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(54) **METHODS FOR USING TRANSCRIPTION-DEPENDENT DIRECTED EVOLUTION OF AAV CAPSIDS**

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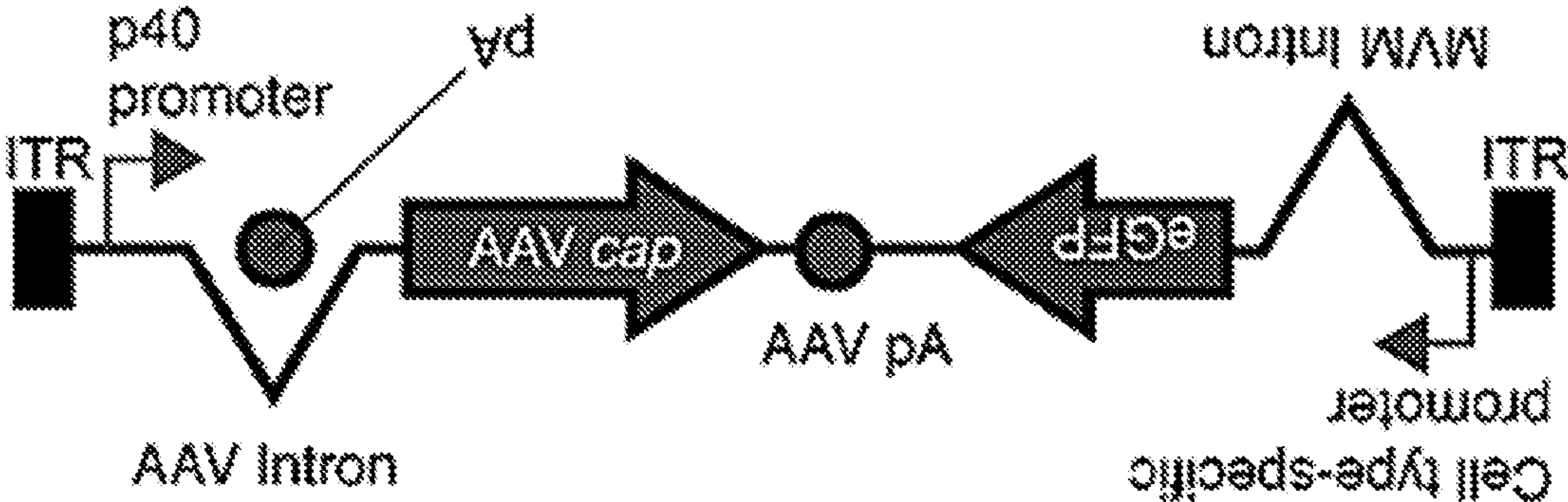
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(57) **ABSTRACT**

Disclosed are methods for performing transcription-dependent directed evolution (TRADE) and novel AAV capsids selected using such methods. This disclosure also provides novel AAV capsid mutants. TRADE technology was used to identify novel AAV vectors that mediate neuronal transduction in the brain following intravenous administration. Application of TRADE in vivo resulted in the identification of new AAV capsids that can transduce neurons more efficiently and more specifically than AAV9 in the brain following administration of the new AAV capsids. The disclosed methods may be used to identify AAV capsids that target various cell populations.

Specification includes a Sequence Listing.



