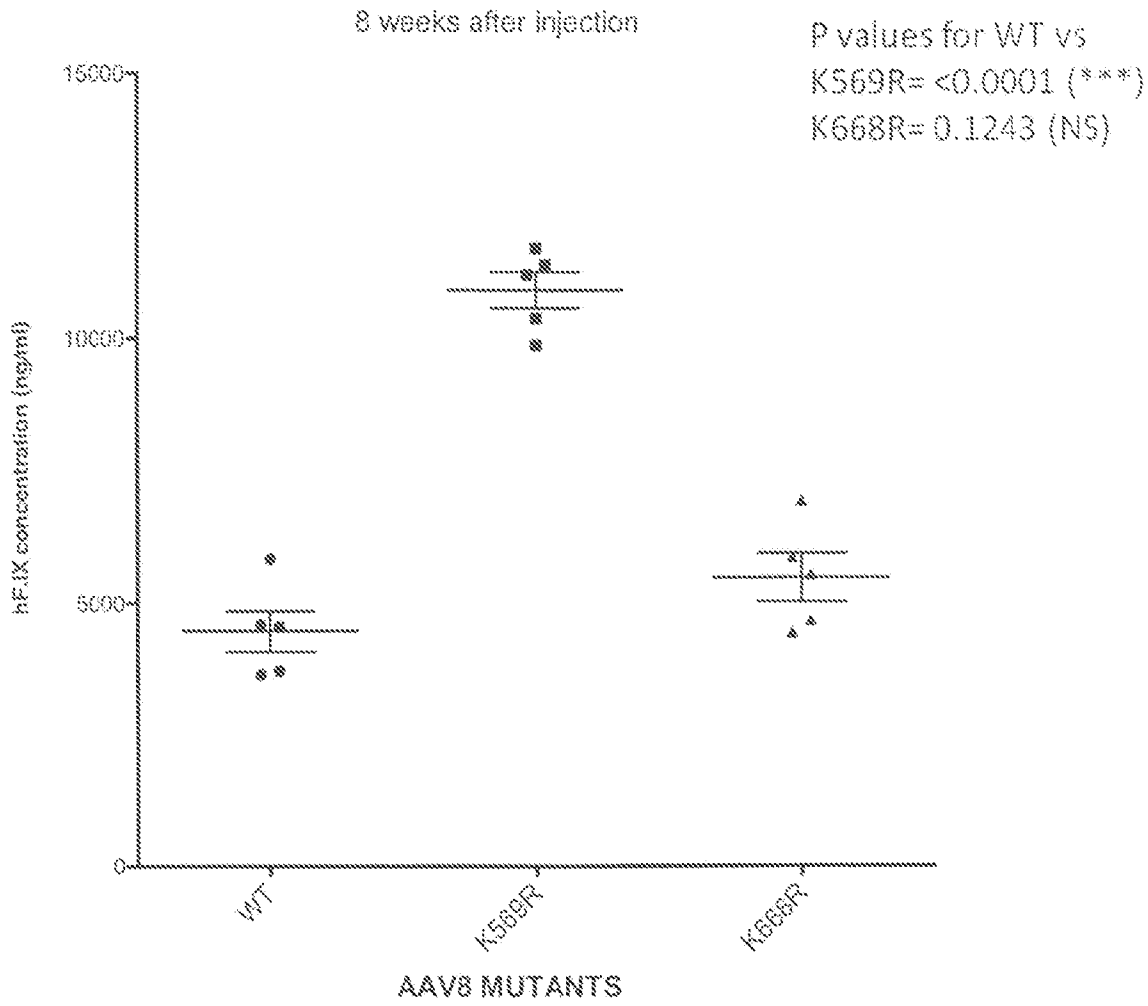
8 weeks after injection
P values for WT vs
K569R= <0.0001 (***)
K668R= 0.1243 (NS)
15000
10000
5000
0
hF.IX concentration (ng/ml)
WT
K568R
K668R
AAV8 MUTANTS

Figure 2B

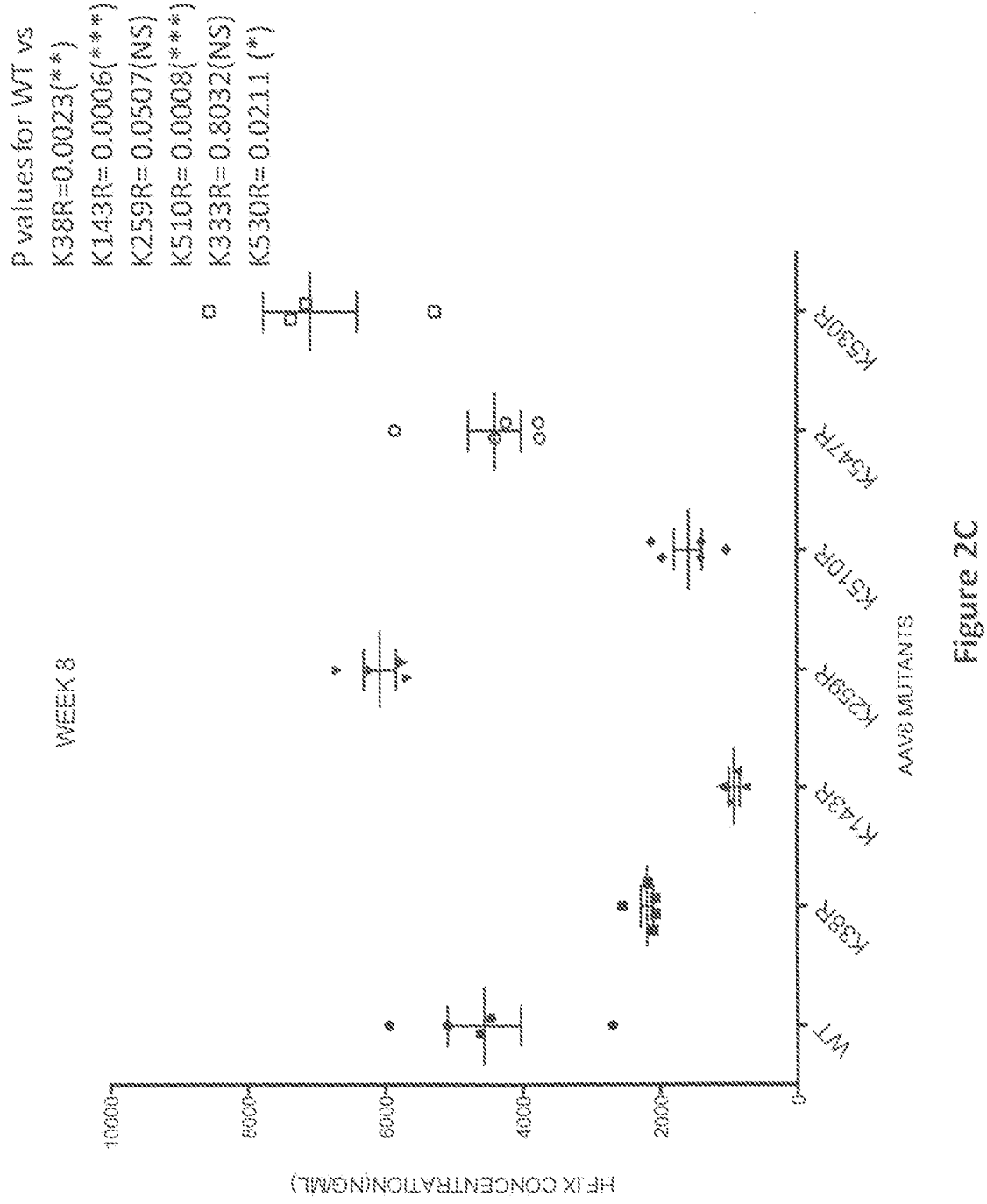
P values for WT vs
K38R=0.0023(**)
K143R= 0.0006(***)
K259R= 0.0507(NS)
K510R= 0.0008(***)
K333R= 0.8032(NS)
K530R= 0.0211 (*)
WEEK 8
HF IX CONCENTRATION(NG/ML)
10000
8000
6000
4000
2000
0
WT
K38R
K143R
K259R
K510R
K547R
K530R
AAV8 MUTANTS

