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(54) Title: ADENO ASSOCIATED VIRUS PLASMIDS AND VECTORS

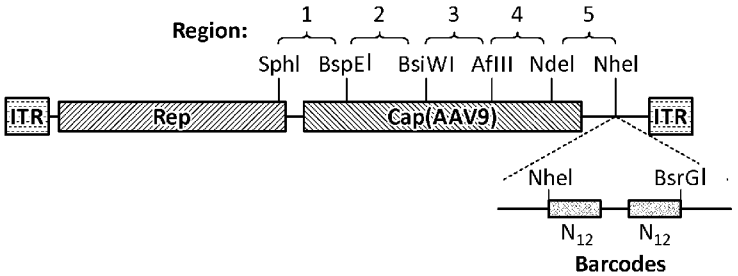

FIG. 1

(57) Abstract: Disclosed herein are adeno associated viral plasmids and viral vectors. Also disclosed are methods of using adeno associated viral vectors.

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ADENO ASSOCIATED VIRUS PLASMIDS AND VECTORS

FIELD

[0001] Generally, the disclosure relates to adeno associated viruses used in gene delivery. More specifically, the disclosure relates to adeno associated viruses used in gene delivery to particular target tissues.

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BACKGROUND

[0003] Recombinant adeno-associated viruses (rAAV) are promising vectors for in vivo gene delivery. A number of naturally occurring serotypes and subtypes have been isolated from human and non-human primate tissues (Gao G et al., J Virol 78, 6381-6388 (2004) and Gao G et al., Proc Natl Acad Sci USA 99, 11854-11859 (2002), both of which are incorporated by reference herein). Among the newly-identified adeno-associated virus (AAV) isolates, AAV serotype 8 (AAV8) and AAV serotype 9 (AAV9) have gained much attention because rAAV vectors derived from these two serotypes can transduce various organs including the liver, heart, skeletal muscles and central nervous system with high efficiency following systemic administration via the periphery (Foust KD et al., Nat Biotechnol 27, 59-65 (2009); Gao et al, 2004, *supra*; Ghosh A et al., Mol Ther 15, 750-755 (2007); Inagaki K et al, Mol Ther 14, 45-53 (2006); Nakai H et al., J Virol 79, 214-224 (2005); Pacak CA et al., Circ Res 99, e3-e9 (2006); Wang Z et al., Nat Biotechnol 23, 321-328 (2005); and Zhu T et al., Circulation 112, 2650-2659 (2005), all of which are incorporated by reference herein).

[0004] The robust transduction by rAAV8 and rAAV9 vectors has been presumed to be ascribed to strong tropism for these cell types, efficient cellular uptake of vectors, and/or rapid uncoating of virion shells in cells (Thomas CE et al., J Virol 78,

3110-3122 (2004), incorporated by reference herein). In addition, emergence of capsid-engineered rAAV with better performance has significantly broadened the utility of rAAV as a vector toolkit (Asokan A et al., Mol Ther, published in advance of press doi:10.1038/mt.2011.287 (2012), incorporated by reference herein). Proof-of-concept for rAAV-mediated gene therapy has been shown in many preclinical animal models of human diseases. Phase I/II clinical studies have been initiated or completed for genetic diseases including hemophilia B (Manno CS et al., Nat Med 12, 342-347 (2006) and Nathwani AC et al., N Engl J Med 365, 2357-2365 (2011), both of which are incorporated by reference herein); muscular dystrophy (Mendell JR et al., N Engl J Med 363, 1429-1437 (2011), incorporated by reference herein); cardiac failure (Jessup M et al., Circulation 124, 304-313 (2011), incorporated by reference herein); blinding retinopathy (Maguire AM et al., Lancet 374, 1597-1605 (2009), incorporated by reference herein); and α1 anti-trypsin deficiency (Flotte TR et al., Hum Gene Ther 22, 1239-1247 (2011), incorporated by reference herein), among others.

[0005] Although rAAV vectors have widely been used in preclinical animal studies and have been tested in clinical safety studies, the current rAAV-mediated gene delivery systems remain suboptimal for broader clinical applications. The sequence of an AAV viral capsid protein defines numerous features of a particular AAV vector. For example, the capsid protein affects capsid structure and assembly, interactions with AAV nonstructural proteins such as Rep and AAP proteins, interactions with host body fluids and extracellular matrix, clearance of the virus from the blood, vascular permeability, antigenicity, reactivity to neutralizing antibodies, tissue/organ/cell type tropism, efficiency of cell attachment and internalization, intracellular trafficking routes, virion uncoating rates, among others. Furthermore, the relationship between a given AAV capsid amino acid sequence and the characteristics of the rAAV vector are unpredictable. Therefore, new rAAV vectors with altered capsid proteins are needed in order to maximize the benefit of AAV based therapies.

SUMMARY

[0006] Disclosed herein are rAAV vectors comprising one or more mutations in the viral capsid. The AAVs comprising the mutations disclosed herein may have altered phenotypes relative to AAVs that do not comprise the mutations. Altered phenotypes that may result from viruses comprising the mutations include, but are not limited to tissue specific targeting and exclusion, enhanced viral transduction, enhanced viral yield, and lack of recognition by immune sera raised against unmutated viruses, etc.

[0007] Also disclosed herein are embodiments of a rAAV9 (AAV9) comprising at least one mutation of one or more of the following residues in the protein sequence of its viral capsid, (alone or in combination) I560, T561, N562, E563, E564, E565, I566, and K567. The rAAVs comprising one or more mutations in the viral capsid disclosed herein, including AAV9, may display efficient viral transduction in certain tissues including the kidney, spleen, and pancreas, and poor transduction in other tissues. Therefore, in certain embodiments, the disclosed rAAVs can, among other things, serve as a vector for gene delivery targeting, for example, the kidney, spleen, and pancreas.

BRIEF DESCRIPTION OF THE FIGURES

[0008] Figure 1 is a viral genome map of AAV9-SBBANN2-BC. Two N12 barcodes (virus barcodes or VBCs) are inserted downstream of the poly A signal (pA). Each VBC can be PCR-amplified with the left or right VBC-specific set of primers. Unique restriction enzyme sites have been introduced for efficient cloning of PCR products with defined mutations.

[0009] Figure 2 is a representation of the bridging PCR strategy to introduce defined mutations into the AAV9 capsid protein in AAV9-SBBANN2-BC.

[0010] Figure 3 is a representation of the AAV Barcode-Seq Procedure disclosed herein. VBC-1 and VBC-2 are individually amplified from a sample using primers that are adjacent to a sample specific barcode (SBC). 6VBC-1 and VBC-2 may be any of hundreds of different VBCs (e.g., VBC1-1, VBC2-1, VBC3-1 and so on as depicted in the figure). SBC-indexed VBC-1 and VBC-2 PCR products from all the samples are mixed together and subjected to Illumina sequencing to profile a total set of barcodes

in a sample. By profiling the total set of barcodes, the viral genomes of each mutant may be quantified in the sample.

[0011] Figure 4 is a scatter plot showing the relationship between the number of Illumina sequence reads and the concentration of DNA template in the AAV Barcode-Seq analysis. Digital sequence read numbers of 100 different DNA concentrations spread over a 4 log range were plotted against the plasmid DNA template concentrations. Normalization was done based on the observed small differences in PCR efficiencies between VBCs determined by a separate control experiment.

[0012] Figure 5 depicts a Monte Carlo simulation study to assess the statistical power of the AAV Barcode-Seq analysis using two plasmid DNA libraries. (Figure 5A and Figure 5B) The two libraries consisted of 118 pAAV9-SBBANN2-BC clones, each clone having a unique VBC1 and VBC2. The library in Figure 5A mimics an AAV library stock and the library in Figure 5B mimics a sample in which mutant clones are present in a range of concentrations. In Figure 5C, different numbers of reference controls in the library are randomly selected and compared to simulated mutants in duplicated experiments. The statistical comparison using a Mann Whitney U-test was done 500 times to determine the power of the analysis.

[0013] Figure 6 depicts a Monte Carlo simulation study to assess the statistical power of the AAV Barcode-Seq analysis of AAV-transduced tissues. A DNA-barcoded AAV library consisting of 100 identical AAV9 clones, each with a pair of unique DNA barcodes, was intravenously administered into mice (n=2), and the liver tissues were harvested 11 days post injection. Total DNA was extracted from the samples and subjected to the AAV Barcode-Seq analysis. The data were used in a Monte Carlo simulation study. Figure 6A shows the results when the experiment is performed in duplicate. Figure 6B shows the results when the experiment is performed in triplicate.

[0014] Figure 7 depicts HEK 293 cell transduction efficiencies of various serotypes and variants. HEK 293 cells were infected with a DNA-barcoded AAV library containing 12 different AAV serotypes and variants. Transduction efficiencies were determined by AAV Barcode-Seq 48 hours post infection. The values are normalized by the transduction efficiency of AAV9. The experiment was done in triplicate. Vertical bars are SEMs.

[0015] Figure 8 depicts blood vector concentration-time curves following intravenous injection of various AAV serotypes or variants in mice. In Figure 8A, recombinant adeno-associated virus serotype 1 (rAAV1), rAAV2, rAAV8 or rAAV9 vector expressing the lacZ gene were injected into mice via the tail vein in bolus at a dose of 1.0×10^{13} vg/kg (n=3-7 per group). Concentrations of rAAV particles in the blood were plotted as a function of time after injection. The data was obtained as described in (Kotchey NM et al., 2011, below) using 20 mice. All concentrations were normalized to that of AAV9. In Figure 8B, the barcoded AAV library ID 394 was administered and blood samples were collected in the same manner as described above and sequenced. The data were limited to those serotypes also assessed in Figure 8A. Figure 8C shows all the pharmacokinetic data obtainable in the experiment shown in Figure 8B. For Figures 8B and 8C, n=2 mice. Vertical bars represent standard errors of the mean.

[0016] Figure 9 is a set of bar graphs depicting the results of an analysis of the indicated serotypes for their reactivity to mouse anti-AAV9 neutralizing antibody as determined by AAV Barcode-Seq. Either naive or AAV9-preimmunized adult mice (n=2 each) were injected with the barcoded AAV library ID 394 via the tail vein, and blood samples were collected 1, 10, 30 and 60 min post injection. Then AAV genomes of each serotype in each sample were quantified by AAV Barcode-Seq. The blue and red lines show the blood vector concentrations relative to that of AAV9 (i.e., the relative concentrations) in naive mice and the anti-AAV9 antibody-harboring mice, respectively. Because AAV9 particles are quickly cleared from the blood circulation in AAV9 antibody-harboring mice, the relative blood concentrations of the AAV serotypes that are not neutralized with anti-AAV9 antibody exhibit a dramatic increase at 10 min and remain thereafter at a high level except for AAV3, which is cleared from the blood circulation very rapidly. In contrast, the AAV serotypes that are neutralized exhibit a pharmacokinetic property similar to that of AAV9. Green lines are ratios of the relative concentrations in the anti- AAV9 antibody-harboring mice to those in naive mice. Vertical bars represent standard errors of the mean.

DETAILED DESCRIPTION

[0017] The term "AAV vector" as used herein means any vector that comprises or derives from components of AAV and is suitable to infect mammalian cells, including human cells, of any of a number of tissue types, such as kidney, spleen, or pancreas, whether in vitro or in vivo. The term “AAV vector” may be used to refer to an AAV type viral particle (or virion) comprising at least a nucleic acid molecule encoding a protein of interest.