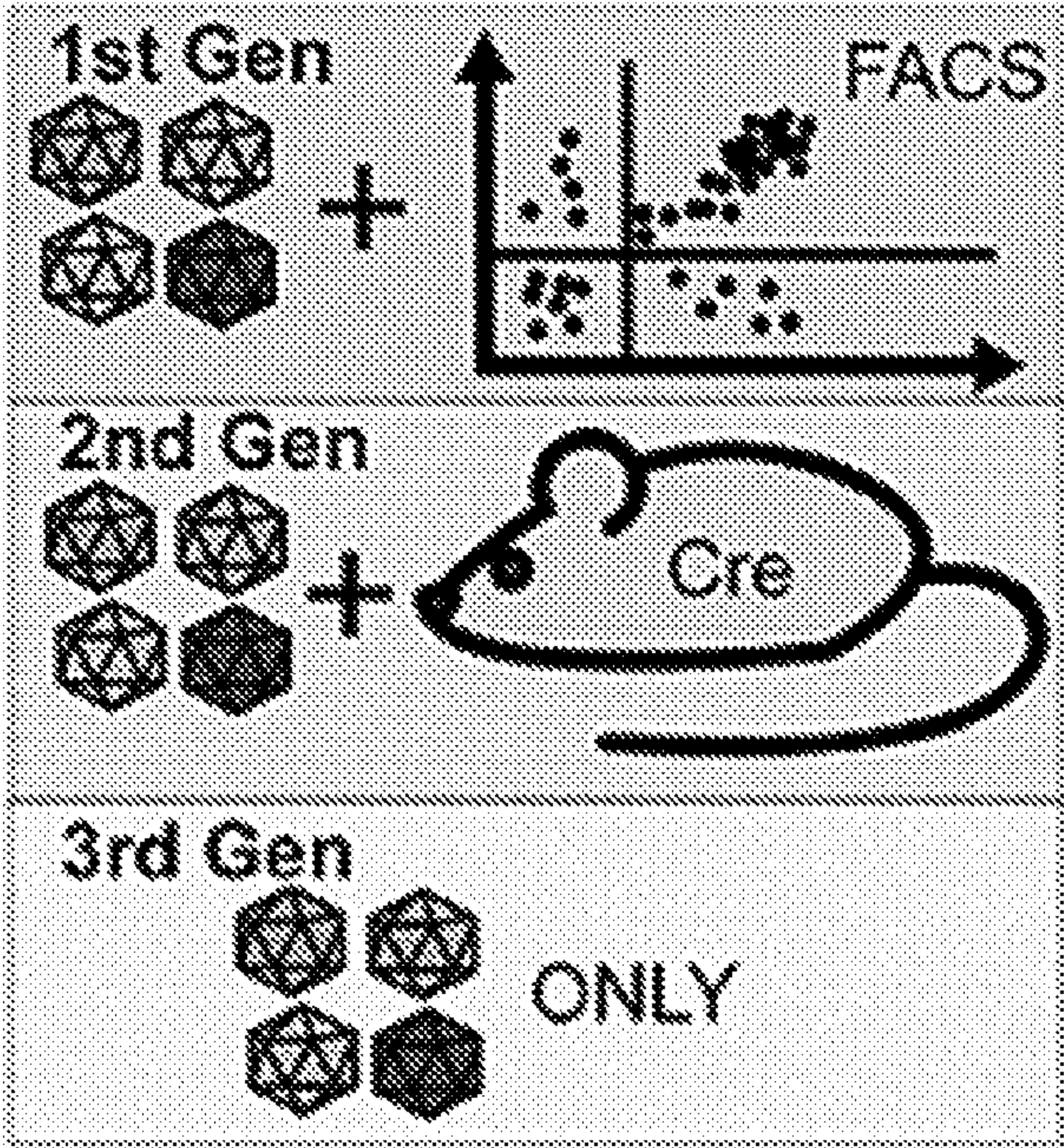
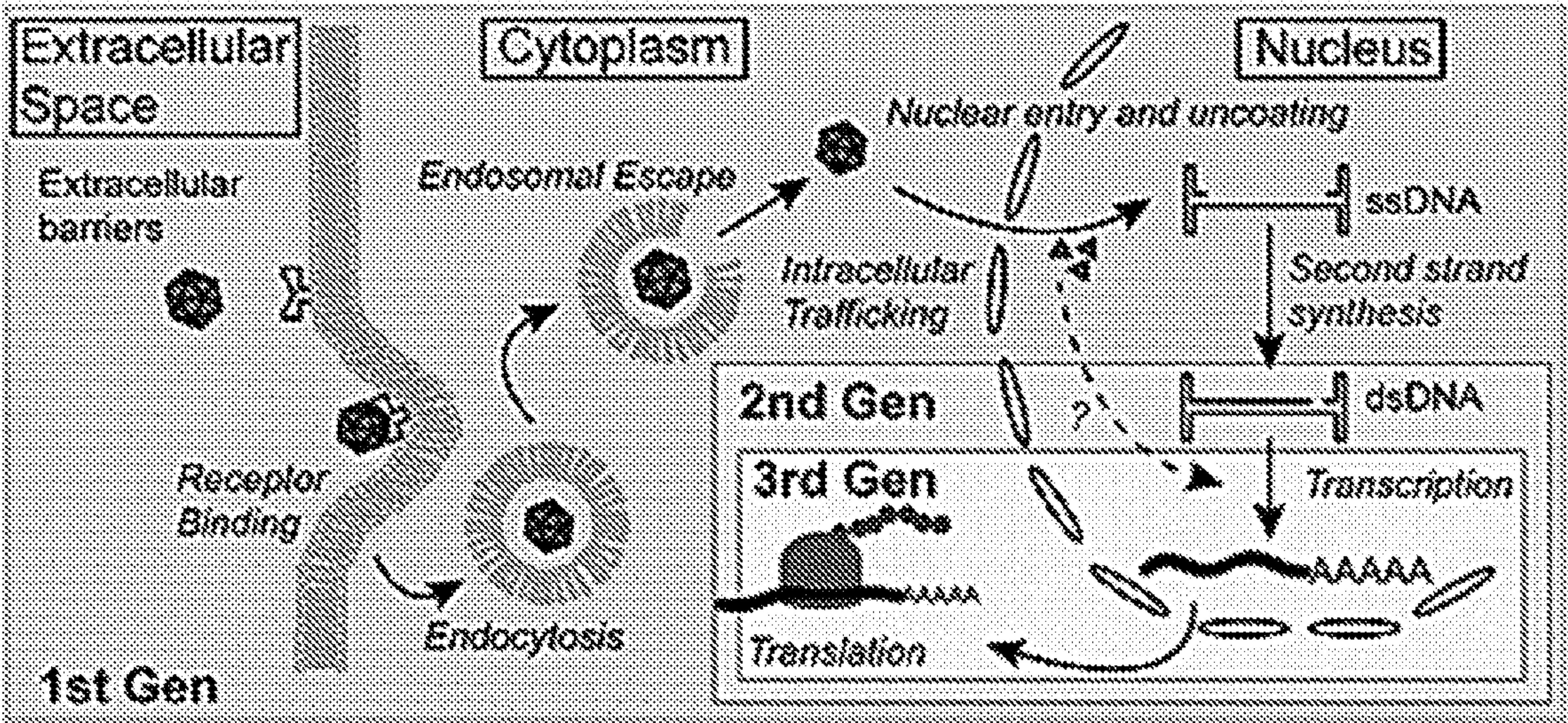