Figure 2C

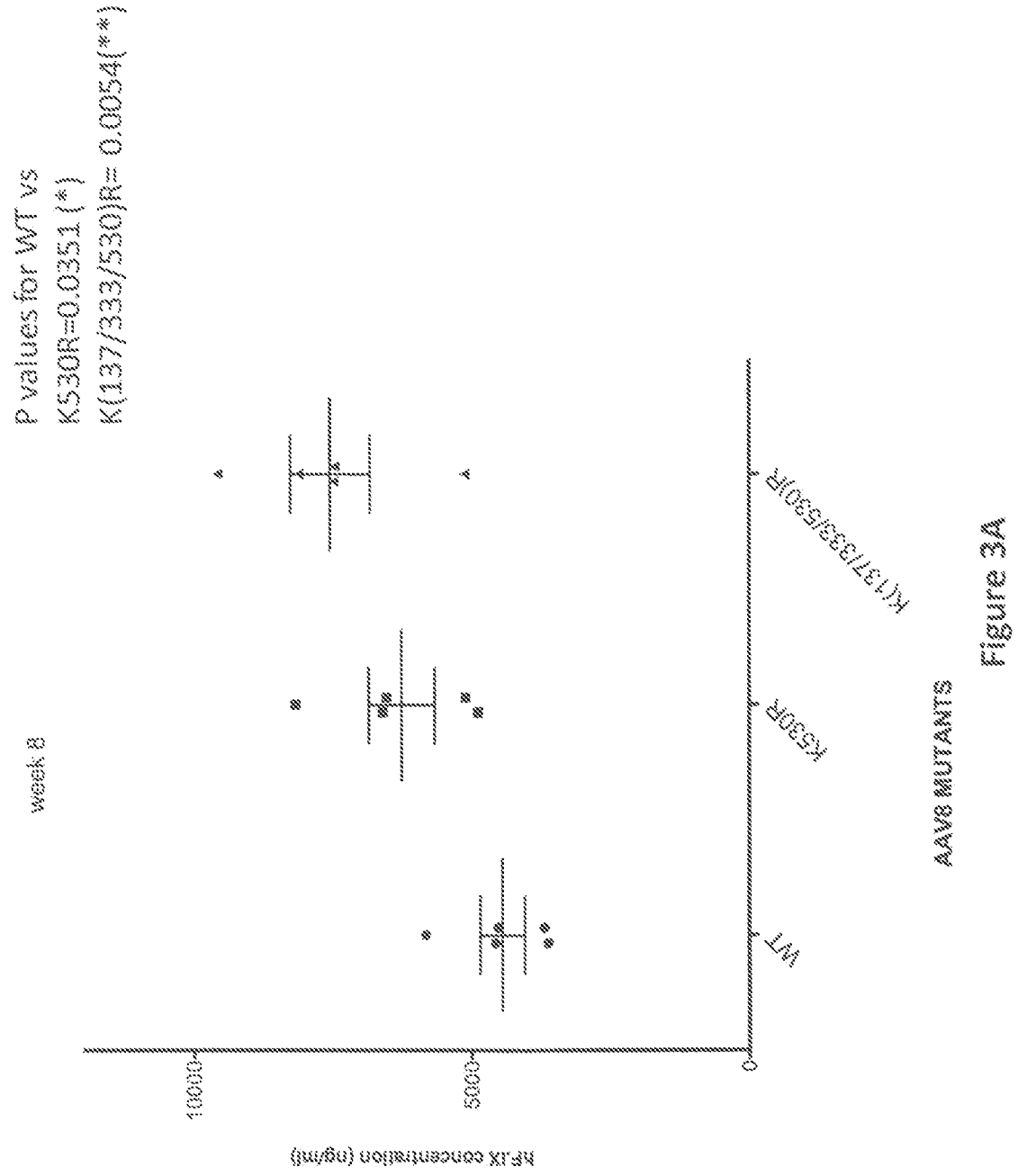
P values for WT vs
KS30R=0.0351(*)
K(137/333/530)R= 0.0054(**)
week 8
hFIX concentration (ng/ml)
10000
5000
0
WT
KS30R
K(137/333/530)R
AAV8 MUTANTS

Figure 3A

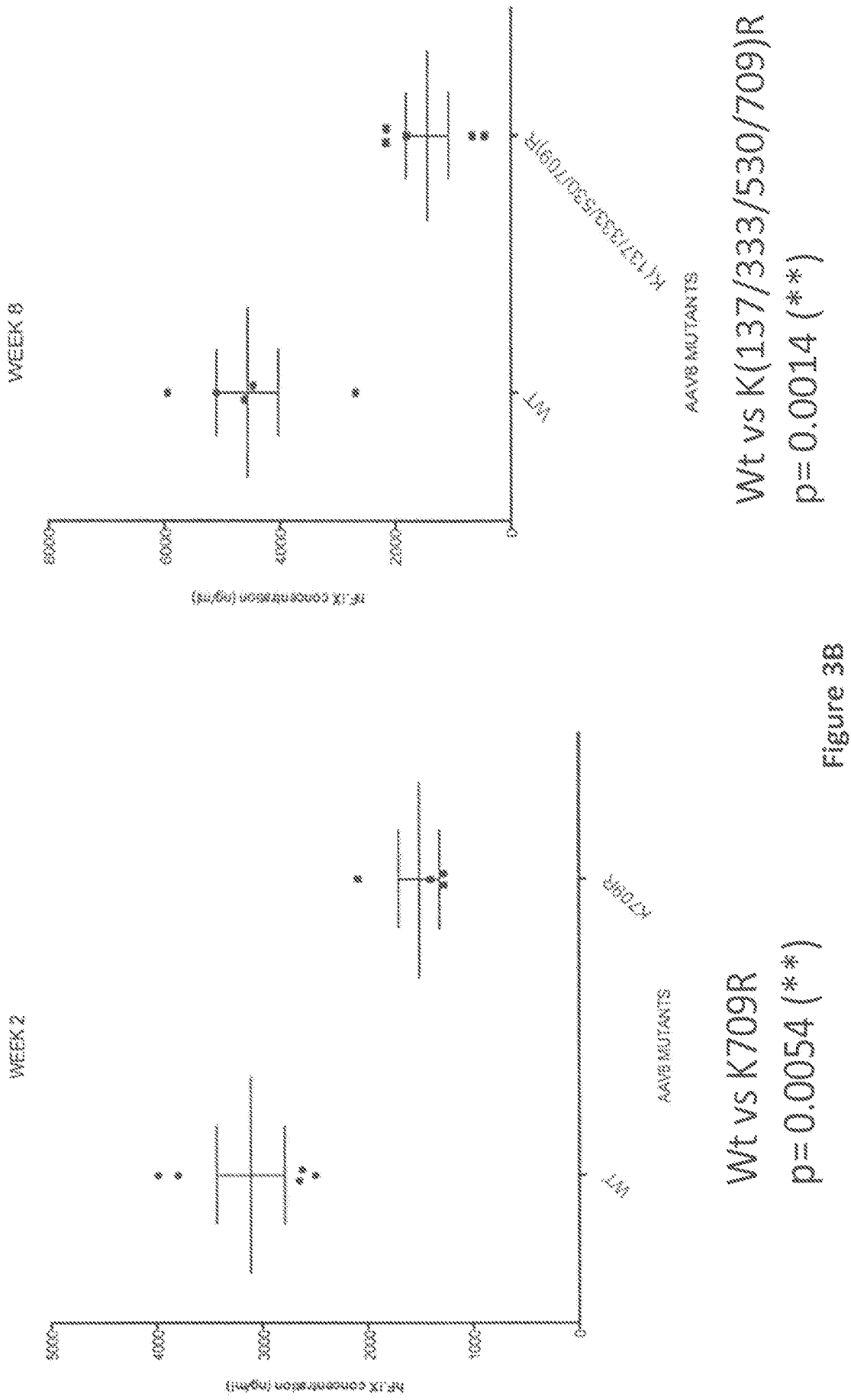
WEEK 8
WEEK 2
AAV8 MUTANTS
WT
K709R
K137/333/530/709R
Wt vs K709R
p=0.0054 (**)
Wt vs K(137/333/530/709)R
p=0.0014 (**)