[0018] Additionally, the AAVs disclosed herein may be derived from various serotypes, including combinations of serotypes (e.g., "pseudotyped" AAV) or from various genomes (e.g., single-stranded or self-complementary). In particular embodiments, the AAV vectors disclosed herein may comprise desired proteins or protein variants. A "variant" as used herein, refers to an amino acid sequence that is altered by one or more amino acids. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, e.g., replacement of leucine with isoleucine. More rarely, a variant may have "nonconservative" changes, e.g., replacement of a glycine with a tryptophan. Analogous minor variations may also include amino acid deletions or insertions, or both. In certain embodiments, the AAV vectors disclosed herein may comprise a protein that differs by one or more amino acids from SEQ ID NO: 1.

[0019] Nucleotide sequences, such as polynucleotides, encoding the proteins of the present disclosure are provided herein. The nucleotides of the present dislcosure can be composed of either RNA or DNA. The disclosure also encompasses those polynucleotides that are complementary in sequence to the polynucleotides disclosed herein.

[0020] Because of the degeneracy of the genetic code, a variety of different polynucleotide sequences can encode the proteins of the present disclosure. In addition, it is well within the skill of a person trained in the art to create alternative polynucleotide sequences encoding the same, or essentially the same, proteins disclosed herein. These variant or alternative polynucleotide sequences are within the scope of the current disclosure. As used herein, references to “essentially the same” sequence refers to sequences which encode amino acid substitutions,

deletions, additions, or insertions which do not eliminate the detectability of the polypeptide encoded by the polynucleotides of the present disclosure.

[0021] The current disclosure also includes variants of the polynucleotides and polypeptides disclosed herein. Variant sequences include those sequences wherein one or more peptides or nucleotides of the sequence have been substituted, deleted, and/or inserted.

[0022] Polynucleotide and polypeptide sequences of the current disclosure can also be defined in terms of particular identity and/or similarity with certain polynucleotides and poypeptides described herein. The sequence identity will typically be greater than 60%, preferably greater than 75%, more preferably greater than 80%, even more preferably greater than 90%, and can be greater than 95%. The identity and/or similarity of a sequence can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence disclosed herein. Unless otherwise specified, as used herein percent sequence identity and/or similarity of two sequences can be determined using the algorithm of Karlin and Altschul (1990), modified as in Karlin and Altschul (1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al. (1990). BLAST searches can be performed with the NBLAST program, score=100, wordlength=12, to obtain sequences with the desired percent sequence identity. To obtain gapped alignments for comparison purposes, Gapped BLAST can be used as described in Altschul et al. (1997). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) can be used.

[0023] Methods of producing AAV vectors as disclosed herein are well known in the art, including methods, for example, using packaging cells, auxiliary viruses or plasmids, and/or baculovirus systems (see, e.g., Samulski et al., J. Virology 63, 3822 (1989); Xiao et al., J. Virology 72, 2224 (1998); Inoue et al., J. Virol. 72, 7024 (1998); WO1998/022607; and WO2005/072364).

[0024] Methods of producing pseudotyped AAV vectors are also known (see, e.g., WO00/28004), as well as various modifications or formulations of AAV vectors, to reduce their immunogenicity upon in vivo administration (see, e.g., WO01/23001; WO00/73316; WO04/112727; WO05/005610; and WO99/06562). In some

embodiments, AAV vectors may be prepared or derived from various serotypes of AAVs which may be mixed together or mixed with other types of viruses to produce chimeric (e.g., pseudotyped) AAV viruses.

[0025] In particular embodiments, the AAV vector may be a human serotype AAV vector. In such embodiments, a human AAV may be derived from any known serotype, e.g., from any one of serotypes 1-11, for instance from AAV1, AAV2, AAV4, AAV6, or AAV9. One specific, non-limiting example of such an AAV vector may include a vector comprising a nucleic acid molecule comprising an ITR and packaging sequence, operatively linked to a nucleic acid encoding an expression cassette for a protein of interest, and a nucleic acid encoding a protein of interest in an AAV9-derived capsid that differs from SEQ ID NO: 1 by one or more amino acids.

[0026] In certain embodiments, the AAV vectors disclosed herein may comprise embodiments of a rAAV9 (AAV9) comprising at least one mutation of one or more of the following residues in the protein sequence of its viral capsid, (alone or in combination) I560, T561, N562, E563, E564, E565, I566, and K567. For example, the AAV vectors disclosed herein may comprise a rAAV9 comprising at least one mutation selected from I560A, T561A, N562A, E563A, E564A, E565A, I566A, and K567A. In further embodiments, the AAV vectors disclosed herein may comprise rAAV9 comprising at least one double mutation selected from I560A/T561A, N562A/E563A, E564A/E565A, and I566A/K567A.

[0027] The rAAVs comprising one or more mutations in the viral capsid disclosed herein, including AAV9, may display efficient viral transduction in certain tissues including the kidney, spleen, and pancreas, and poor transduction in other tissues. Therefore, in certain embodiments, the disclosed rAAVs can, among other things, serve as a vector for gene delivery targeting, for example, the kidney, spleen, and pancreas.

[0028] The AAV vectors disclosed herein may include a nucleic acid encoding a protein of interest. In various embodiments, the nucleic acid also may include one or more regulatory sequences allowing expression and, in some embodiments, secretion of the protein of interest, such as e.g., a promoter, enhancer, polyadenylation signal, an internal ribosome entry site (IRES), a sequence encoding a protein transduction domain (PTD), and the like. Thus, in some embodiments, the nucleic acid may comprise a promoter region operably linked to the coding sequence

to cause or improve expression of the protein of interest in infected cells. Such a promoter may be ubiquitous, cell- or tissue-specific, strong, weak, regulated, chimeric, etc., for example to allow efficient and stable production of the protein in the infected tissue. The promoter may be homologous to the encoded protein, or heterologous, although generally promoters of use in the disclosed methods are functional in human cells. Examples of regulated promoters include, without limitation, Tet on/off element-containing promoters, rapamycin-inducible promoters, tamoxifen-inducible promoters, and metallothionein promoters. Other promoters that may be used include promoters that are tissue specific for tissues such as kidney, spleen, and pancreas. Examples of ubiquitous promoters include viral promoters, particularly the CMV promoter, the RSV promoter, the SV40 promoter, etc., and cellular promoters such as the PGK (phosphoglycerate kinase) promoter and the β-actin promoter.

[0029] In some embodiments of the AAV vectors disclosed herein, one or more feedback elements may be used to dampen over-expression of the protein of interest. For example, some embodiments of the AAV vectors may include one or more siRNA sequences that would target the exogenous transcript. In other embodiments, the AAV vector may include one or more additional promoters that may be recognized by inhibitory transcription factors. In various embodiments, the AAV vectors disclosed herein may comprise a construct that may create a homoeostatic feedback loop that may maintain expression levels of the protein of interest at a physiological level.

[0030] In various embodiments, the AAV vectors disclosed herein can comprise a nucleic acid that may include a leader sequence allowing secretion of the encoded protein. In some embodiments, fusion of the transgene of interest with a sequence encoding a secretion signal peptide (usually located at the N-terminal of secreted polypeptides) may allow the production of the therapeutic protein in a form that can be secreted from the transduced cell. Examples of such signal peptides include the albumin, the β-glucuronidase, the alkaline protease or the fibronectin secretory signal peptides.

[0031] As described herein, effective and long term expression of therapeutic proteins of interest in kidney, spleen, or pancreas may be achieved with non-invasive techniques, through peripheral administration of certain AAV vectors, such as an

AAV9 vector. Such peripheral administration may include any administration route that does not necessitate direct injection into kidney, spleen, or pancreas. More particularly, peripheral administration may include systemic injections, such as intramuscular, intravenous, intraperitoneal, intra-arterial, or subcutaneous injections. In some embodiments, peripheral administration also may include oral administration (see, for instance, WO96/40954), delivery using implants, (see, for instance, WO01/91803), or administration by instillation through the respiratory system, e.g., using sprays, aerosols or any other appropriate formulations.

[0032] In various embodiments, the desired doses of the AAV vectors may be easily adapted by the skilled artisan, e.g., depending on the disease condition, the subject, the treatment schedule, etc. In some embodiments, from 10^5 to 10^{12} viral genomes are administered per dose, for example, from 10^6 to 10^{11}, from 10^7 to 10^{11}, or from 10^8 to 10^{11}. In other embodiments, exemplary doses for achieving therapeutic effects may include virus titers of at least about 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} or 10^{11} viral genomes or more. Virus titer may also be expressed in terms of transducing units, which may be readily calculated by those of skill in the art.

[0033] In various embodiments, the AAV vectors disclosed herein may be administered in any suitable form, for instance, either as a liquid solution or suspension, as a solid form suitable for solution or suspension in liquid prior to injection, as a gel or as an emulsion. The vectors may be formulated with any appropriate and pharmaceutically acceptable excipient, carrier, adjuvant, diluent, etc. For instance, for injection, a suitable carrier or diluent may be an isotonic solution, a buffer, sterile and pyrogen-free water, or, for instance, a sterile and pyrogen-free phosphate-buffered saline solution. For inhalation, the carrier may be in particulate form.

[0034] The vectors may be administered in a "therapeutically-effective" amount, e.g., an amount that is sufficient to alleviate (e.g., decrease, reduce) at least one of the symptoms associated with a disease state, or to provide improvement in the condition of the subject. In some embodiments, repeated administrations may be performed, for instance using either the same or a different peripheral administration route and/or the same vector or a distinct vector such as a different mutant form of AAV9.

SEQUENCES

SEQ ID NO: 1 is the amino acid sequence of the unmutated AAV9 capsid.

SEQ ID NO: 2 is the nucleic acid sequence of the unmutated AAV9 capsid.

SEQ ID NO: 3 is the nucleic acid sequence of the pAAV9-SBBANN2-BC plasmid.

SEQ ID NO: 4 is the amino acid sequence of an I560/T561 double mutation.

SEQ ID NO: 5 is the amino acid sequence of an N562/E563 double mutation.

SEQ ID NO: 6 is the amino acid sequence of an E564/E565 double mutation.

SEQ ID NO: 7 is the amino acid sequence of an I566/K567 double mutation.

EXAMPLES

[0035] The following examples are illustrative of disclosed methods. In light of this disclosure, those of skill in the art will recognize that variations of these examples and other examples of the disclosed method would be possible without undue experimentation.

Example 1 – AAV Barcode-Seq

[0036] pAAV9-SBBANN2-BC is a wild-type AAV plasmid designed to facilitate high-throughput site directed mutagenesis of the AAV9 capsid. The parental plasmids used to synthesize pAAV9-SBBANN2-BC were the wild-type AAV2 plasmid pUC620 (Avigen Inc.) and the AAV9 helper plasmid p5E18-VD2/9 (Gao et al. 2004, *supra*).