Fig. 1

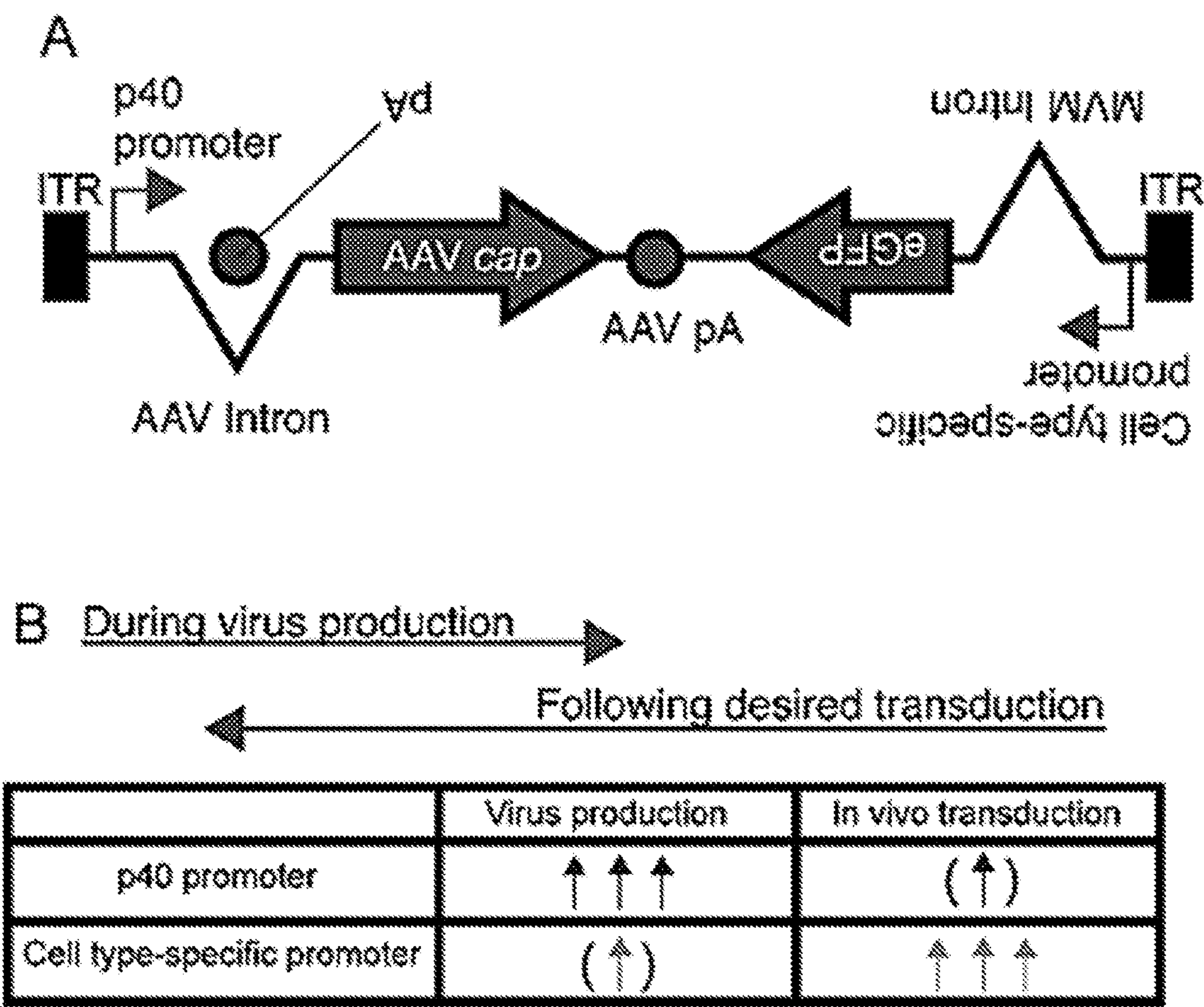
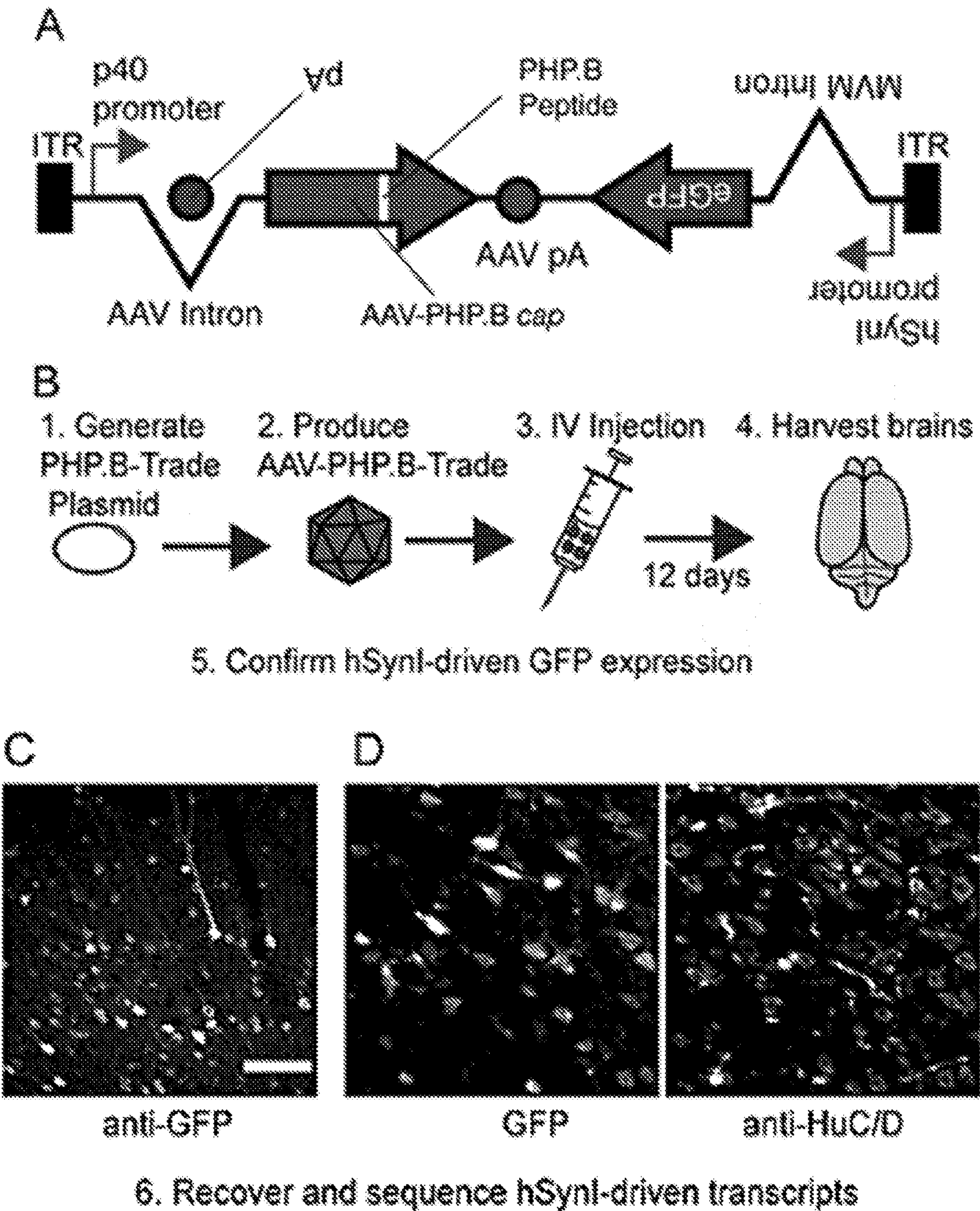