Figure 3B

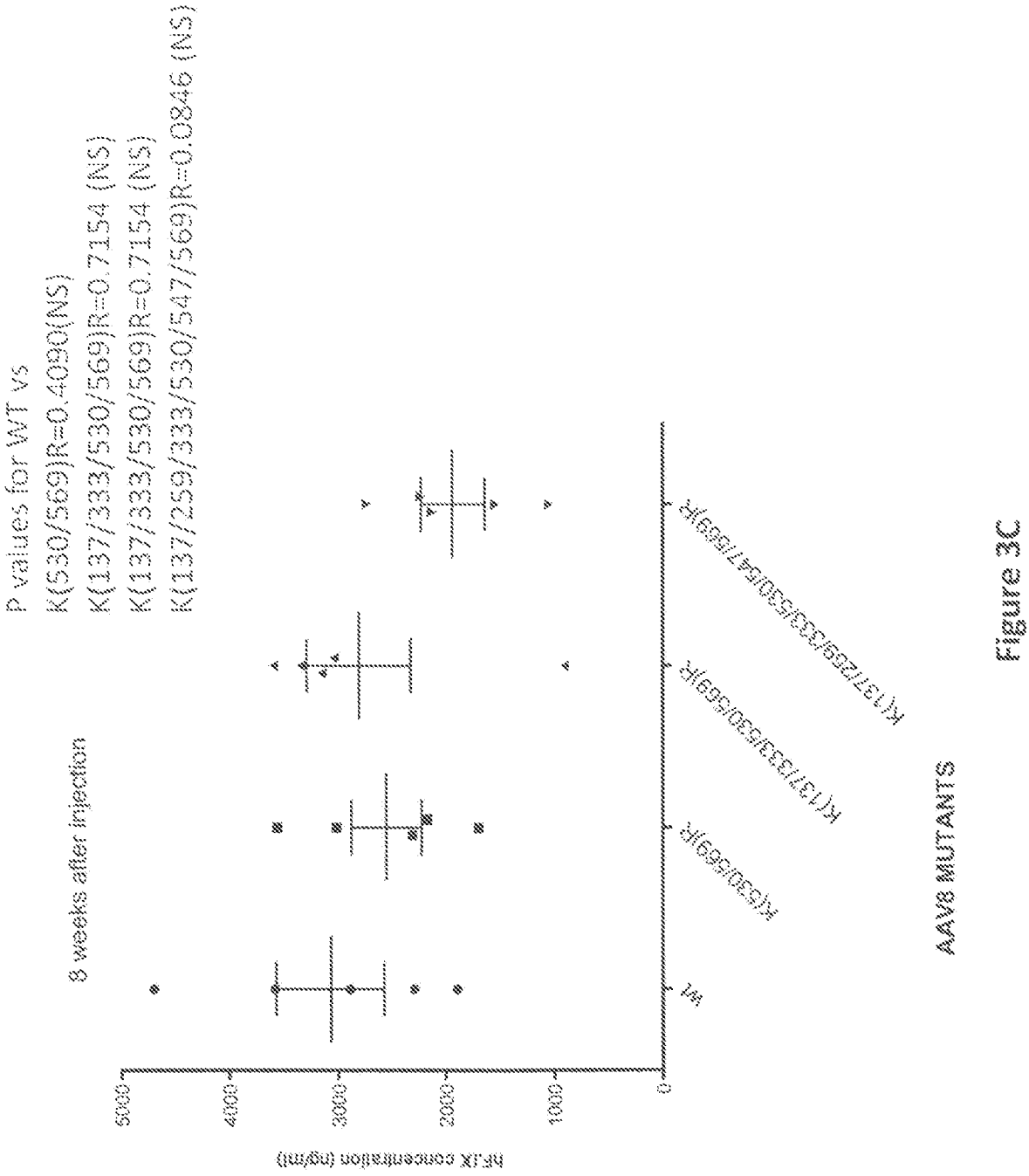
P values for WT vs
K(530/569)R=0.4090(NS)
K(137/333/530/569)R=0.7154 (NS)
K(137/333/530/569)R=0.7154 (NS)
K(137/259/333/530/547/569)R=0.0846 (NS)
8 weeks after injection
hF.IX concentration (ng/ml)
6000
4000
2000
1000
0
WT
K(530/569)R
K(137/333/530/569)R
K(137/259/333/530/547/569)R
AAV8 MUTANTS

Figure 3C

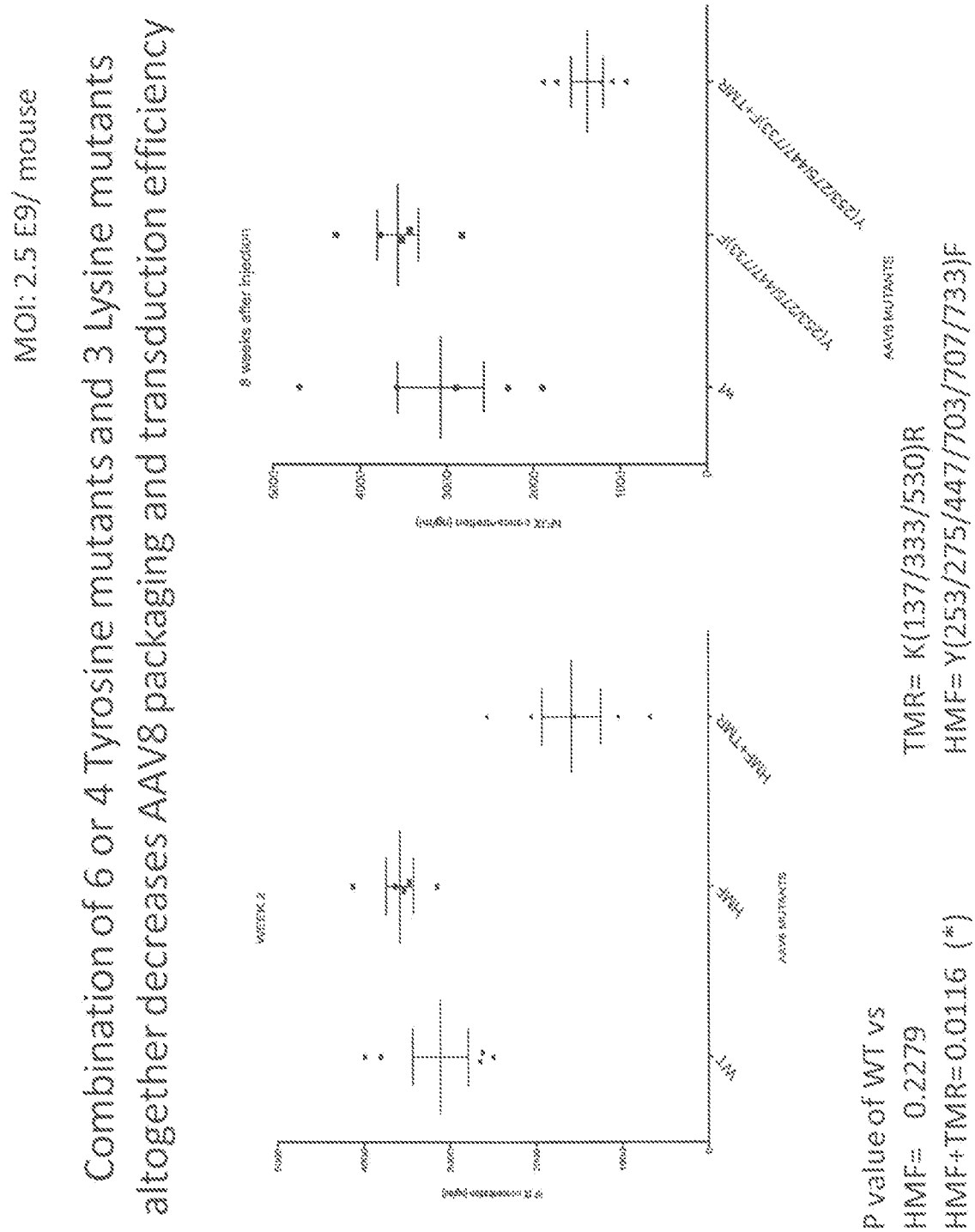
MOI: 2.5 E9/ mouse
Combination of 6 or 4 Tyrosine mutants and 3 Lysine mutants altogether decreases AAV8 packaging and transduction efficiency
8 weeks after injection
AAV8 MUTANTS
P value of WT vs
HMF= 0.2279
HMF+TMR=0.0116 (*)
TMR= K(137/333/530)R
HMF=Y(253/275/447/703/707/733)F