[0037] The pAAV9-SBBANN2-BC plasmid was synthesized as follows:

[0038] The AAV2 capsid gene in pUC620 was replaced with the AAV9 cap gene from p5E18-VD2/9 (SEQ ID NO: 2). A Bgl II site was also introduced downstream of the capsid gene open reading frame (ORF). Additionally, a silent mutation was introduced in the AAV2 rep gene to create a new Sph I site. Also, silent mutations were introduced within the AAV9 cap gene to create new BspEI, Afl II and Nde I sites for convenient molecular cloning of AAV9 capsid mutants. Finally, a cassette comprising a set of two 12-nucleotide virus DNA barcodes (VBCs), three primer binding sites to efficiently PCR-amplify the VBCs, and a probe sequence that

facilitates quantitative PCR, was subcloned 3' of the polyadenylation signal of the AAV genome (Fig. 1).

[0039] A DNA-barcoded pAAV9-SBBANN2-BC based plasmid library was created by inserting double stranded oligonucleotides with random sequences between the NheI and BsrGI restriction sites in pAAV9-SBBANN2-BC. The libraries were then used as platforms to creating DNA-barcoded capsid mutant AAV plasmid libraries. A pAAV9-SBBANN2-BC based library could include as many as 7 X 10^6 individual plasmids.

[0040] DNA-barcoded pAAV9-SBBANN2-BC plasmid constructs with double alanine mutations at defined locations were synthesized. A bridging PCR technique illustrated in Figure 2 is used to introduce a double alanine mutation at a defined location in the AAV9 capsid protein (SEQ ID NO: 1). Referring now to Figure 2, left and right side DNA fragments are individually amplified by corresponding PCR primer sets and are treated with Dpn I. The amplified fragments are then used as a template for a bridging PCR reaction. The asterisk in the figure indicates a mutation to be introduced. The amplified fragments are then cut with restriction enzymes that cut at the sites indicated by RE1 and RE2. The amplified and restricted fragments are then inserted into the corresponding restriction enzyme sites of pAAV9-SBBANN2-BC. Mutagenesis is performed using a PCR primer comprising a GCTGCT sequence that is used to introduce two consecutive alanine mutations into the capsid protein.

[0041] Plasmid libraries were created by mixing on the order of 10 products of the bridged PCR reaction, adding them to a single plasmid cut at the corresponding restriction sites, and ligating them into the plasmid. Confirmation of incorporation of the plasmid insert and/or the presence of the desired mutation was performed by DNA sequencing and by restriction enzyme digestion followed by gel electrophoresis of DNA.

[0042] From the plasmids, DNA-barcoded AAV virus libraries were then produced. AAV production and purification is as described in Grimm D et al., Blood 102, 2412-2419 (2003), which is incorporated by reference herein, except that AAV helper plasmid is not used at the AAV production. This is due to the AAVs in the library carrying both the rep and cap genes. Therefore AAV helper plasmid is not required to produce virus from the library plasmid. Each library may contain

hundreds of different AAV capsid mutants, each of which has a unique set of DNA barcodes in its viral genome.

[0043] When an AAV library is produced using a plasmid DNA pool that contains multiple different mutant plasmids, genotype-phenotype dissociation or capsid mosaicism may occur where a viral genome may not necessarily code the capsid protein of the virion (Muller OJ et al., Nat Biotechnol 21, 1040-1046 (2003), incorporated by reference herein). To preclude this possibility, each AAV clone of the controls and mutants are produced individually and then mixed after the completion of the virus production. Then the viral clone pool is purified according to the procedure described in Grimm, et al., 2003, *supra*.

[0044] Figure 3 outlines the AAV Barcode-Seq analysis. AAV viral genomes were extracted from samples of interest. The AAV viral genomes in this case were those derived from various AAV clones present in a library. The viral genome of each AAV clone has a clone-specific set of two viral DNA barcodes (shown as VBCx-1 and VBCx-2 in Figure 3, wherein x represents the clone number). All the VBCx-1s and VBCx-2s present in a sample can be PCR amplified with VBC-1- and VBC-2-specific primer sets, respectively. Each primer is tagged with sample-specific barcode (SBC) for multi-sample indexing for Illumina sequencing. The SBCs used were 5-nucleotide-long SBCs previously reported by Craig et al. (Craig DW et al., Nat Methods 5, 887-893 (2008), incorporated by reference herein). However, longer or shorter barcodes may be used. The maximum number of SBC-indexed PCR products used per lane in Illumina sequencing was 96 (48 SBCs x 2 VBCs). Once all the VBC-1 and VBC-2 PCR products from all the samples of interest were obtained, they were mixed together, and subjected to Illumina sequencing. Although a low sequence diversity of PCR products in reference image construction (Bentley DR et al., Nature 456, 53-59 (2008), incorporated by reference herein) may occur in while using Illumina sequencing of PCR products, the use of frame shifting primers, overcame this potential problem.

[0045] Illumina sequencing was performed using an Illumina GAIIx or HiSeq2000 generating approximately 10-100 million reads per lane. Sequencing reads were sorted according to SBCs and VBCs by an algorithm implemented in Perl.

Example 2 – Using AAV Barcode-Seq to quantify AAV genomes in a sample

[0046] AAV Barcode-Seq faces particular challenges in that a viral genome may account for only less than 0.0001% of DNA molecules in a sample. As a result, the viral genomes present in a sample are PCR-amplified prior to sequencing.

[0047] To address how accurately AAV Barcode-Seq could quantify viral genomes in a sample, three plasmid DNA pools were created. Each pool consisted of 100 pAAV9-SBBANN2-BC clones, and each clone had a unique VBC1 and VBC2. Pool 1 contained pAAV9-SBBANN2-BC clones at the same concentration, while pools 2 and 3 were a mixture of pAAV9-SBBANN2-BC clones at various but known concentrations that differed by a maximum of 10000 fold. In Pool 2, Clone No. 1 and Clone No. 100 were set at the lowest and highest concentrations, respectively, while in Pool 3, the concentrations of clone no. 1 and no. 100 were set as the highest and lowest concentrations, respectively. Using the three pools as PCR templates, VBC1 and VBC2 were amplified independently by 35 cycles of PCR with SBC-tagged primers. The PCR reactions were performed in quadruplicate and the resulting PCR products were sequenced.

[0048] Sequence read numbers were obtained in a range from 400K-3M per one PCR product (that is, one SBC-indexed VBC1 or VBC2 PCR product). When using Pool 1, the averages of the coefficients of variation (a normalized measure of dispersion of the distribution) of the 100 VBC sequence read numbers in the quadruplicated PCR products were 0.63 and 0.57 for VBC1 and VBC2, respectively, while the averages of the coefficients of variation of the globally normalized sequence read numbers of the same VBC in the quadruplicated PCR products were 0.13 and 0.19 for VBC1 and VBC2. The relatively high coefficients of variation between VBCs indicate that PCR amplification efficiencies could vary to some degree depending on the sequence of VBCs. The small degree of dispersion among the normalized sequence read numbers for the same VBC in the four independent PCR reactions indicates high reproducibility. Pools 2 and 3 showed that the sequence read numbers are linearly correlated with the concentrations of each barcode in at least 3 log range with the Pearson's coefficients of 0.93 and 0.96 for VBC1 and VBC2, respectively, when the read numbers are globally normalized and

corrected with relative PCR amplification efficiencies determined by the experiment using Pool 1 (see, Figure 4).

[0049] In AAV Barcode-Seq, DNA-barcoded AAV libraries may contain reference AAV clones with wild type AAV9 capsid as well as the capsid from heparin binding mutant AAV2R585E in addition to mutant clones. Additionally, multiple clones per reference and/or multiple clones per mutant may be included in the libraries. Each of these multiple clones may differ in their individual VBC's such that two reference clones that are otherwise identical may have different VBC's.

[0050] To investigate the sensitivity and power of the analysis, two plasmid DNA pools, each containing 118 pAAV9-SBBANN2-BC clones were generated. Each clone within the pool had a unique set of VBC1 and VBC2 (see, Figure 5). One pool mimics viral genomes recovered from an AAV library stock in which reference controls and simulated mutants are mixed at an equimolar ratio (see, Figure 5A). The second pool mimics viral genomes recovered from an experimental sample in which the mutant clones are at varying concentrations - for example, given two genomes having a representation in the pool at 1x, the remaining clones can be represented at 1/16x, 1/8x, 1/4x, 1/2x, 2x, 4x, 8x or 16x, with two genomes per concentration (see, Figure 5B). The pools were analyzed using AAV-Barcode Seq. Monte Carlo simulation combined with Mann Whitney U-test indicated high sensitivity and power for the AAV-Barcode Seq (see, Figure 5C).

Example 3 -- Using AAV Barcode-Seq to examine distribution of AAV libraries in vivo

[0051] AAV library 394 was injected in two mice, and the liver tissues were harvested 11 days post-injection. VBCs were PCR-amplified by PCR from total DNA extracted from the liver tissue and sequenced. The AAV library used (Library ID 394) consisted of a mixture of 100 AAV9 virus preparations that are genetically identical but were synthesized in separate culture dishes. Each of the 100 AAV9 clones can be individually identified because each has a clone-specific set of DNA barcodes incorporated in its viral genome. The composition of AAV9 is summarized in Table 1 below. Liver transduction efficiencies of each clone exhibited a coefficient of variation of 0.25 in one mouse and 0.42 in the other. When analyzed by a Monte Carlo simulation approach, a power of nearly 0.8 in detecting 2-fold differences (2

fold increase and decrease) with a p value of <0.05 was attainable by the inclusion of 8 or more reference clones in an AAV library (see, Figure 6).

Table 1 – composition of AAV library 394, total of 132 individually barcoded clones.

Serotype	**Description**	**Number of barcoded clones**
AAV9	Control	100
AAV2R585E	Control	10
AAV1	Wild Type	2
AAV2	Wild Type	2
AAV3	Wild Type	2
AAV4	Wild Type	2
AAV5	Wild Type	2
AAV6	Wild Type	2
AAV7	Wild Type	2
AAV8	Wild Type	2
AAVrh10	Wild Type	2
AAV1&9 hybrid 1	Wild type	2
AAV1&9 hybrid 2	Wild type	2

Example 4 – Using AAV Barcode-Seq to assess in vitro transduction efficiency of AAV variants

[0052] A population of HEK 293 cells was infected with a DNA barcoded AAV library 394 at a multiplicity of infection (MOI) of 10^5. The library contained the indicated AAV serotypes and variants. Results are shown in Figure 7.

Example 5 – Using AAV Barcode-Seq to assess blood clearance of AAV variants

[0053] It has been reported that AAV9 exhibits distinctively delayed blood clearance compared to other serotypes when infused intravenously in mice. In addition, AAV1 is rapidly eliminated from the blood circulation and AAV2 is cleared very rapidly for the first 30 minutes following intravenous administration and slowly

cleared thereafter (see, Figure 8A and Kotchey NM et al., Mol Ther 19, 1079-1089 (2011), incorporated by reference herein).

[0054] In Figure 8B, the same pharmacokinetic features were also observed using AAV Barcode-Seq. Figure 8C shows that the results were observed after using only two mice. Without AAV Barcode-Seq, at least 33 mice and a significant amount of time would be required to obtain the same information.

Example 6 – Using AAV Barcode-Seq to assess reactivity to neutralizing antibodies AAV variants

[0055] Reactivity to neutralizing antibodies against the AAV capsid was investigated by injecting intravenously the AAV library ID 394 (see, Table 2) into naive mice and mice that had been previously immunized with an intravenous injection of 1.0×10^{11} vector genomes (vg) of AAV9-CMV-lacZ 3, or more, weeks prior to the intravenous infusion of AAV library ID 394. Blood concentrations of each AAV serotype or variant were then quantified by the AAV Barcode-Seq and normalized to the blood concentration of AAV9. Results are shown in Figure 9.