Fig. 2



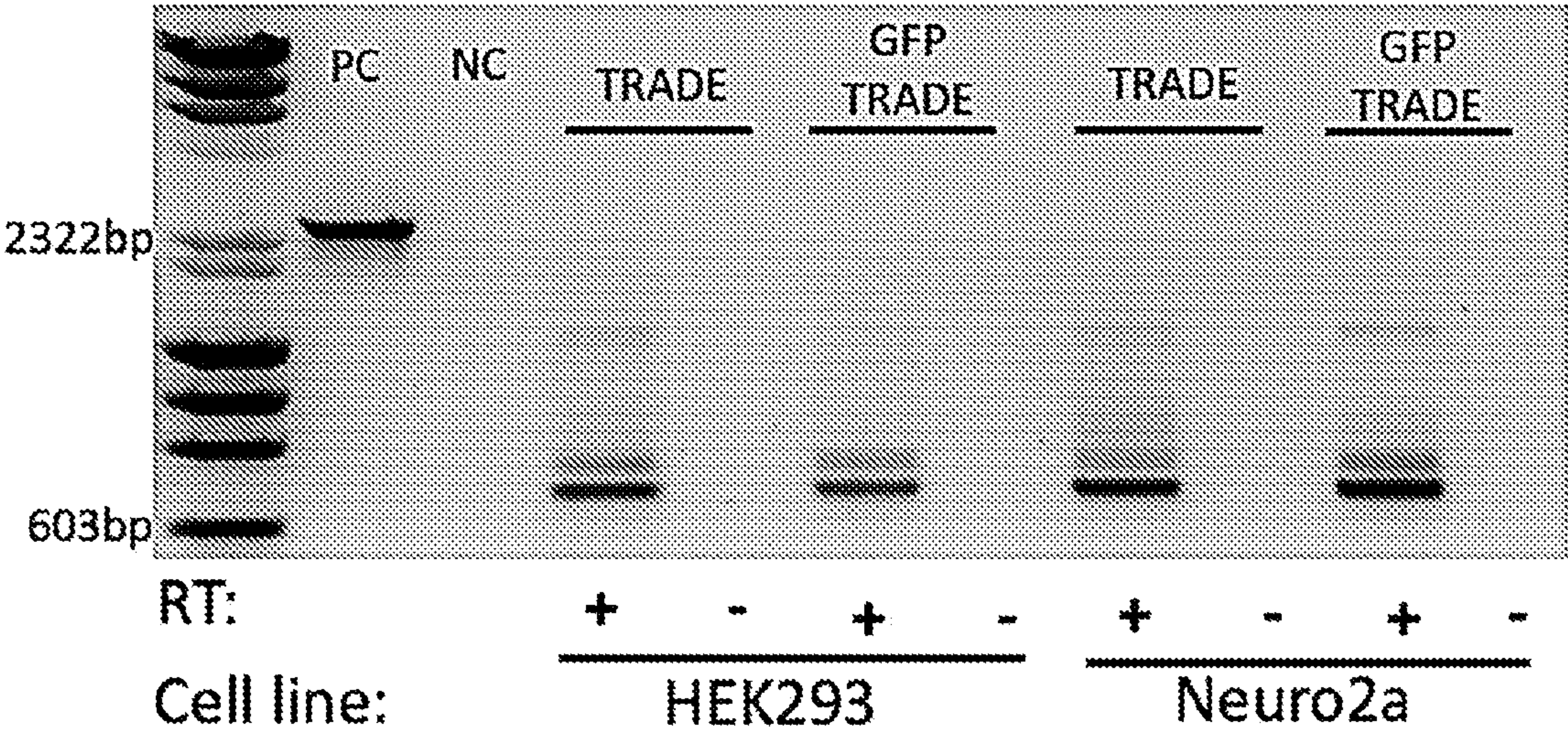


Fig. 4

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Fig. 5