Figure 3D

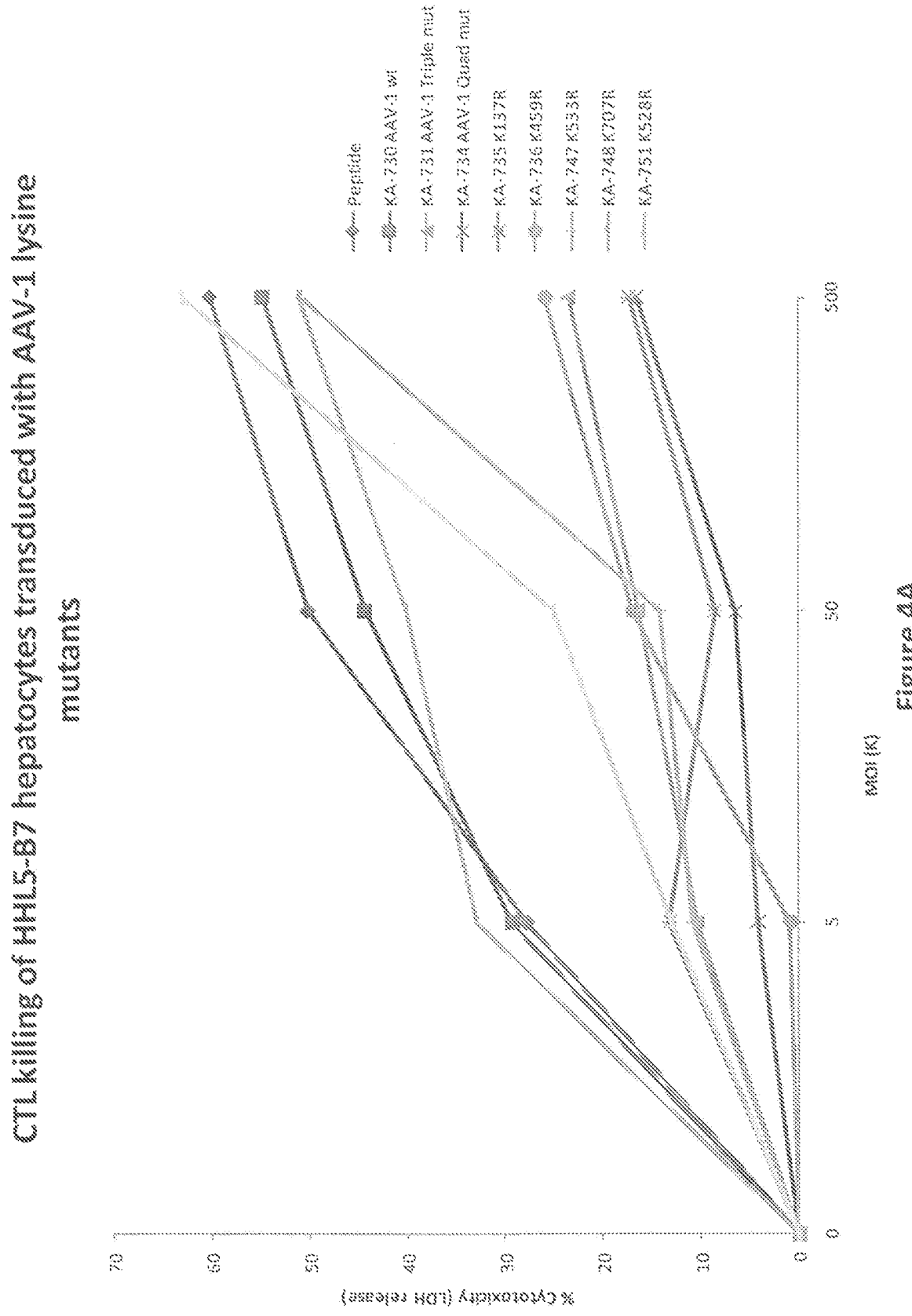
CTL killing of HHL5-87 hepatocytes transduced with AAV-1 lysine mutants
% Cytotoxicity (LDH release)
MOI (K)
Peptide
KA-730 AAV-1 wt
KA-731 AAV-1 Triple mut
KA-734 AAV-1 Quad mut
KA-735 K137R
KA-736 K459R
KA-747 K533R
KA-748 K707R
KA-751 K528R

Figure 4A

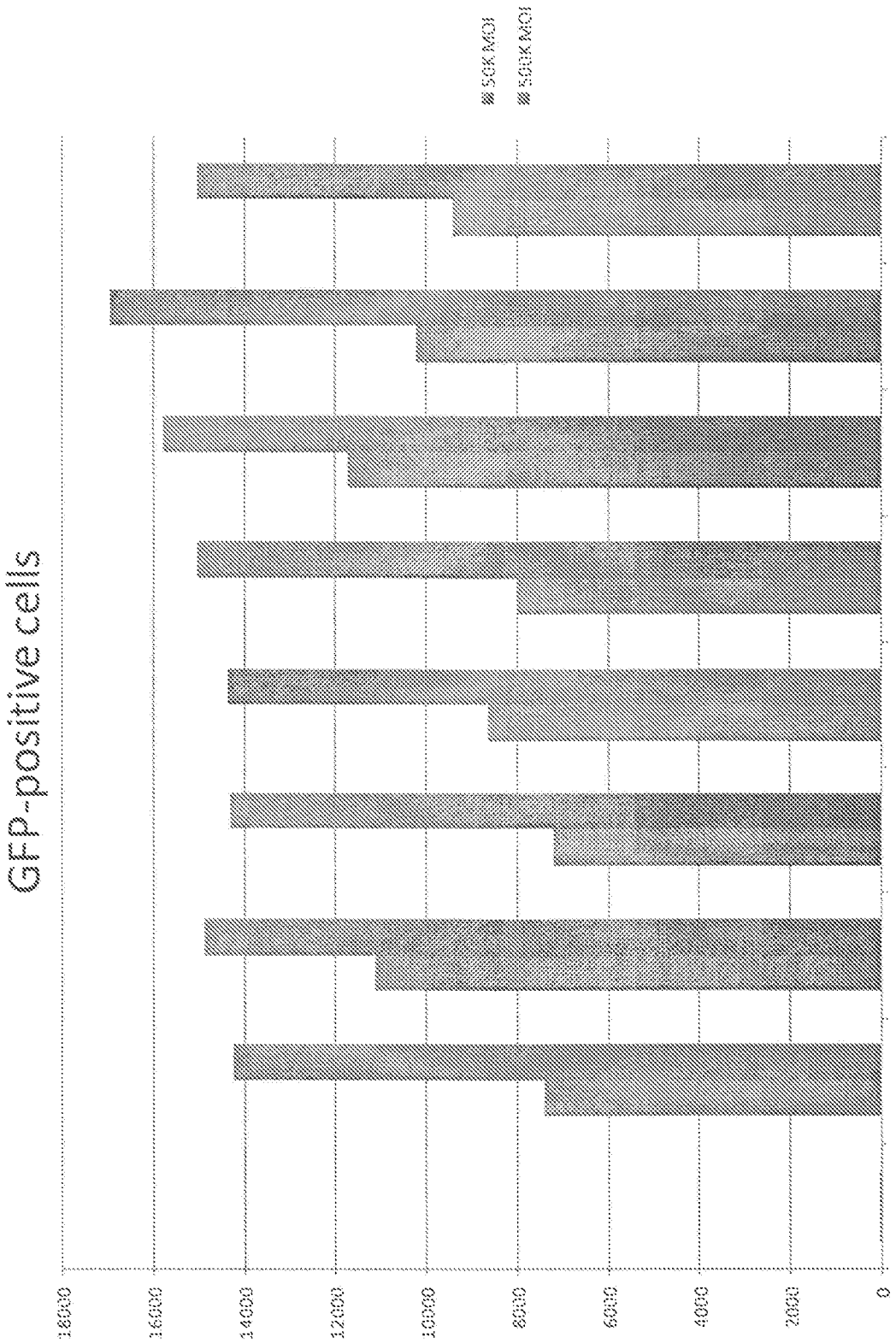
GFP-positive cells
16000
14000
12000
10000
8000
6000
4000
2000
0
None
wt
Triple mutant
Quadruple mutant
137R
459R
533R
707R
529R
5e3K MOI
5e4K MOI