[0056] It has been shown previously that the level of AAV neutralizing antibody in the blood circulation of mice previously immunized with AAV as described above is sufficient to eliminate a majority of infused AAV viral particles within 30 minutes (Kotchey NM et al., 2011, *supra*). In mice previously immunized with AAV9, the AAV9-normalized relative blood concentration of a serotype not recognized by anti-AAV9 antibodies would significantly increase for the first hour following AAV injection. The increase does not occur with a serotype that is recognized by anti-AAV9 antibodies. When the relative pharmacokinetic profiles of each serotype or variant were compared between the naive animals and AAV9 antibody-positive animals, a clear distinction was observed between the AAV serotypes or variants that are neutralized with anti-AAV9 antibody (i.e., AAV8, AAVrh10, and AAV1.9-1) and those that are not neutralized. This observation on the cross-reactivity of anti-AAV9 neutralizing antibody shows that the results from AAV Barcode-Seq recapitulate results obtained by other methods (Gao G et al., 2004, *supra*).

Example 7 -- Using AAV Barcode-Seq to assess in vivo transduction of AAV variants

[0057] Three mice were injected with AAV library ID 394 via the tail vain at a dose of 1.0 x 10^{12} vg/mouse. Six to eight weeks post-injection, various tissues were harvested. Total DNA was extracted from each tissue sample and sequenced. AAV Barcode-Seq revealed that AAV8, AAV9 and AAVrh10, known to have high transduction efficiency also exhibit higher transduction efficiency compared to other serotypes in many tissues using AAV Barcode-Seq. Similarly, AAV3 is known to have poor transduction and that result is recapitulated using AAV Barcode-Seq.

Example 8 – Mutations in AAV9 capsid assessed by AAV Barcode-Seq

[0058] The first atomic structure of an AAV determined was that of AAV2 in 2002 (Xie Q et al., Proc Natl Acad Sci USA 99, 10405-10410 (2002), incorporated by reference herein). Since then, the three dimensional structures of other serotypes, AAV1, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8 and AAV9 have been determined completely or partially by X-ray crystallography and cryo-electron microscopy combined with image reconstruction (DiMattia M et al., Acta Crystallogr Sect F Struct Biol Cryst Commun 61, 917-921 (2005); Govindasamy L et al., J Virol 80, 11556-11570 (2006); Lerch TF et al., Virology 403, 26-36 (2010); Miller EB et al., Acta Crystallogr Sect F Struct Biol Cryst Commun 62, 1271-1274 (2006); Nam H et al., J Virol 81, 12260-12271 (2007); Padron E et al., J Virol 79, 5047-5058 (2005); Quesada O et al., Acta Crystallogr Sect F Struct Biol Cryst Commun 63, 1073-1076 (2007); Xie Q et al., Virology 420, 10-19 (2008), all of which are incorporated by reference herein).

[0059] The structures above have significantly advanced knowledge of AAV capsid structure-function relationships, and accelerated the research on AAV capsid engineering aimed at creating novel AAV capsids with better biological performance or altered tropism (Asokan A et al., 2012, *supra*, and Girod A et al., Nat Med 5, 1438 (1999), incorporated by reference herein). The main strategic paradigm in protein research is to obtain and use protein structural information to identify potential structural domains, predict their phenotypic roles, and then perform targeted mutagenesis on the structural domains to elucidate the phenotypes. Structural biology approaches provides a comprehensive picture of the whole protein or protein

complex of interest and takes advantage of a wealth of structural information of various types of proteins to predict functions (Thornton JM et al, Nat Struct Biol 7, 991-994 (2000). That said, this approach may not be the most efficient way to interrogate the roles of structural domains of viral capsids. Phenotypic outcomes of capsids depend on their quaternary structure, and therefore, viral capsids are structurally, functionally and often coevolutionarily constrained in a manner specific to each viral species. As a result, it is difficult to understand functional roles and the significance of each amino acid or group thereof in a region of interest solely through structural studies without complementing data obtained by mutagenesis experiments. With regard to the AAV capsid it has been shown that only one or a few amino acid mutations can significantly change the phenotype of the viral capsid and that the correlation between a given mutation and the resulting phenotype is highly unpredictable (Excoffon KJ et al., Proc Natl Acad Sci USA106, 3865-3870 (2009); Pulicherla N et al., Mol Ther 19, 1070-1078 (2011); and Vandenberghe LH et al., Gene Ther 16, 1416-1428 (2009); all of which are incorporated by reference herein).

[0060] AAV Barcode-Seq was used to perform a double alanine mutagenesis of the carboxy-terminal half of the AAV9 capsid, spanning amino acid positions from 356 to 736 of the AAV9 capsid. One hundred ninety-one double alanine mutants of AAV9 that entirely cover the 381 amino acids in the carboxy-terminal half of the AAV9 capsid were tested for intact particle production. A total of 382 AAV9 mutant clones (2 independent clones per mutant), 15 reference control wild type AAV9 clones, and 15 reference control AAV2R585E clones were prepared separately to generate a mutant library.

[0061] Viral particles were produced by calcium phosphate transfection of HEK 293 cells with each AAV plasmid and an adenovirus helper plasmid (pHelper®, Agilent). Viral particles were recovered from the cells and 4 AAV viral clone pools, each of which contained the equal fraction of each viral preparation obtained from one culture, were created. The viral DNA was extracted from the 4 viral clone pools, and amplified and sequenced as per the AAV Barcode-Seq method described above.

Example 9 – AAV9 clones with mutations at N562, E563, E564, E565, I566, and/or K567 transduce HEK293, Pro5, and Lec2 cells less efficiently than wild type AAV9

[0062] HEK 293, CHO Pro5 and Lec2 cells were infected with DNA-barcoded AAV9 double alanine mutant libraries. The transduction efficiency of each mutant was assessed at 48 hours post-infection using AAV Barcode-Seq. Table 2 summarizes AAV9 double alanine mutations that resulted in increased or decreased transduction with statistical significance compared to that of the wild type AAV9 (Mann Whitney U-test, $p<0.05$) in either of HEK293, CHO Pro5 and CHO Lec2 cells.

Table 2 – transduction efficiencies of AAV9 clones with the indicated mutations in the indicated cell lines. Transduction efficiency is normalized to wild type AAV9 (SEQ ID NO: 1), where wild type AAV9 transduction = 1.

	Relative Transduction Efficiency		
Mutations	HEK 293	CHO Pro5	CHO Lec2
I560A and T561A	1	0.2	0.23
N562A and E563A	1	0.21	0.11
E564A and E565A	0.25	0.49	0.32
I566A and K567A	1	0.29	0.15

[0063] The I560A/T561A, N562A/E563A, E564A/E565A, and I566A/K567A double mutant clones exhibit moderately reduced transduction efficiencies in Pro5 and Lec2 cells (51-89% reduction, $p<0.05$). E564A/E565A also showed a statistically significant decrease in transduction in HEK293 cells ($p<0.05$). Among the 8 amino acids in this sequence (ITNEEEIK), ITN-EE-K are surface-exposed in the deep 2-fold symmetry axis valley. The N562A/E563A double mutant binds to Lec2 cells at a level comparable to the wild type AAV9, but transduction efficiency was 10 fold lower than the wild type. A similar trend was also observed in the other 3 mutants.

Example 10 -- AAV9 clones with mutations at N562, E563, E564, E565, I566, and/or K567 influence tissue transduction efficiencies in vivo

[0064] Adult male mice (n=3) were intravenously injected with DNA-barcoded AAV9 mutant libraries containing double alanine mutant viral capsids. Six to 8 weeks after the injection, the following 12 tissues; brain, heart, lung, liver, kidney, spleen, intestine, pancreas, testis, muscle, fat and skin were collected. Total DNA was extracted from each tissue and analyzed according to AAV Barcode-Seq. Results are shown in Table 3.

Table 3 –Transduction efficiencies in vivo, normalized to transduction efficiency of wild type AAV9 (SEQ ID NO: 1). Abbreviations: Br = Brain, H = Heart, Lu = Lung, Ki = Kidney, Sp = Spleen, I = Intestine, Pa = Pancreas, T = Testis, Mu = Muscle, Sk = Skin

Mutations	Br	H	Lu	Lv	Ki	Sp	I	Pa	T	Mu	Fat	Sk
I560A/T561A	0.53	1.00	0.51	0.41	1.00	1.00	0.48	0.60	1.00	1.00	0.51	0.25
N562A/E363A	0.02	0.17	0.09	0.00	1.00	1.00	0.04	0.79	0.12	0.37	0.06	0.05
E564A/E565A	0.08	0.18	0.08	0.02	0.39	0.71	0.02	0.53	0.08	0.11	0.04	0.04
I566A/K567A	0.01	0.09	0.07	0.02	1.00	1.00	0.07	0.46	0.09	0.18	0.09	0.02

[0065] The mutants display a substantial decrease in transfection relative to wild type AAV, except with regard to kidney, spleen, and pancreas, where they were similar if not identical to transduction efficiency to wild type.

Example 11 -- AAV9 clones with mutations at N562 and E563 display delayed blood clearance

[0066] When AAV vectors are infused into mice via the tail vein, AAV9 persists in the bloodstream for a long period of time while many other AAV serotypes are cleared from the blood circulation relatively rapidly. Here we investigated how double alanine mutations on the AAV9 capsid could influence the blood clearance rates when infused into mice. To this end, we injected DNA-barcoded libraries into mice (n=2 per library), and performed the AAV Barcode-Seq using the blood samples collected at 1, 10, 30 min, 1, 4, 8, 24 and 72 h following injection. Results for selected mutations are shown in Table 4. Amount of each mutant in serum is expressed relative to wild type AAV9 (AAV9 = 1).

Table 4

	Sample collection time (min post injection)							
Mutant	1	10	30	60	240	480	1440	4320
AAV9	1	1	1	1	1	1	1	1
I560A/T561A	1	0.6	1	0.7	1	1	1	0.2
N562A/E363A	1	1	1	1	1.3	1.7	2.2	2
E564A/E565A	1	0.7	1	0.6	0.7	1	1	1
I566A/K567A	0.4	0.4	0.3	0.4	0.4	0.6	1	0.2

CLAIMS

1. An adeno associated virus plasmid comprising:

a polynucleotide that encodes a protein with the amino acid sequence of SEQ ID NO: 1, or a fragment thereof, the protein comprising a mutation at an amino acid selected from at least one of I560, T561, N562, E563, E564, E565, I566, and K567.

2. The plasmid of claim 1, wherein the protein comprises a mutation at I560 and T561.

3. The plasmid of claim 1, wherein the protein comprises a I560A mutation and a T561A mutation.

4. The plasmid of claim 1, wherein the protein comprises a mutation at N562 and a mutation at E563.

5. The plasmid of claim 1, wherein the protein comprises a N562A mutation and a E563A mutation.

6. The plasmid of claim 1, wherein the protein comprises a mutation at E564 and a mutation at E565.

7. The plasmid of claim 1, wherein the protein comprises a E564A mutation and a E565A mutation.

8. The plasmid of claim 1, wherein the protein comprises a mutation at I566 and a mutation at K567.

9. The plasmid of claim 1, wherein the protein comprises a I566A mutation and a K567A mutation.

10. The plasmid of claim 1, wherein the protein has a sequence selected from at least one of the amino acid sequence of SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6; and SEQ ID NO:7.