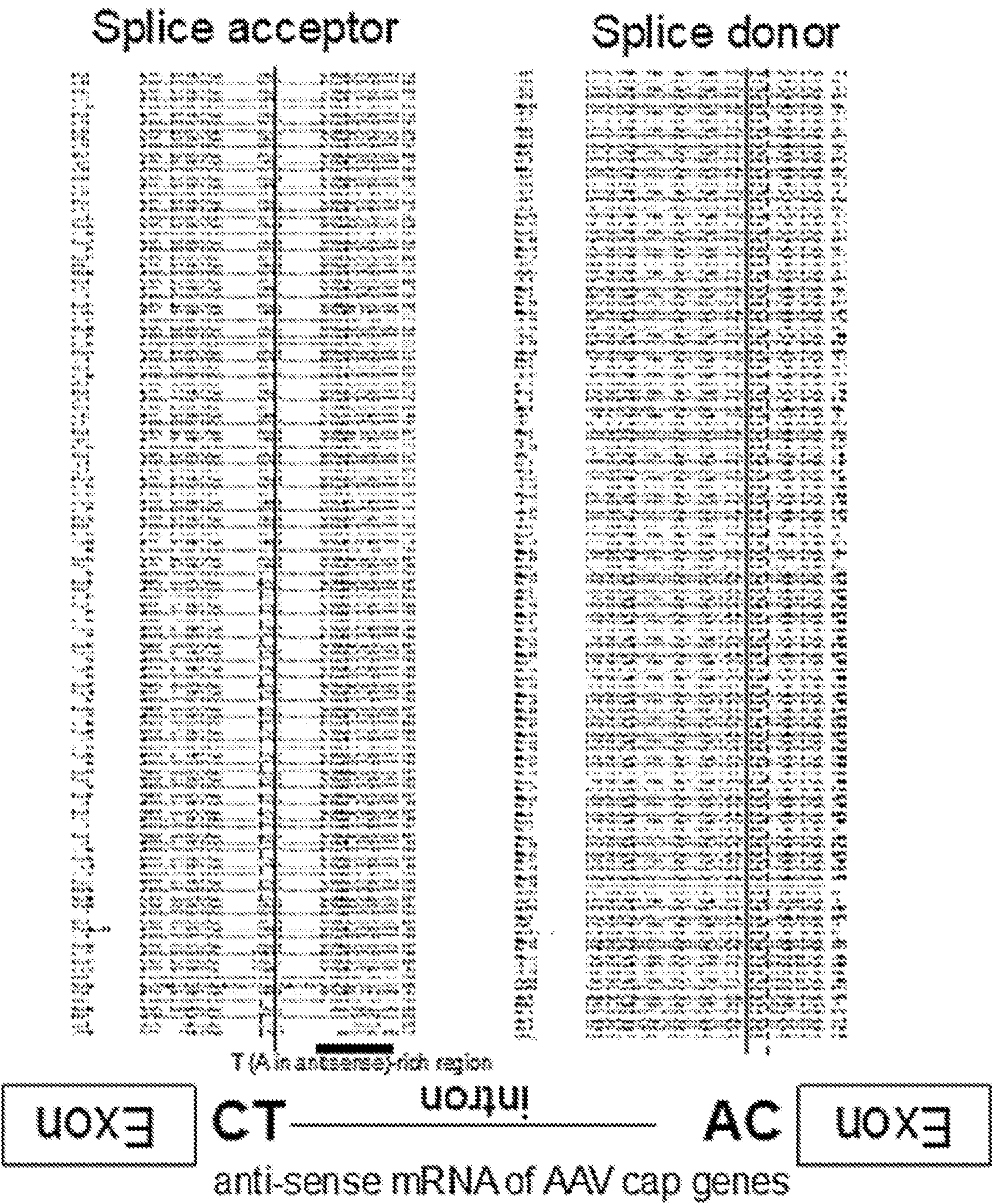


Fig. 6

(A)

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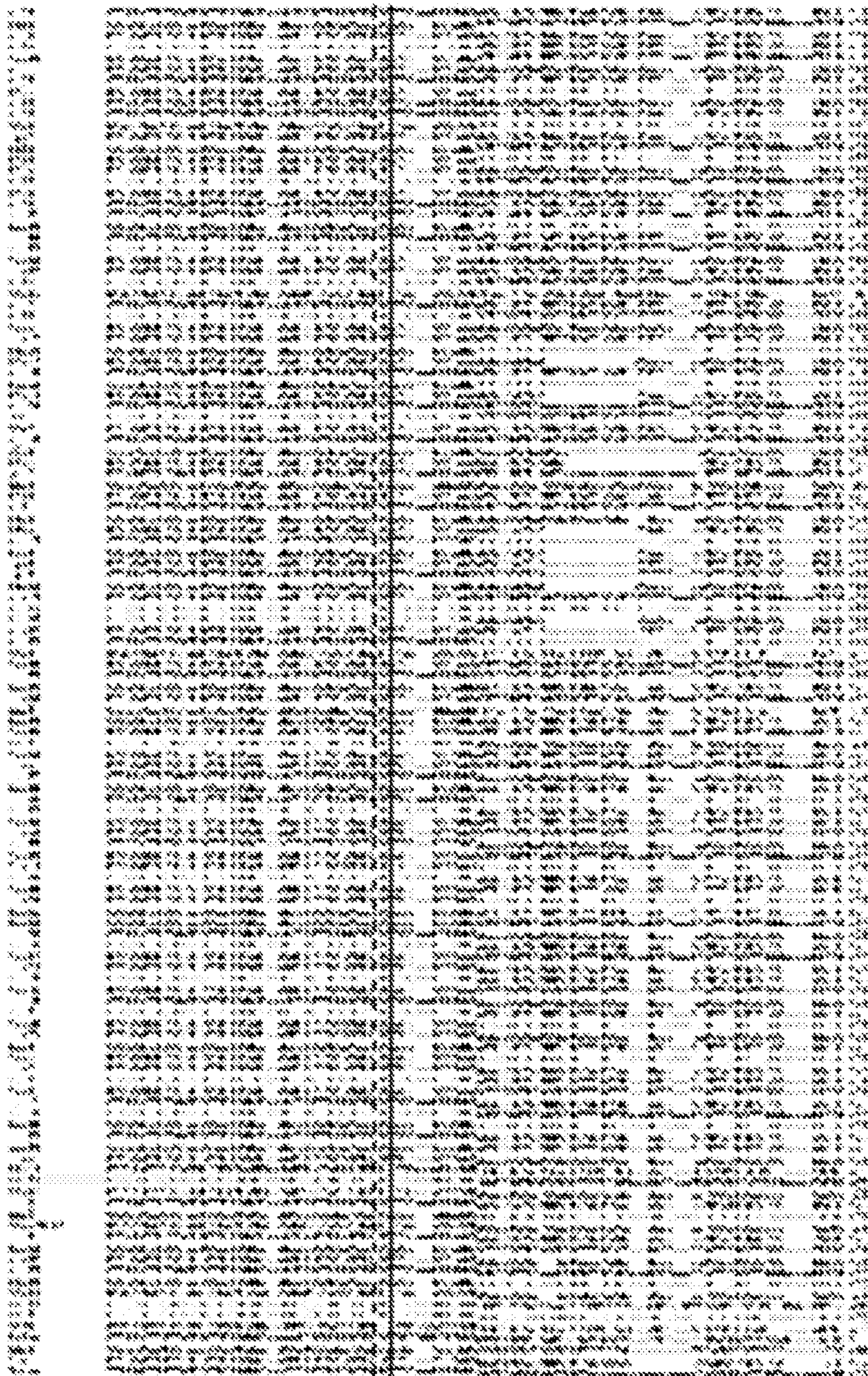
(B)