Figure 4B

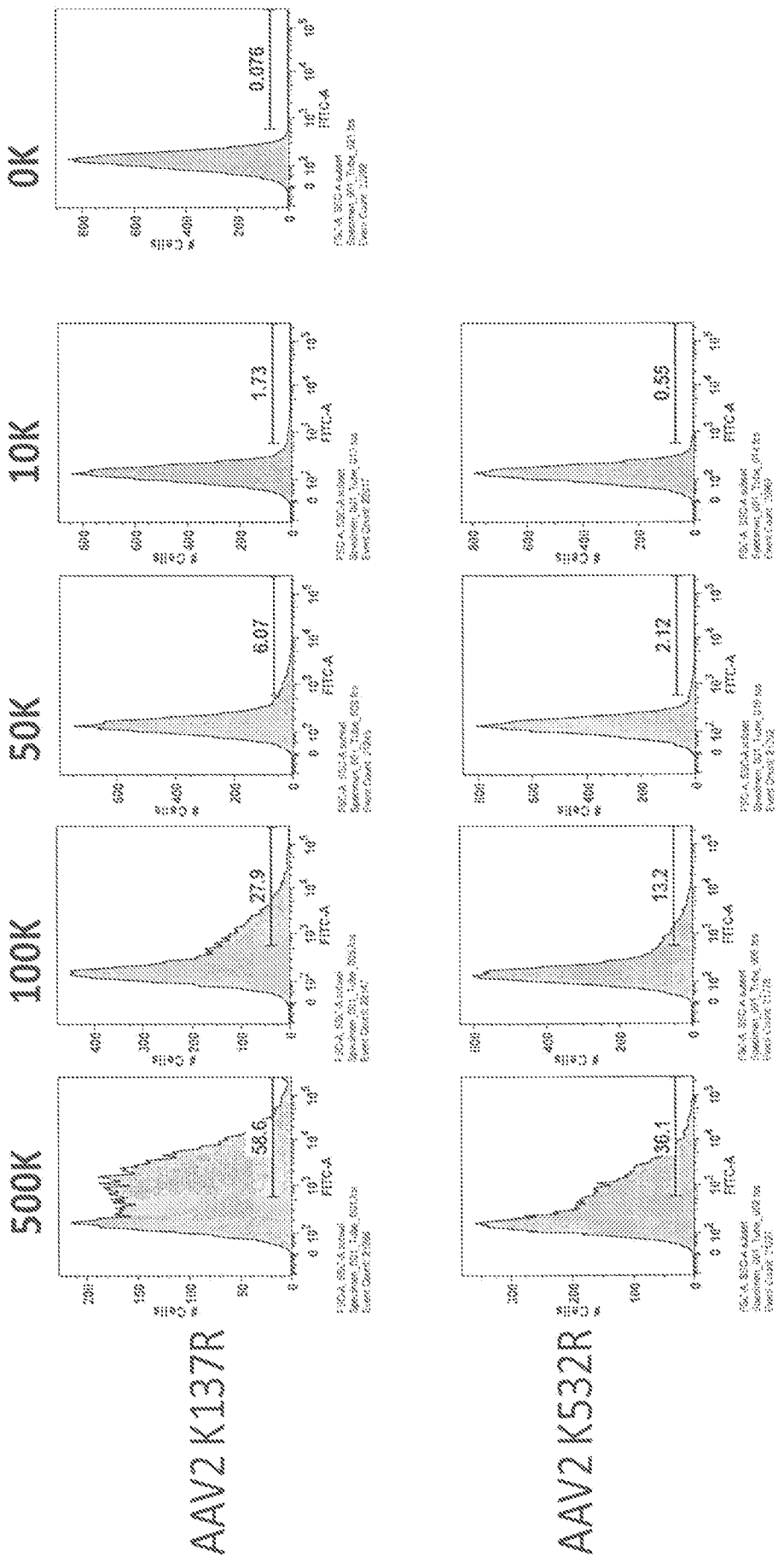
0K
10K
50K
100K
500K
AAV2 K137R
AAV2 K532R
0.076
1.73
6.07
27.9
58.6
0.55
2.12
13.2
36.1
FITC-A
Cells

Figure 5A

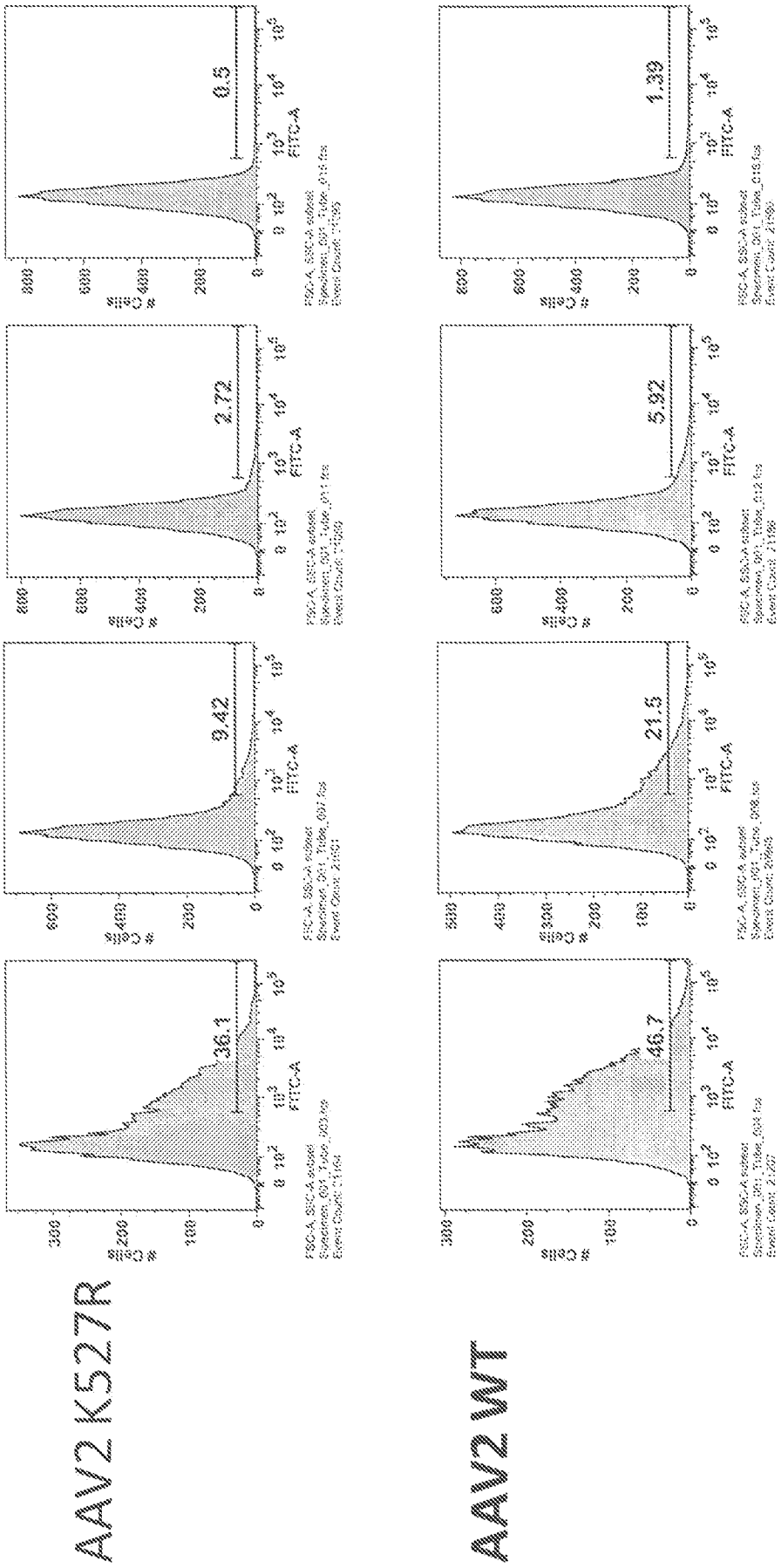

Figure 5A (cont'd)

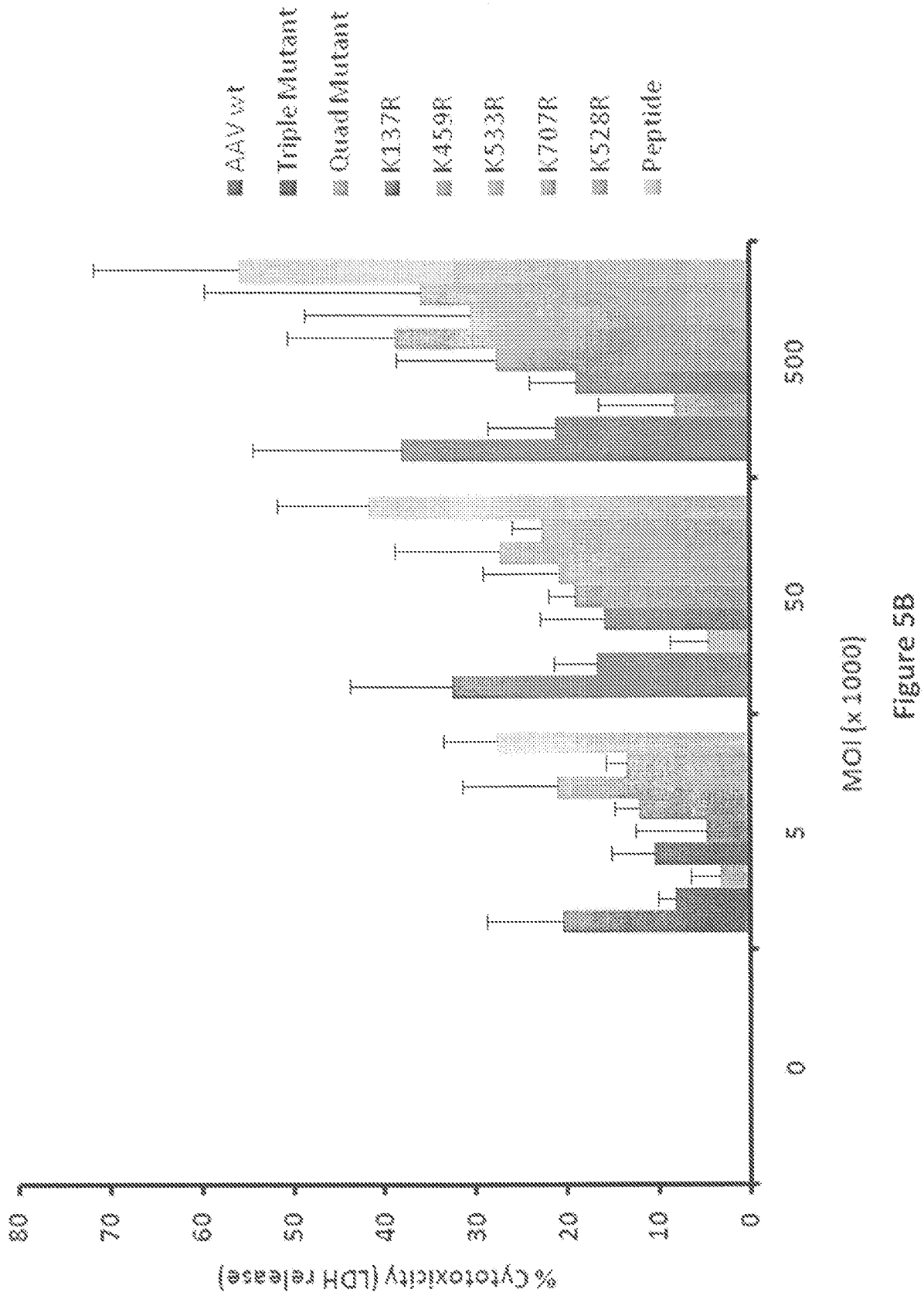
AAV wt
Triple Mutant
Quad Mutant
K137R
K459R
K533R
K707R
K528R
Peptide
% Cytotoxicity (LDH release)
0
10
20
30
40
50
60
70
80
0
5
50
500
MOI (x 1000)