11. The plasmid of claim 1, wherein the polynucleotide encodes a protein comprising a mutation at an additional amino acid.

12. The plasmid of claim 1, wherein the protein comprises at lease one mutation selected from I560A, T561A, N562A, E563A, E564A, E565A, I566A, or K567A.

13. An adeno associated virus vector derived from any one of the plasmids of claims 1-12.

14. A method of delivering a polynucleotide that encodes a protein of interest to a tissue in a subject, the method comprising:

administering an effective amount of an adeno associated virus vector of claim 8 to the subject; wherein the adeno associated virus vector comprises the polynucleotide that encodes the protein of interest; and wherein the tissue is selected from kidney, spleen, and pancreas.

15. The method of claim 14 comprising minimizing delivery of the polynucleotide to a tissue selected from at least one of brain, heart, lung, liver, intestine, testis, muscle, fat, or skin.

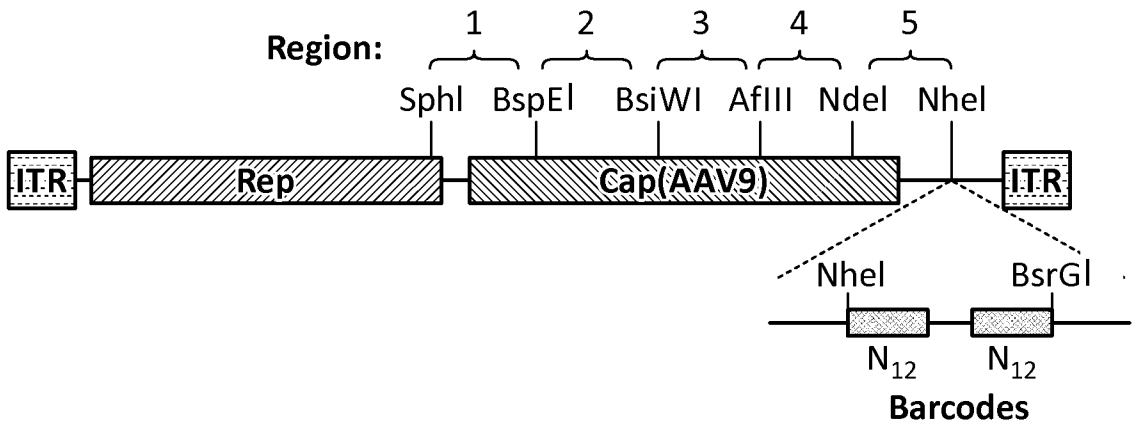
Region:
1
2
3
4
5
SphI
BspEI
BsiWI
AflII
NdeI
NheI
ITR
Rep
Cap(AAV9)
ITR
NheI
BsrGI
N12
N12
Barcodes

FIG. 1

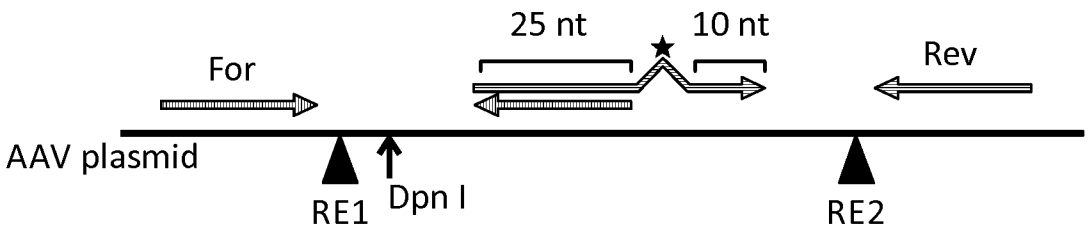
25 nt
10 nt
For
Rev
AAV plasmid
RE1
Dpn I
RE2

FIG. 2

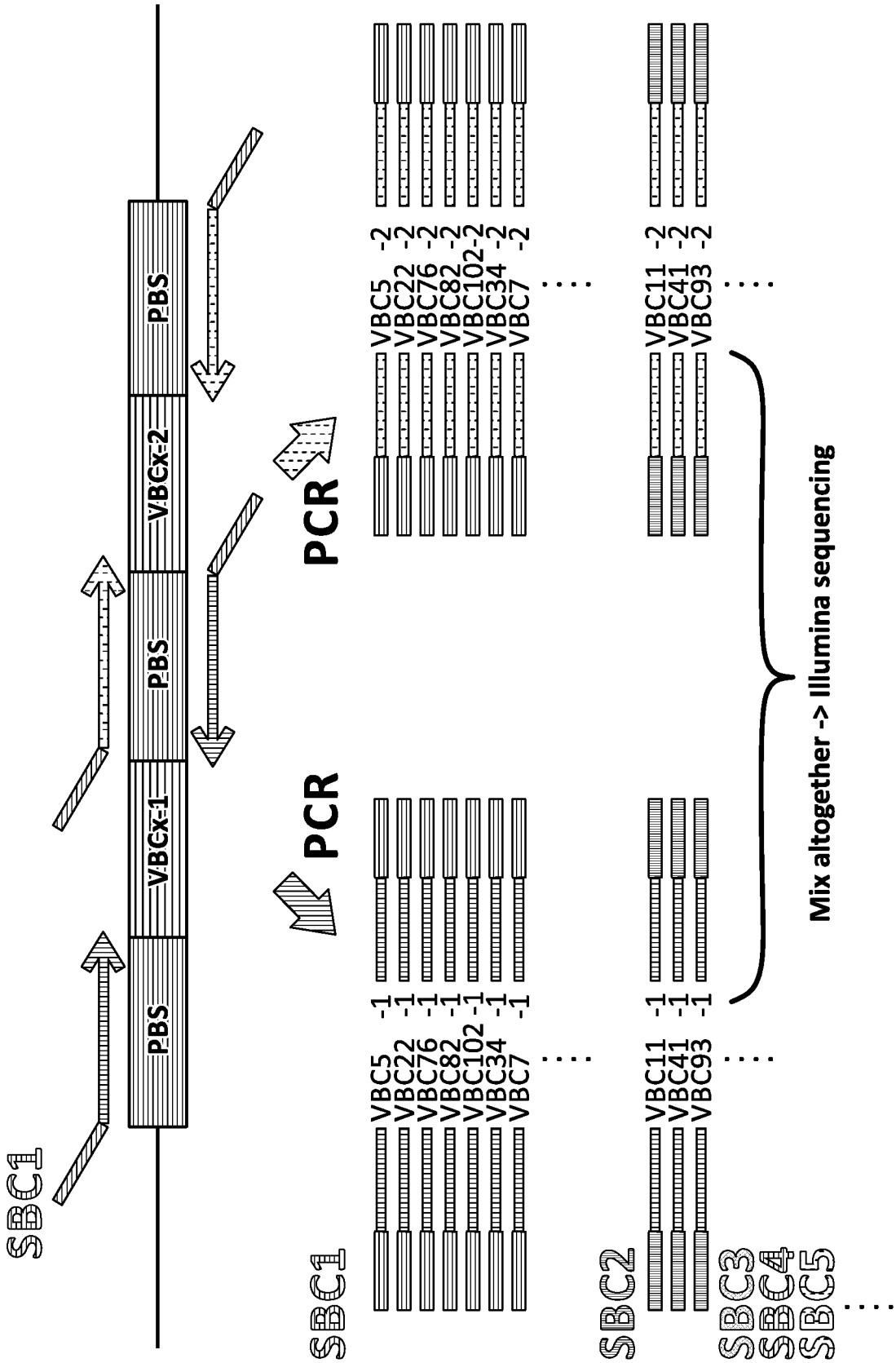
SBC1
PBS
VBCx-1
PBS
VBCx-2
PBS
PCR
PCR
VBC5 -1
VBC22 -1
VBC76 -1
VBC82 -1
VBC102 -1
VBC34 -1
VBC7 -1
VBC11 -1
VBC41 -1
VBC93 -1
VBC5 -2
VBC22 -2
VBC76 -2
VBC82 -2
VBC102 -2
VBC34 -2
VBC7 -2
VBC11 -2
VBC41 -2
VBC93 -2
Mix altogether -> Illumina sequencing
SBC1
SBC2
SBC3
SBC4
SBC5

FIG. 3

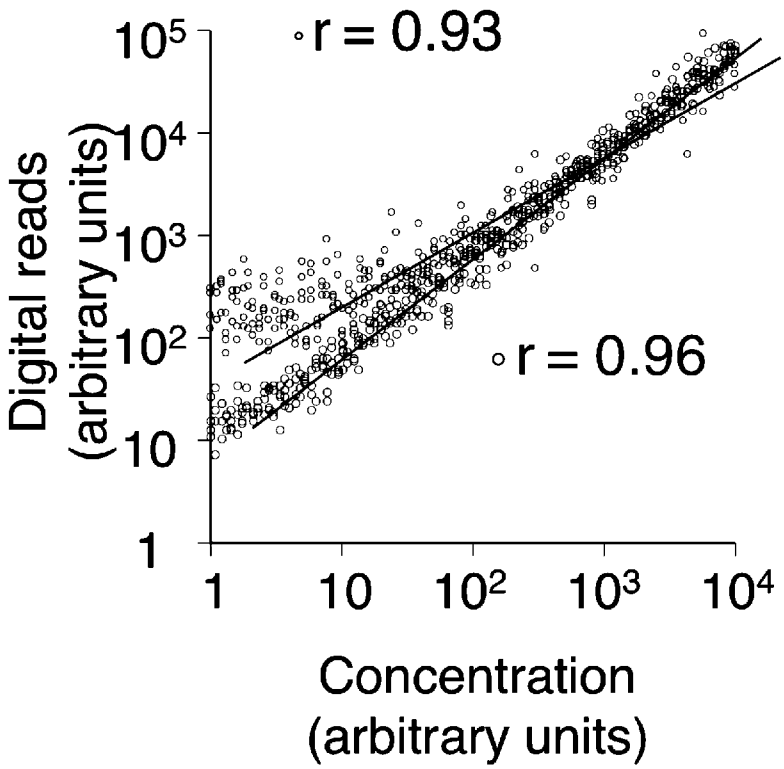
r = 0.93
r = 0.96
Digital reads
(arbitrary units)
Concentration
(arbitrary units)
10^5
10^4
10^3
10^2
10
1
1
10
10^2
10^3
10^4