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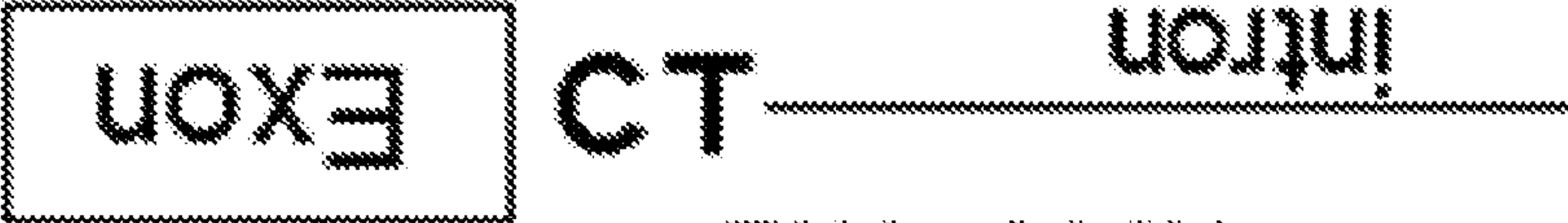
Fig. 7

Fig 8A

Splice acceptor



T(A in antisense)-rich region



anti-sense mRNA of AAV cap genes

Fig. 8B

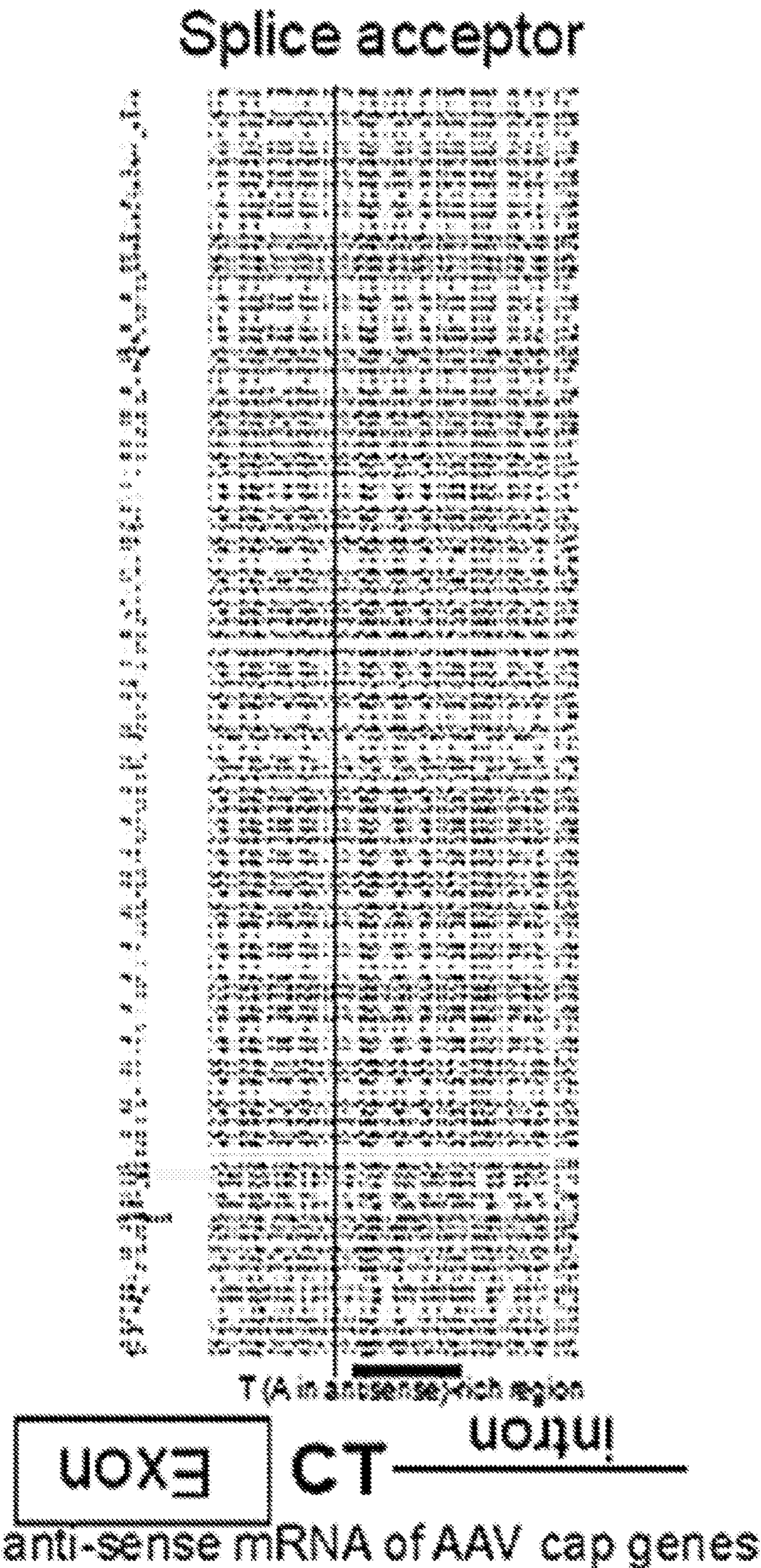


Fig. 8

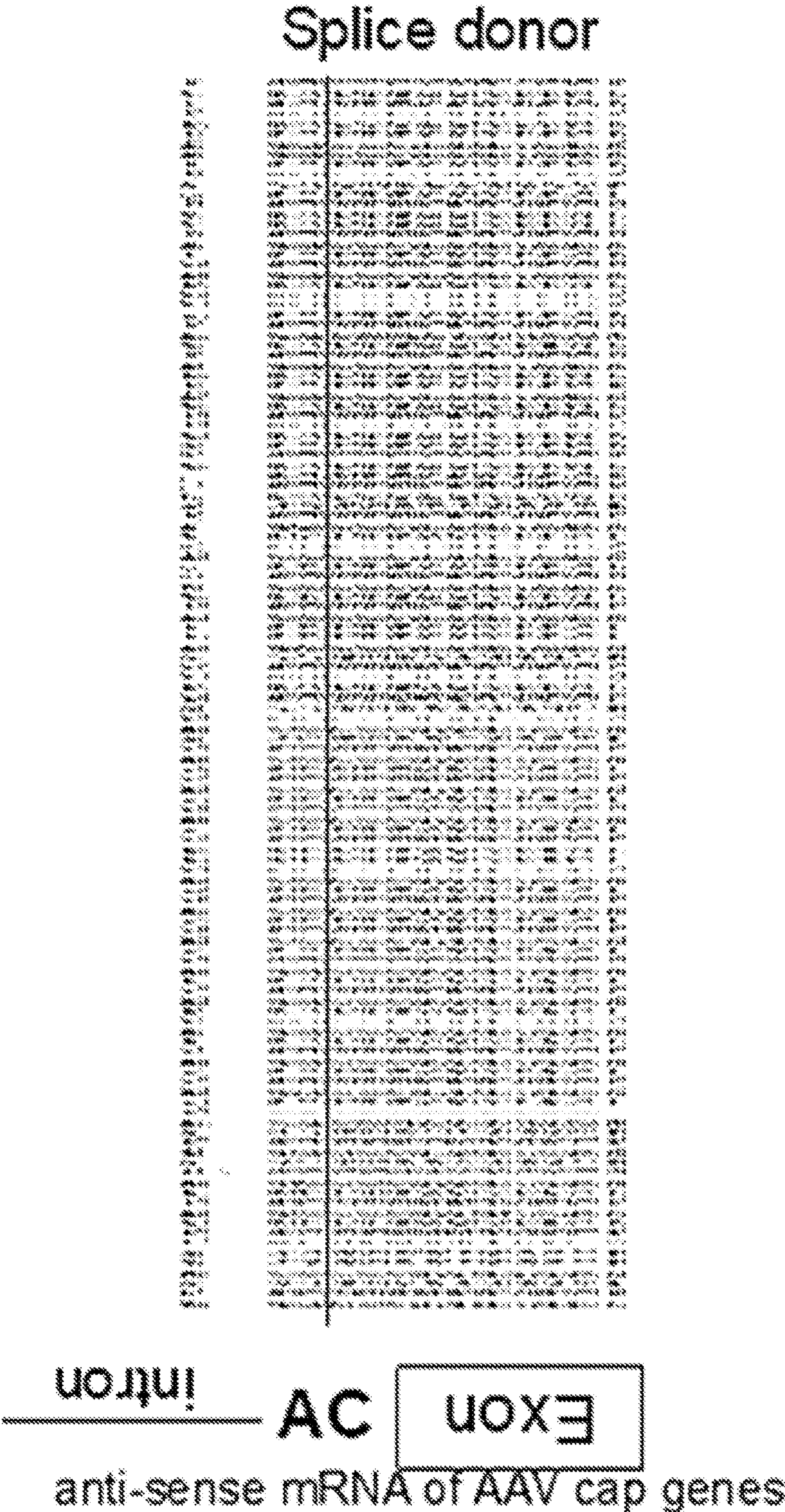


Fig. 9

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Fig. 