Figure 5B

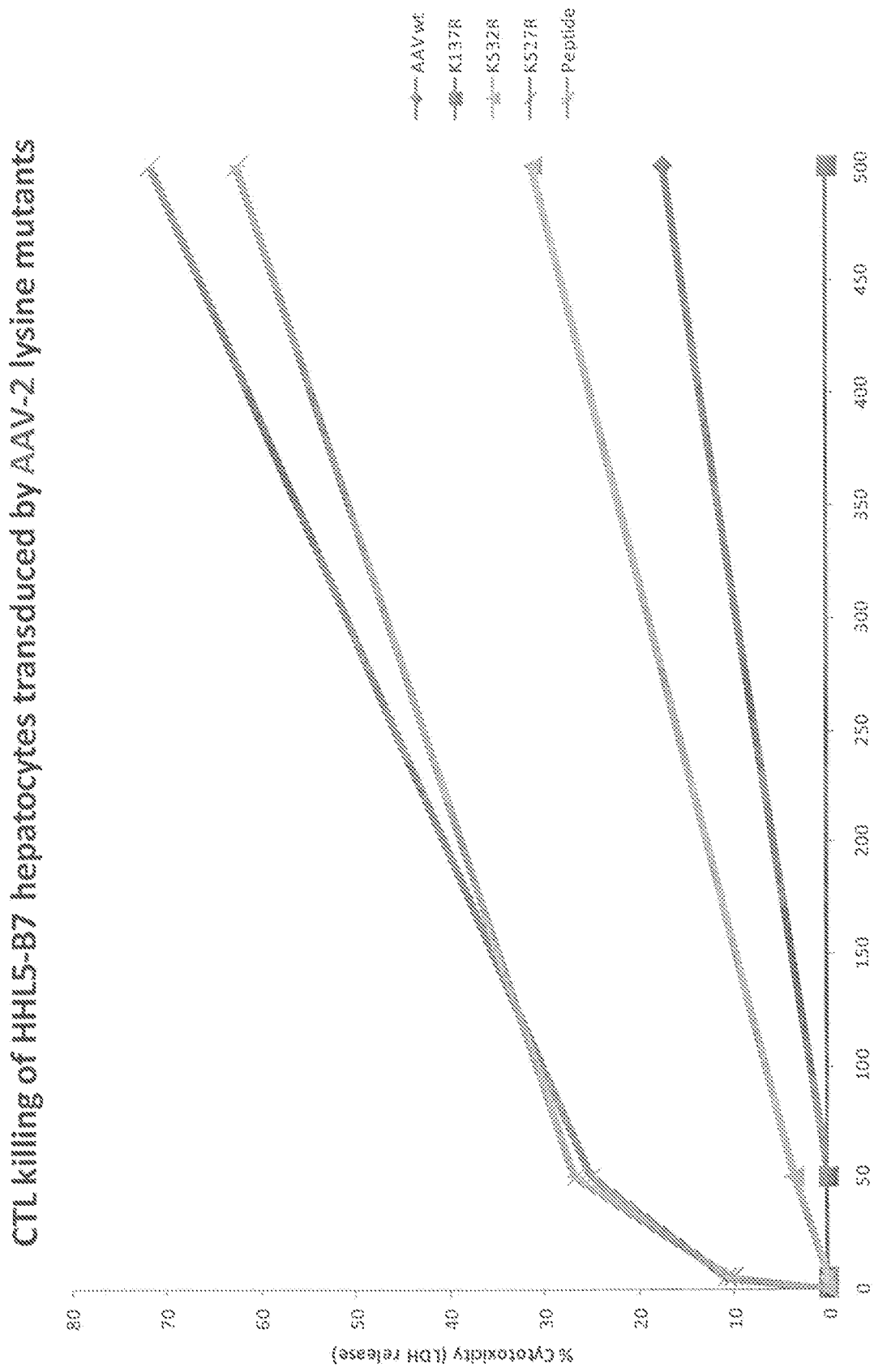
CTL killing of HHL5-B7 hepatocytes transduced by AAV-2 lysine mutants
AAVwt
K137R
K532R
K527R
Peptide
% Cytotoxicity (LDH release)
MOI (x 1000)
0
10
20
30
40
50
60
70
80
0
50
100
150
200
250
300
350
400
450
500

Figure 6

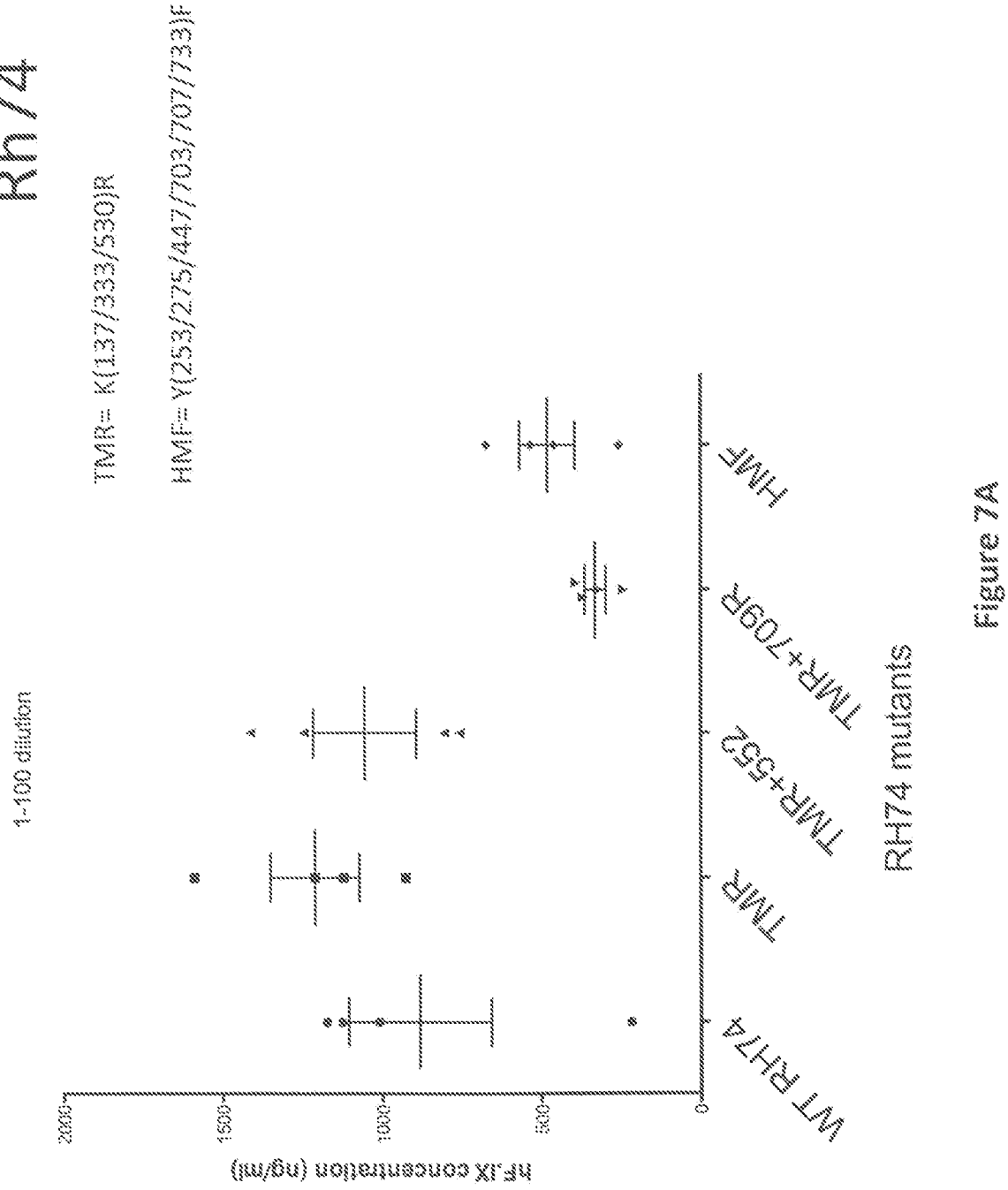
Rh74
TMR= K(137/333/530)R
HMF= Y(253/275/447/703/707/733)F
1-100 dilution
hF.IX concentration (ng/ml)
2000
1500
1000
500
0
WT RH74
TMR
TMR+652
TMR+709R
HMF
RH74 mutants