FIG. 4

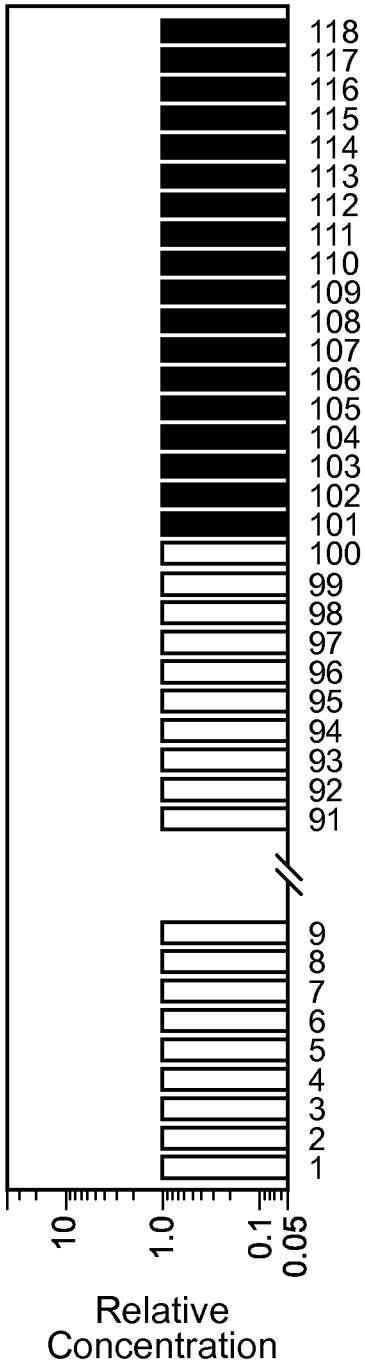
118
117
116
115
114
113
112
111
110
109
108
107
106
105
104
103
102
101
100
99
98
97
96
95
94
93
92
91
9
8
7
6
5
4
3
2
1
10
1.0
0.1
0.05
Relative
Concentration

FIG. 5A

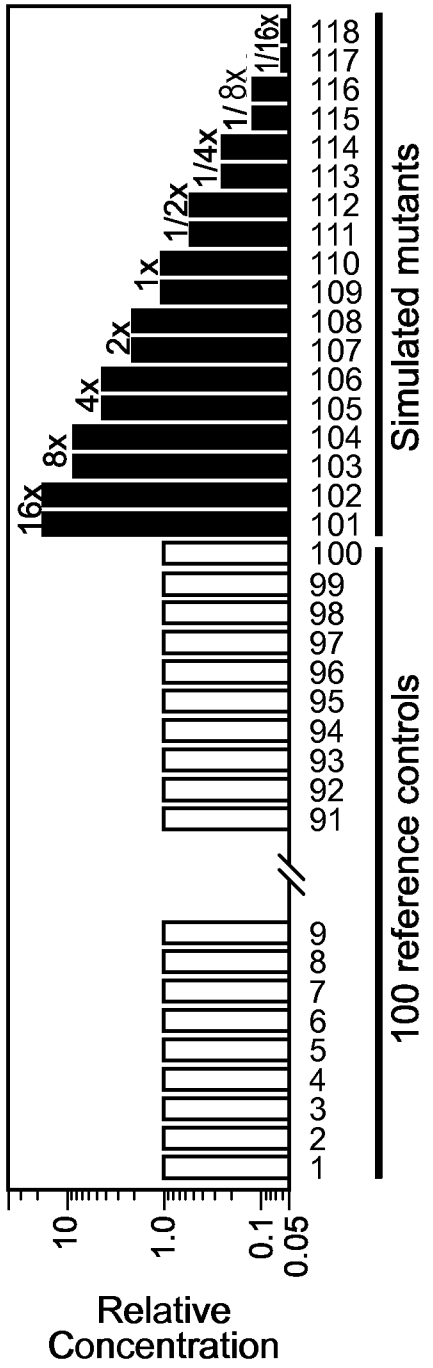
16x
8x
4x
2x
1x
1/2x
1/4x
1/8x
1/16x
Simulated mutants
100 reference controls
118
117
116
115
114
113
112
111
110
109
108
107
106
105
104
103
102
101
100
99
98
97
96
95
94
93
92
91
9
8
7
6
5
4
3
2
1
10
1.0
0.1
0.05
Relative
Concentration

FIG. 5B

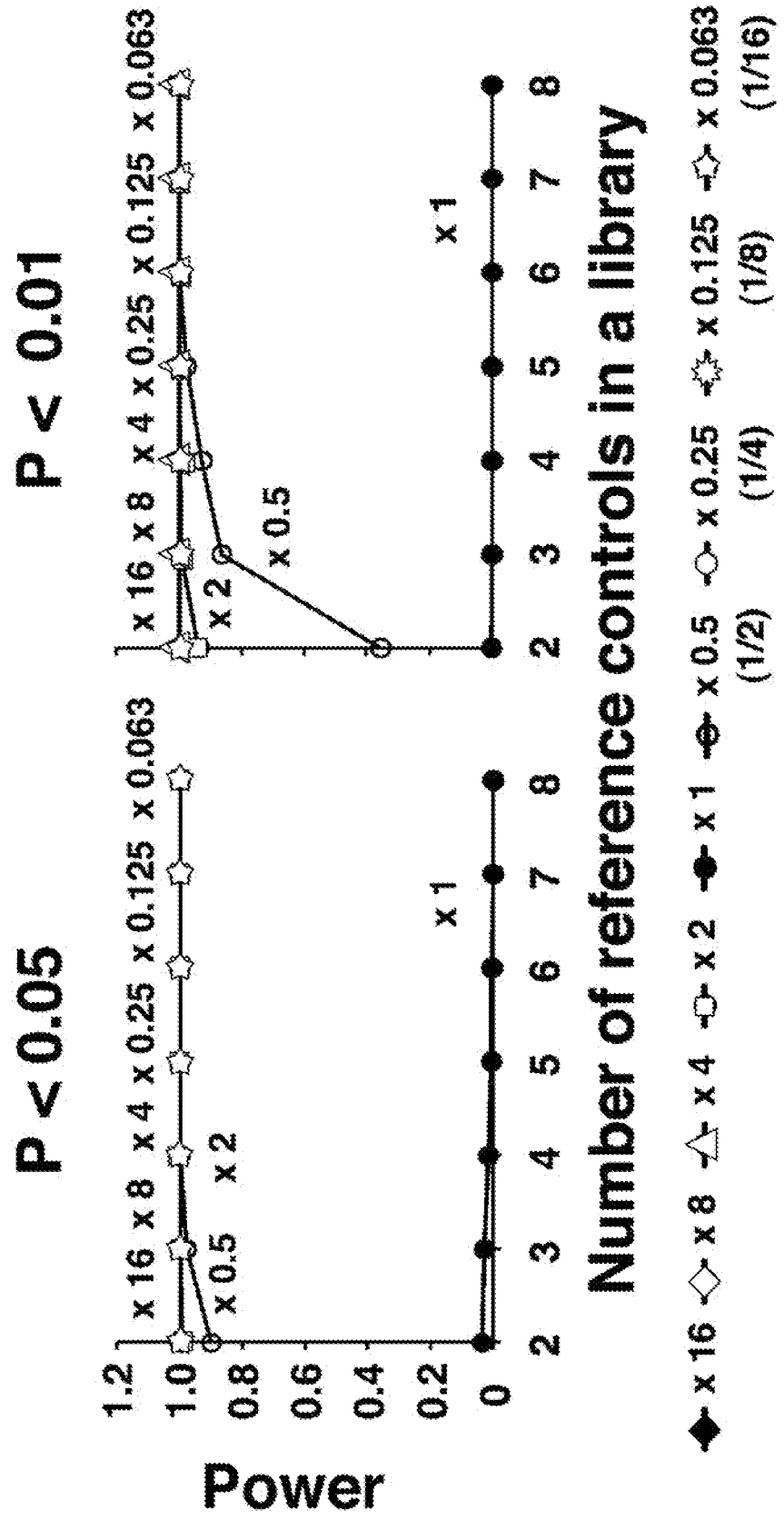
P < 0.05
P < 0.01
Power
Number of reference controls in a library
x 16
x 8
x 4
x 2
x 1
x 0.5
x 0.25
x 0.125
x 0.063
(1/2)
(1/4)
(1/8)
(1/16)

FIG. 5C

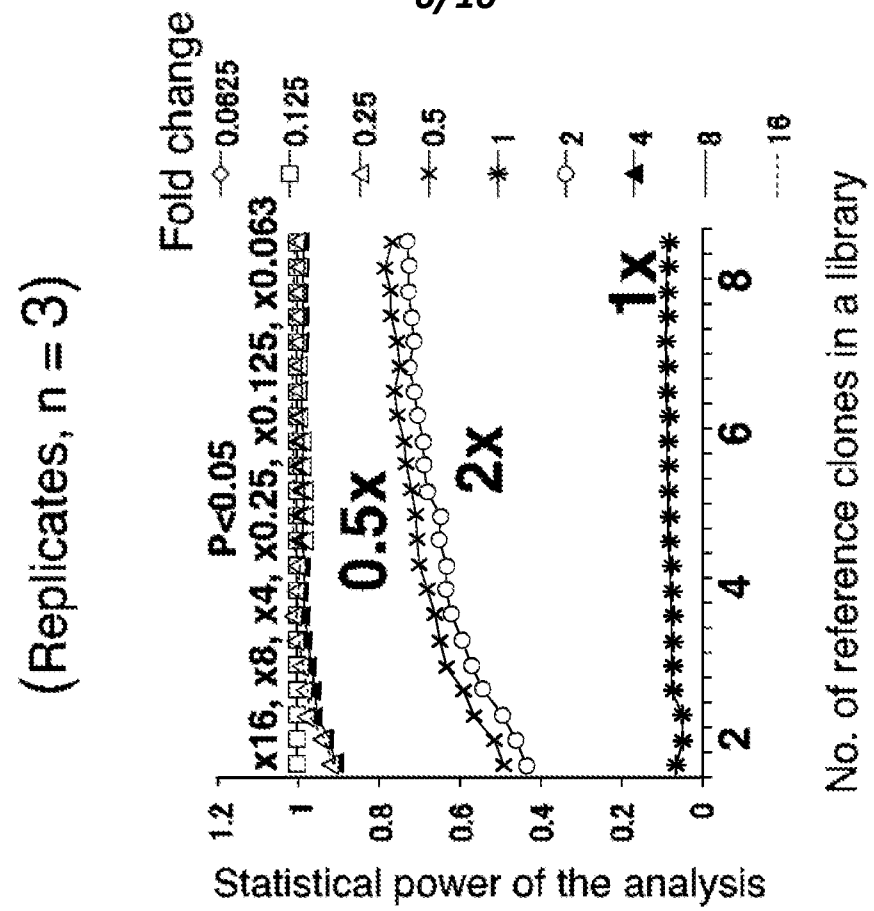
(Replicates, n = 3)
Fold change
0.0625
0.125
0.25
0.5
1
2
4
8
16
P<0.05
x16, x8, x4, x0.25, x0.125, x0.063
0.5x
2x
1x
Statistical power of the analysis
No. of reference clones in a library

FIG. 6B

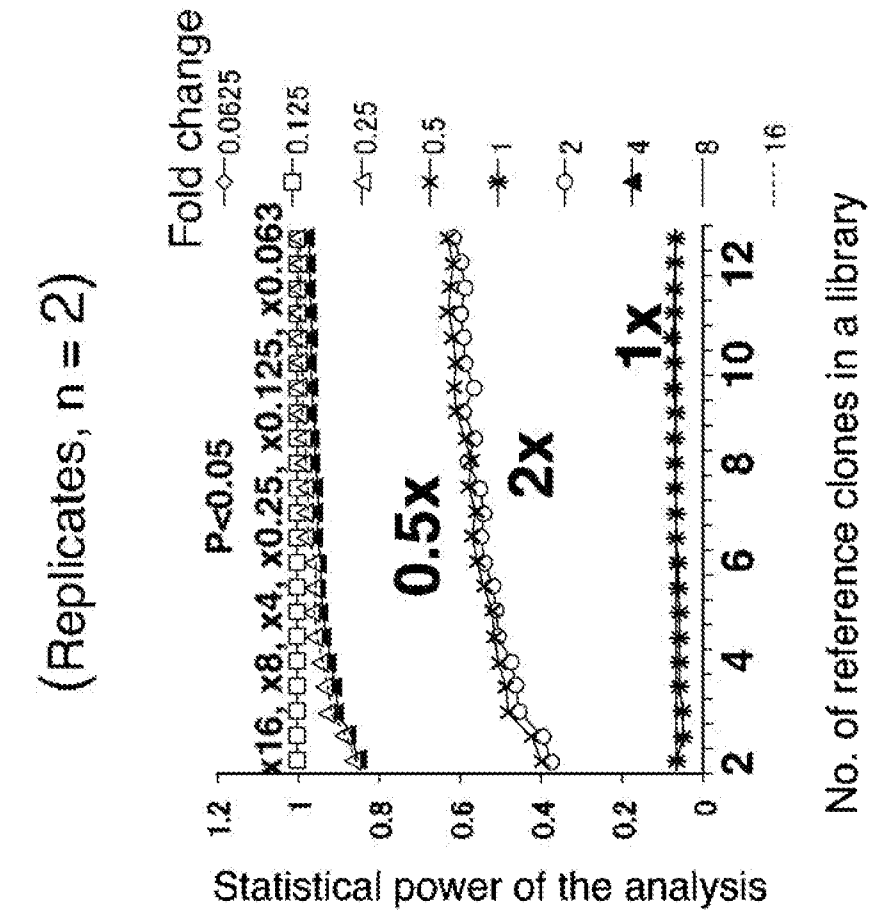
(Replicates, n = 2)
Fold change
0.0625
0.125
0.25
0.5
1
2
4
8
16
P<0.05
x16, x8, x4, x0.25, x0.125, x0.063
0.5x
2x
1x
Statistical power of the analysis
No. of reference clones in a library

FIG. 6A

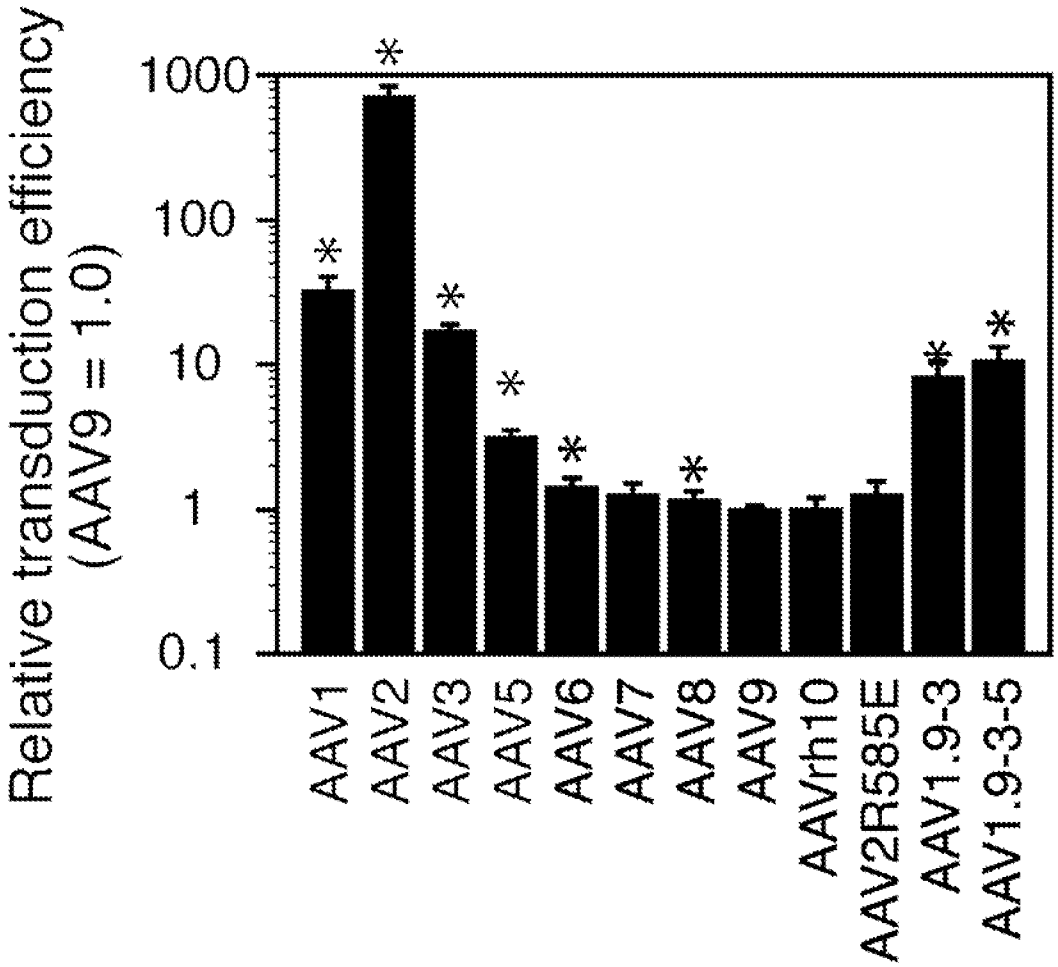
Relative transduction efficiency
(AAV9 = 1.0)
1000
100
10
1
0.1
*
AAV1
AAV2
AAV3
AAV5
AAV6
AAV7
AAV8
AAV9
AAVrh10
AAV2R585E
AAV1.9-3
AAV1.9-3-5

FIG. 7

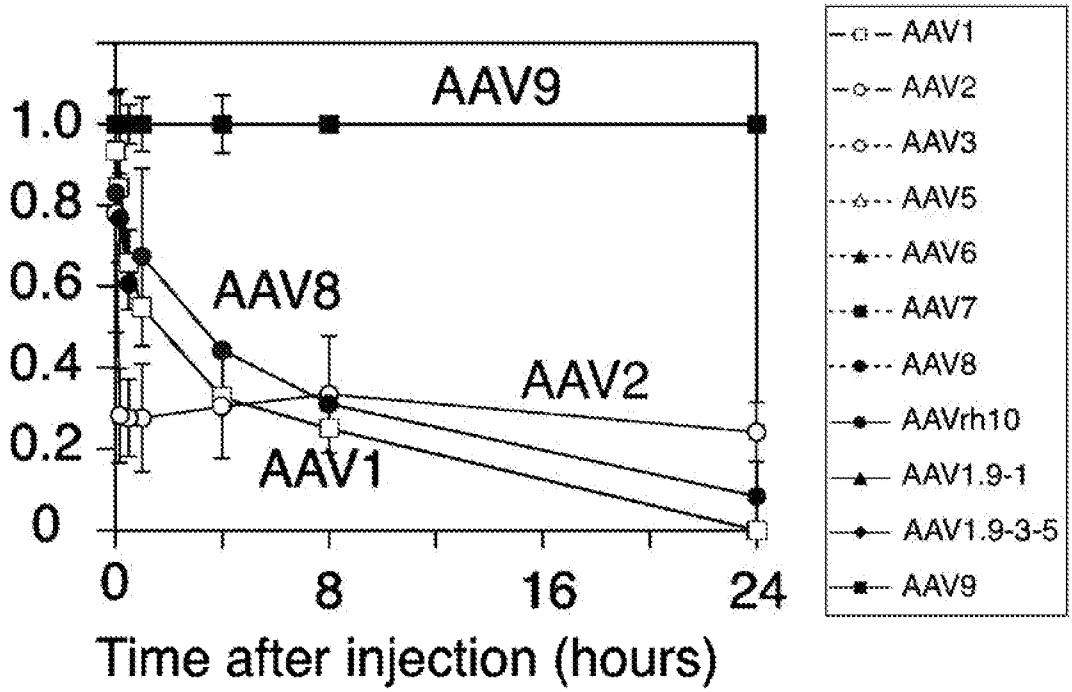
AAV9
AAV8
AAV2
AAV1
1.0
0.8
0.6
0.4
0.2
0
0
8
16
24
Time after injection (hours)
AAV1
AAV2
AAV3
AAV5
AAV6
AAV7
AAV8
AAVrh10
AAV1.9-1
AAV1.9-3-5
AAV9

FIG. 8A

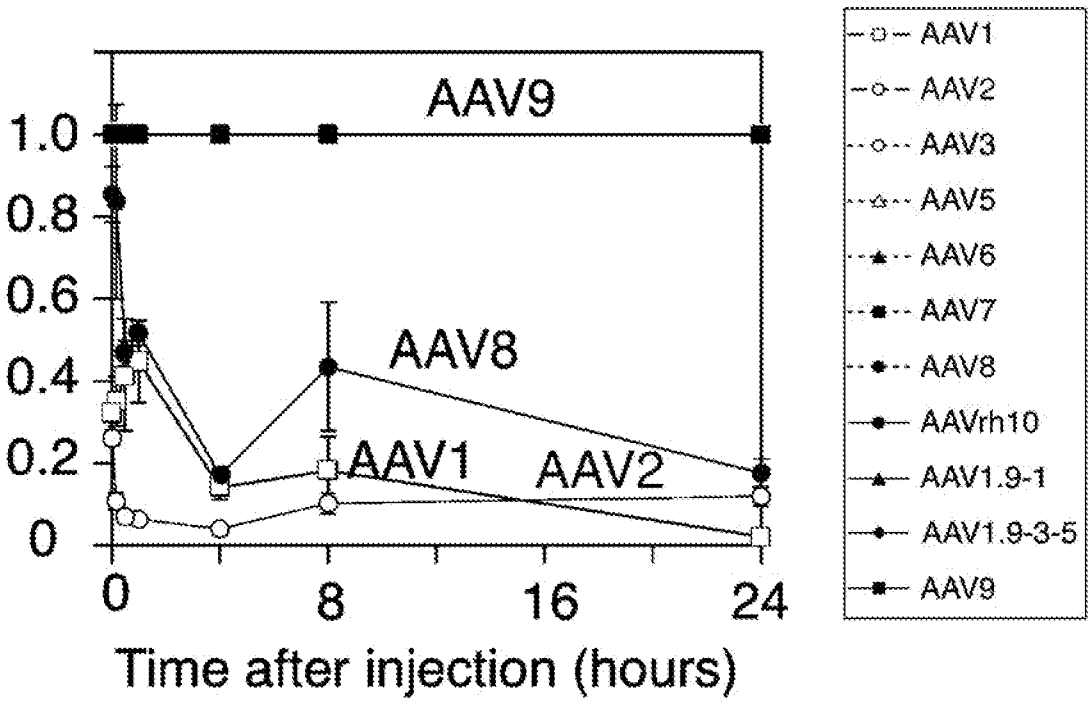
AAV9
AAV8
AAV1
AAV2
1.0
0.8
0.6
0.4
0.2
0
0
8
16
24
Time after injection (hours)
AAV1
AAV2
AAV3
AAV5
AAV6
AAV7
AAV8
AAVrh10
AAV1.9-1
AAV1.9-3-5
AAV9