Figure 7A

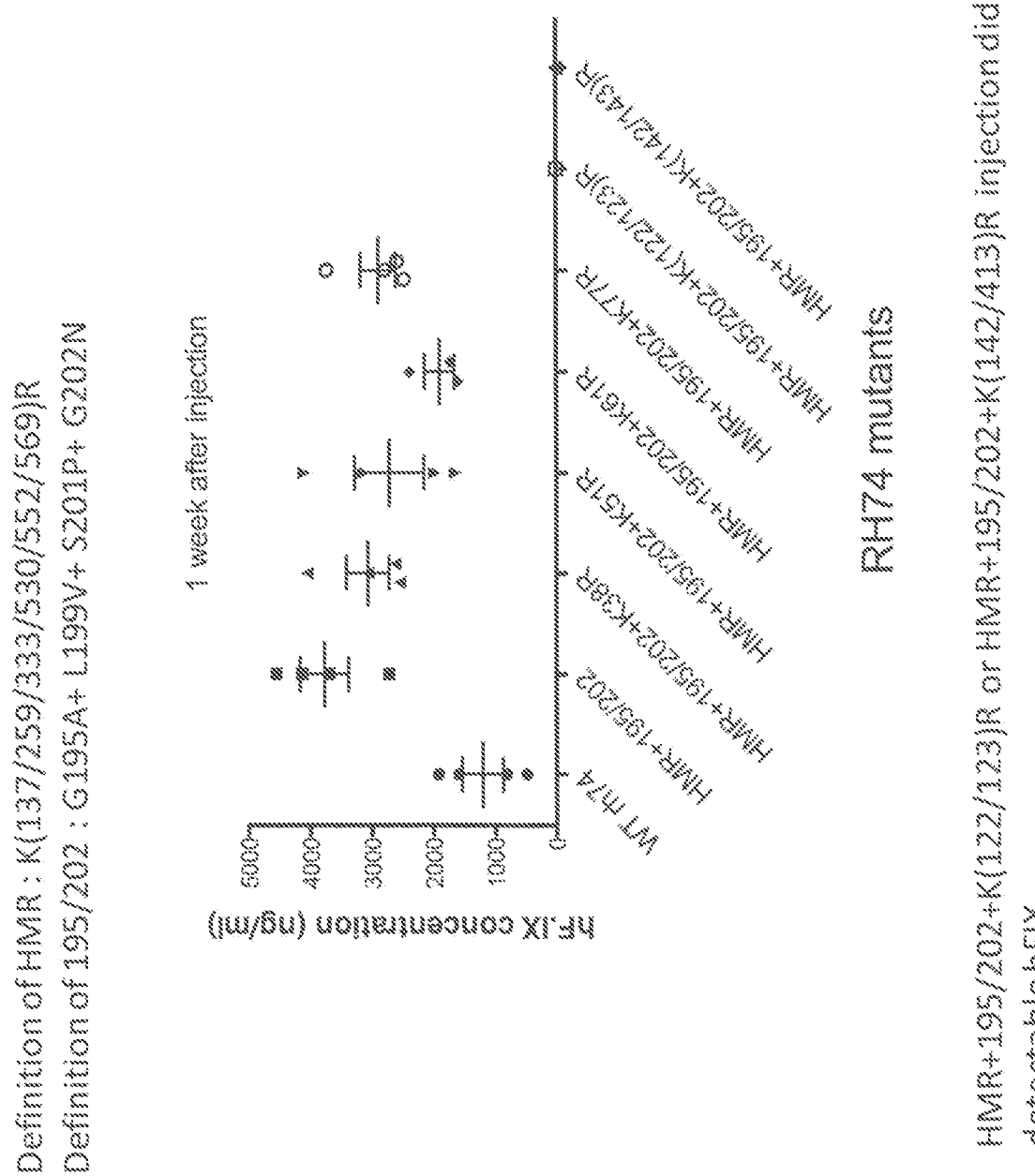
Rh74
Definition of HMR : K(137/259/333/530/552/569)R
Definition of 195/202 : G195A+ L199V+ S201P+ G202N
1 week after injection
hF.IX concentration (ng/ml)
5000
4000
3000
2000
1000
0
WT rh74
HMR+195/202
HMR+195/202+K62R
HMR+195/202+K63R
HMR+195/202+K67R
HMR+195/202+K(122/123)R
HMR+195/202+K(142/143)R
RH74 mutants
HMR+195/202+K(142/413)R injection did not produce any detectable hFIX

Figure 7B

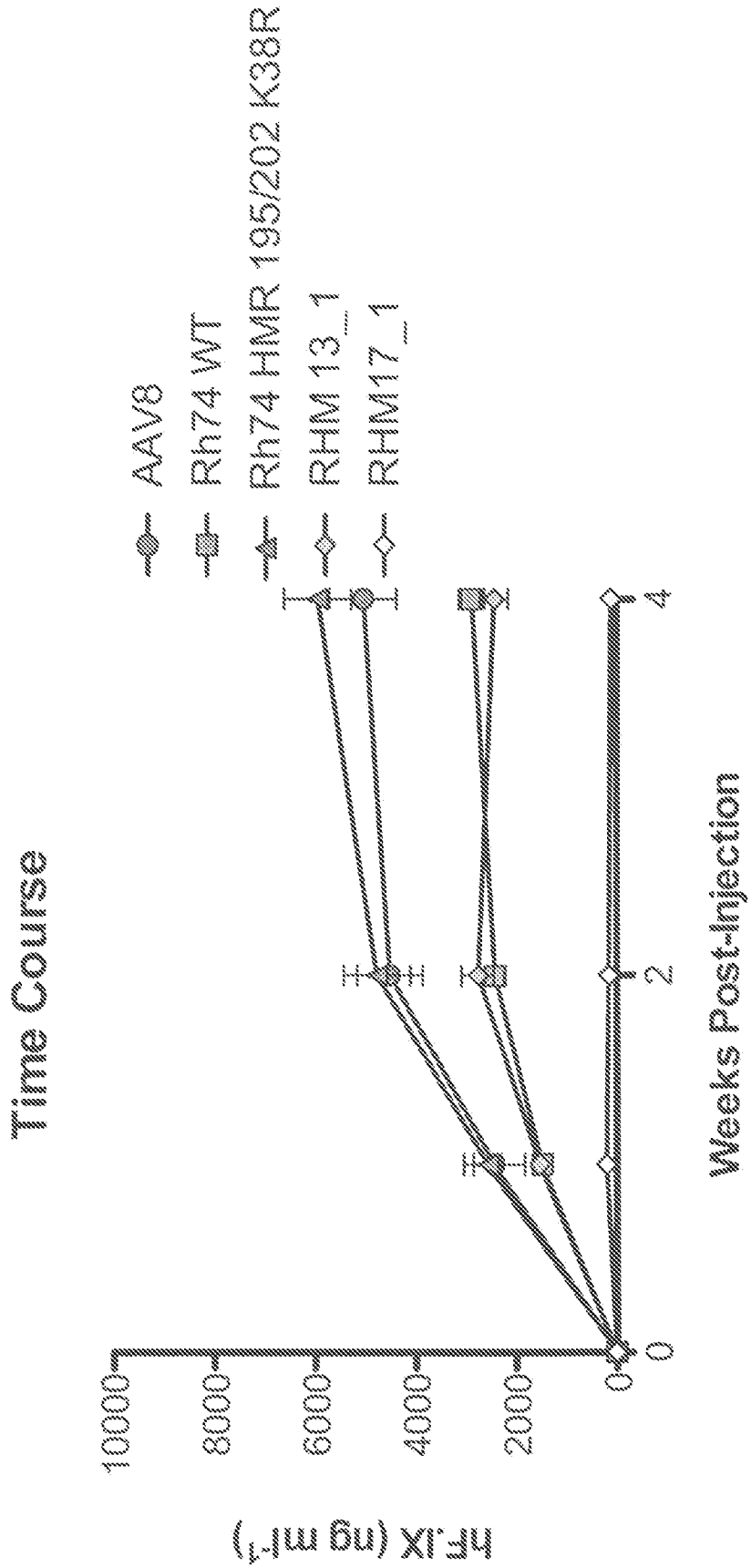
Time Course
AAV8
Rh74 WT
Rh74 HMR 195/202 K38R
RHM13_1
RHM17_1
hF.IX (ng ml-1)
10000
8000
6000
4000
2000
0
0
2
4
Weeks Post-Injection
RH74 HMR 195/202 K38R = K(137/259/333/530/552/569/38)R G195A-L199V-S201P-G202N
RHM13_1 = K(137/259/333/530/552/569/38/51/77)R G195A-L199V-S201P-G202N
RHM17_1 = K(137/259/333/530/552/569/38/51/77/169/547/668/163)R G195A-L199V-S201P-G202N

Figure 7C

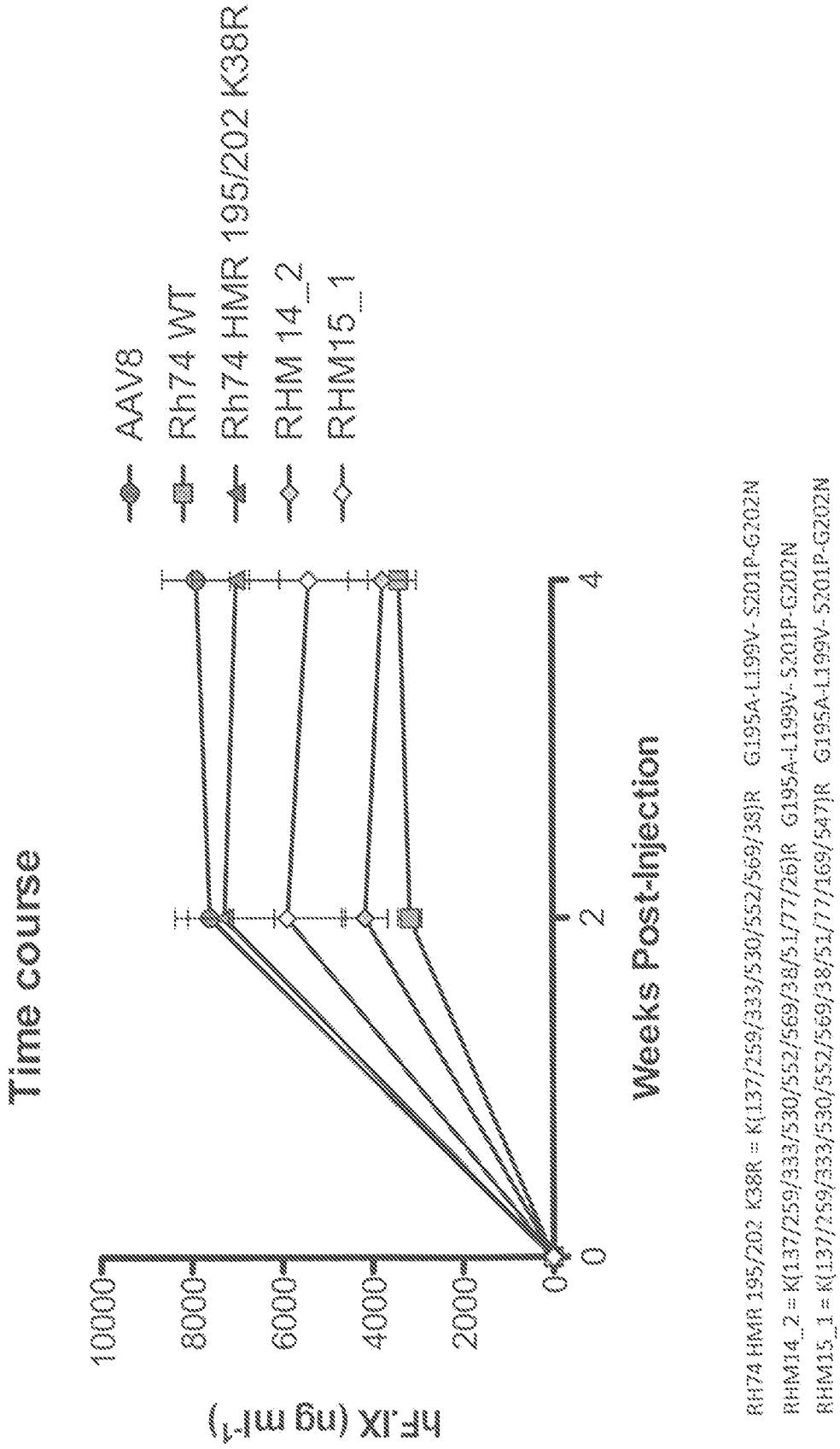
Time course
AAV8
Rh74 WT
Rh74 HMR 195/202 K38R
RHM14_2
RHM15_1
hFLIX (mg ml-1)
10000
8000
6000
4000
2000
0
0
2
4
Weeks Post-Injection
RH74 HMR 195/202 K38R = K(137/259/333/530/552/569/38)R G195A-L199V-S201P-G202N
RHM14_2 = K(137/259/333/530/552/569/38/51/77/26)R G195A-L199V-S201P-G202N
RHM15_1 = K(137/259/333/530/552/569/38/51/77/169/547)R G195A-L199V-S201P-G202N

Figure 7D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US13/37170

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/864, 7/01; C07K 14/015; A61K 48/00; C07H 21/04 (2013.01)
USPC - 435/320.1, 115; 424/93.2, 233.1, 199,1; 514/44R
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C12N 15/00, 15/864, 15/861, 15/86, 15/85, 15/79, 15/63; A61K 35/70, 35/713, 48/00 (2013.01)
USPC: 435/320.1, 948, 457, 456, 455, 440, 91.1, 173.3, 235.1, 115, 71.1, 69.1; 424/93.2, 93.21, 93.1, 93.6, 204.1, 205.1, 233.1, 192.1

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google; Google Scholar; DialogPRO; Pubmed; Google; Google Scholar; DialogPRO; PubMed; 'AAV,' 'rAAV,' adeno*, virue, lysine, ubiquitin, proteasom*, 'factor VIII,' 'factor IX,' substitut*, 'vp1,' vector, transduc, serotype

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2009/0197338 (VANDENBERGHE, L et al.), August 6, 2009; abstract; paragraphs [0005], [0006], [0008], [0013], [0026], [0066], [0076], [0169]	1-4, 5/1-5/4, 6/5/1-6/5/4, 7-9, 10/5/1-10/5/4, 11/10/5/1-11/10/5/4, 12/10/5/1-12/10/5/4, 13, 17-20 --- 14-16, 21-23
Y	WO 2011/126808 A1 (WILSON, JM et al.), October 13, 2011; page 2, lines 12-30; page 29, lines 12-26	14
Y	US 7629322 B2 (KLEINSCHMIDT, J et al.), December 8, 2009; column 1, lines 7-14; lines 16-30; column 5, lines 46-52	15
Y	US 6127525 A (CRYSTAL, RG et al.), November 28, 2011; column 3, lines 59-67 to column 4, lines 1-12	16, 21-23
A	SANLIOGLU, S et al. Rate Limiting Steps Of AAV Transduction And Implications For Human Gene Therapy. Current Gene Therapy. 2001, Vol. 1, No. 2, pp 137-147; page 140, column 1, paragraph 2 to page 141, column 1, paragraph 1.	1
A	CIECHANOVER, A. The Ubiquitin-Proteasome System: Death Of Proteins Is Required For Life Of Cells. Celltransmissions. September 2003, Vol. 19, No. 3; pp 3-10; <URL="http://www.sigmaaldrich.com/technical-service-home/publications/cell-transmissions.html">; page 3, column 1, paragraph 3 to column 2, paragraph 1.	1

☐ Further documents are listed in the continuation of Box C. ☐

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

Date of the actual completion of the international search	Date of mailing of the international search report
13 August 2013 (13.08.2013)	21 AUG 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)