FIG. 8B

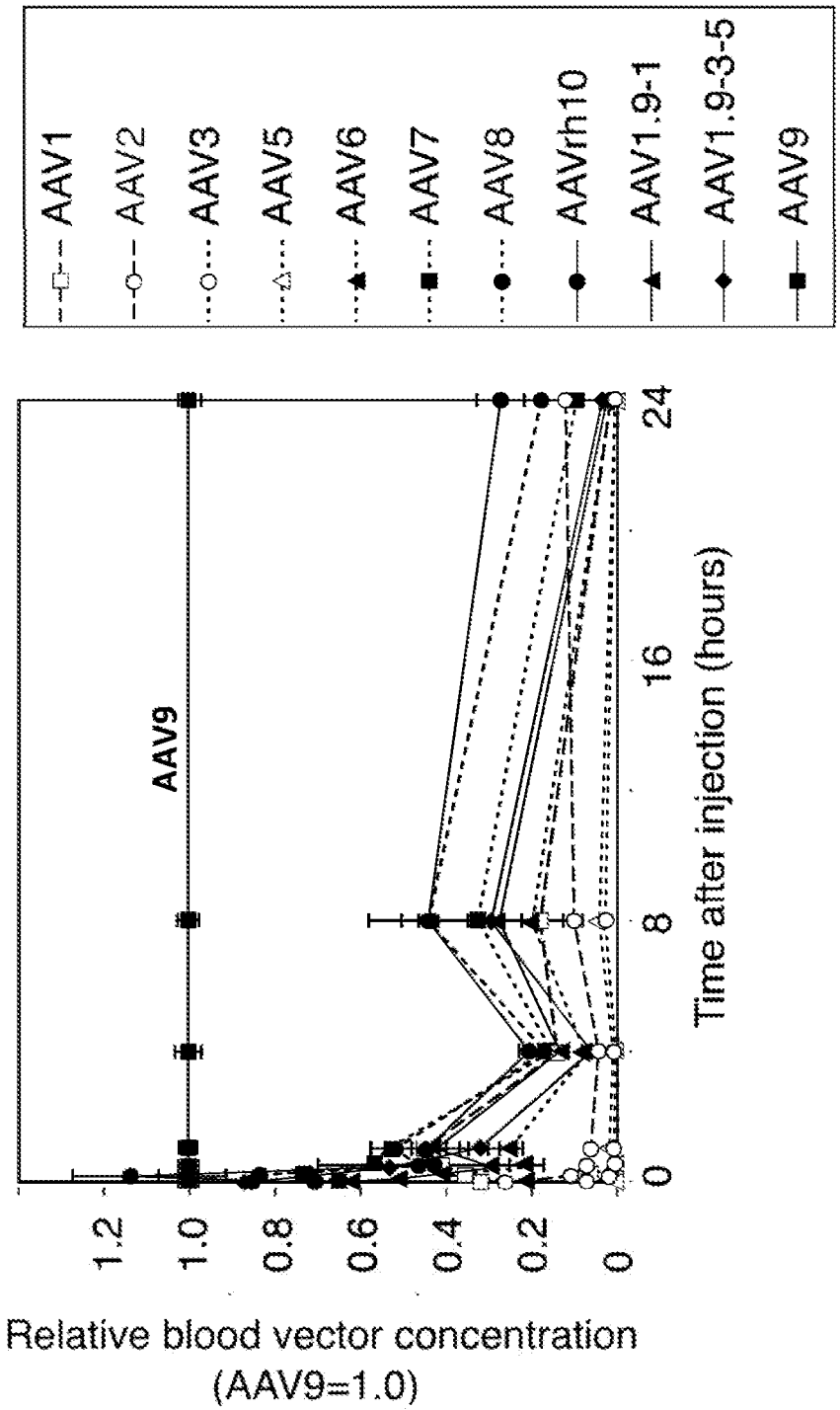
AAV1
AAV2
AAV3
AAV5
AAV6
AAV7
AAV8
AAVrh10
AAV1.9-1
AAV1.9-3-5
AAV9
AAV9
1.2
1.0
0.8
0.6
0.4
0.2
0
0
8
16
24
Time after injection (hours)
Relative blood vector concentration
(AAV9=1.0)

FIG. 8C

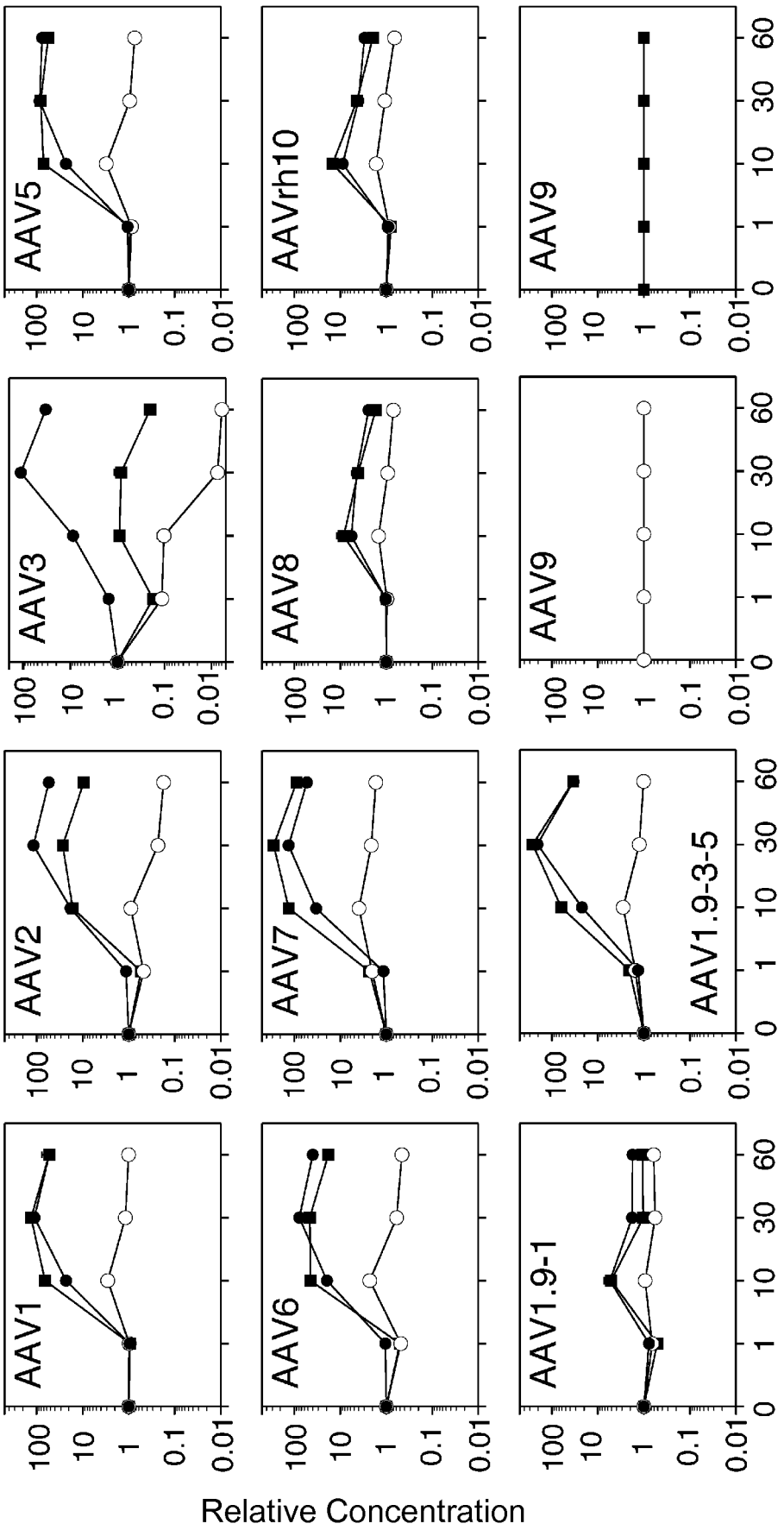
AAV1
AAV2
AAV3
AAV5
AAV6
AAV7
AAV8
AAVrh10
AAV1.9-1
AAV1.9-3-5
AAV9
AAV9
100
10
1
0.1
0.01
0
1
10
30
60
Relative Concentration
Time after injection (min)

FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/037440

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/861(2006.01)i, C12N 15/63(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 15/861; C12N 7/01; A61K 48/00; C12N 15/63

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: adeno associated virus, AAV, mutation, plasmid, vector, I560, T561, N562, E563, E565, K567

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ADACHI, KEI et al., `A New Recombinant Adeno-Associated Virus (AAV)-based random peptide display library system: infection-defective AAV1.9-3 as a novel detargeted platform for vector evolution`, Gene Therapy and Regulation, 31 October 2010, Vol. 5, No. 1, pp. 31-55 See the whole document.	1-13
A	US 2007-0196338 A1 (SAMULSKI, RICHARD JUDE et al.) 23 August 2007 See claims 1 and 37; paragraphs [0065]-[0077]; Table 2.	1-13
A	US 2007-0036757 A1 (KLEINSCHMIDT, JURGEN et al.) 15 February 2007 See claims 1-6; paragraphs [0056]-[0059].	1-13
A	WO 2010-011404 A2 (EOS NEUROSCIENCE, INC., et al.) 28 January 2010 See claims 1, 11, 16, 18-20, 70 and 77-81; paragraphs [0042]-[0055] and [0066]-[0078].	1-13
A	WO 2006-119150 A2 (BETH ISRAEL DEACONESS MEDICAL CENTER) 9 November 2006 See claims 1, 3, 6, 12-15 and 21-24; page 23, line 10 - page 24, line 21.	1-13

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of 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
19 August 2013 (19.08.2013)	**20 August 2013 (20.08.2013)**
Name and mailing address of the ISA/KR Korean Intellectual Property Office 189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea Facsimile No. +82-42-472-7140	Authorized officer HEO Joo Hyung Telephone No. +82-42-481-8150

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/037440

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 14,15
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 14 and 15 pertain to methods for treatment of the human body by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/037440

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KOTCHEY, NICOLE M. et al, `A potential role of distinctively delayed blood clearance of recombinant adeno-associated virus serotype 9 in robust cardiac transduction`, Molecular Therapy, June 2011, Vol. 19, No. 6, pp. 1079-1089 See the whole document.	1-13

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2013/037440

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2007-0196338 A1	23/08/2007	US 7867484 B2	11/01/2011
		WO 2007-089632 A2	09/08/2007
		WO 2007-089632 A3	11/12/2008
US 2007-0036757 A1	15/02/2007	AT 409750 T	15/10/2008
		DE 602004016871 D1	13/11/2008
		EP 1486567 A1	15/12/2004
		EP 1664314 A2	07/06/2006
		EP 1664314 B1	01/10/2008
		US 7629322 B2	08/12/2009
		WO 2004-111248 A2	23/12/2004
		WO 2004-111248 A3	07/07/2005
WO 2010-011404 A2	28/01/2010	AU 2009-274482 A1	28/01/2010
		CA 2762118 A1	28/01/2010
		CN 102159713 A	17/08/2011
		EP 2315833 A2	04/05/2011
		EP 2315833 A4	04/01/2012
		GB 201021748 D0	02/02/2011
		GB 2472964 A	23/02/2011
		GB 2472964 B	24/04/2013
		MX 2010012592 A	05/05/2011
		US 2012-0093772 A1	19/04/2012
		WO 2010-011404 A3	24/02/2011
WO 2006-119150 A2	09/11/2006	US 2009-0215870 A1	27/08/2009
		WO 2006-119150 A3	24/05/2007