10

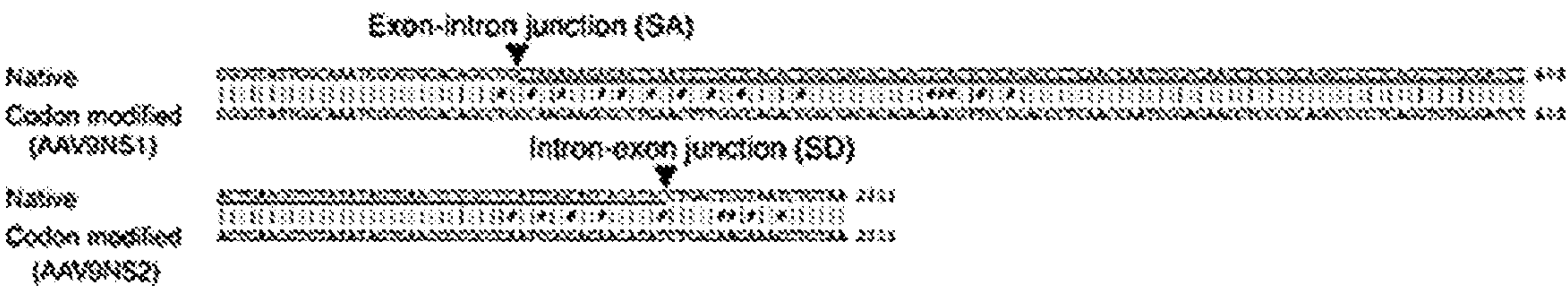


Fig. 11

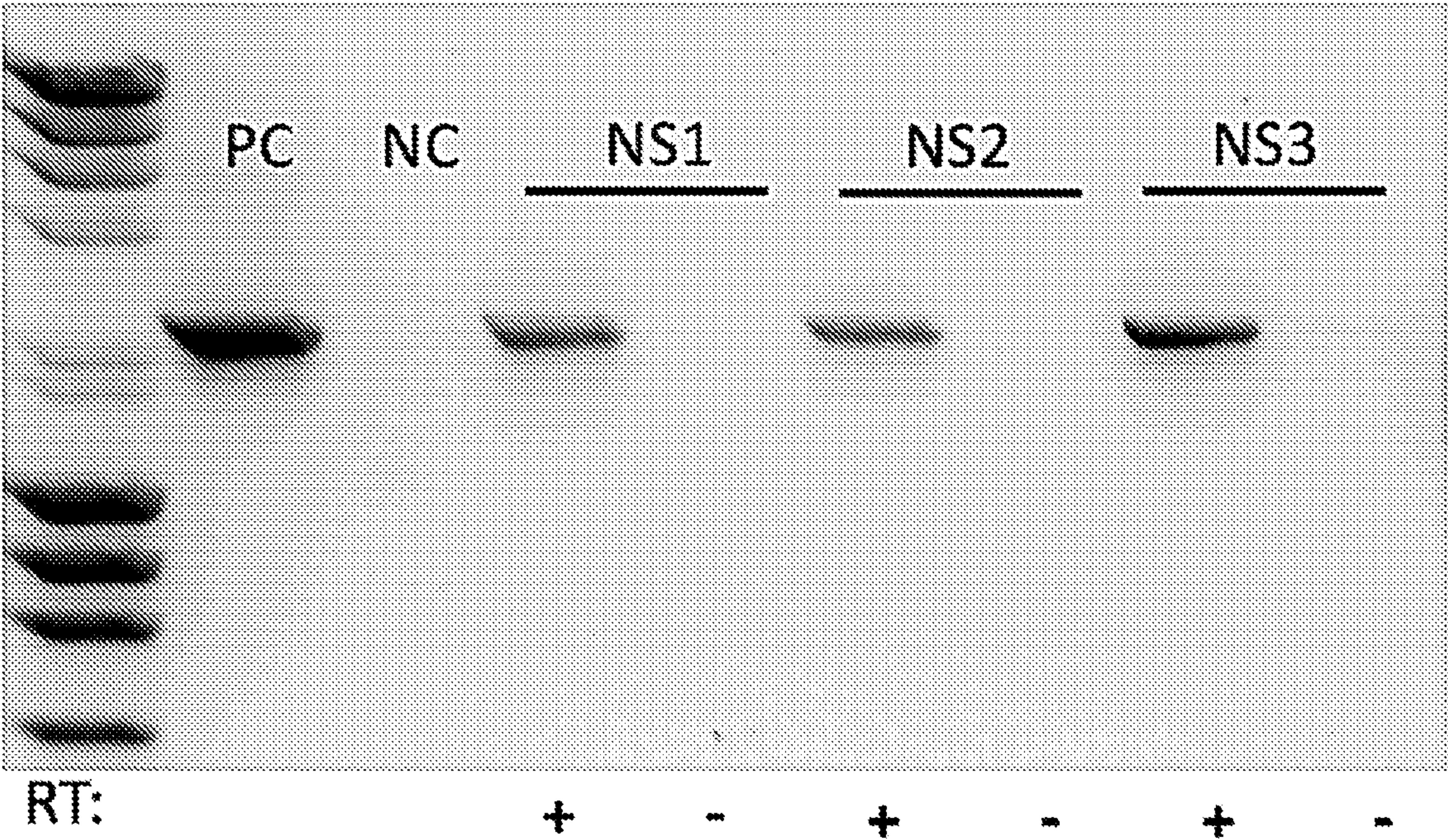


Fig. 12

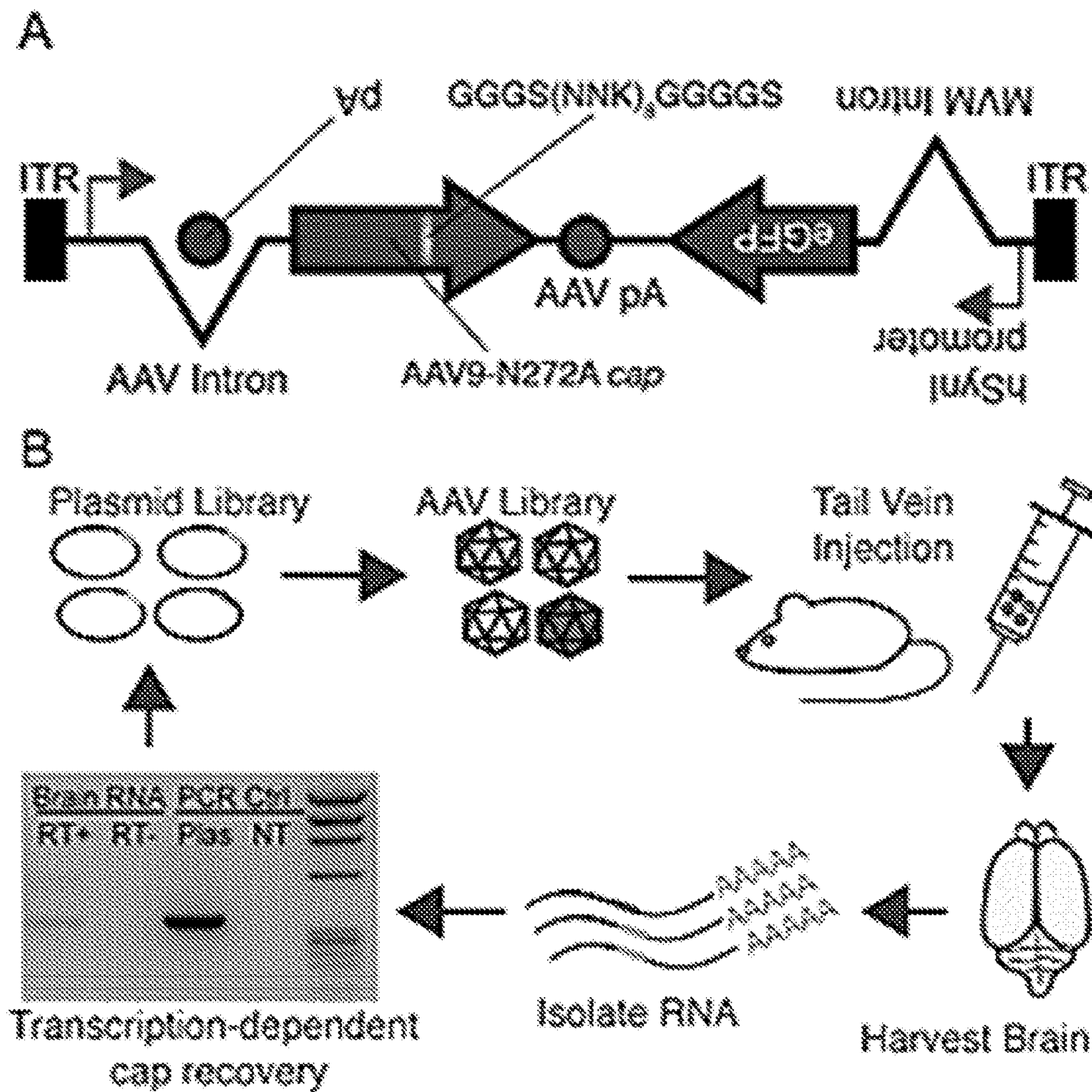
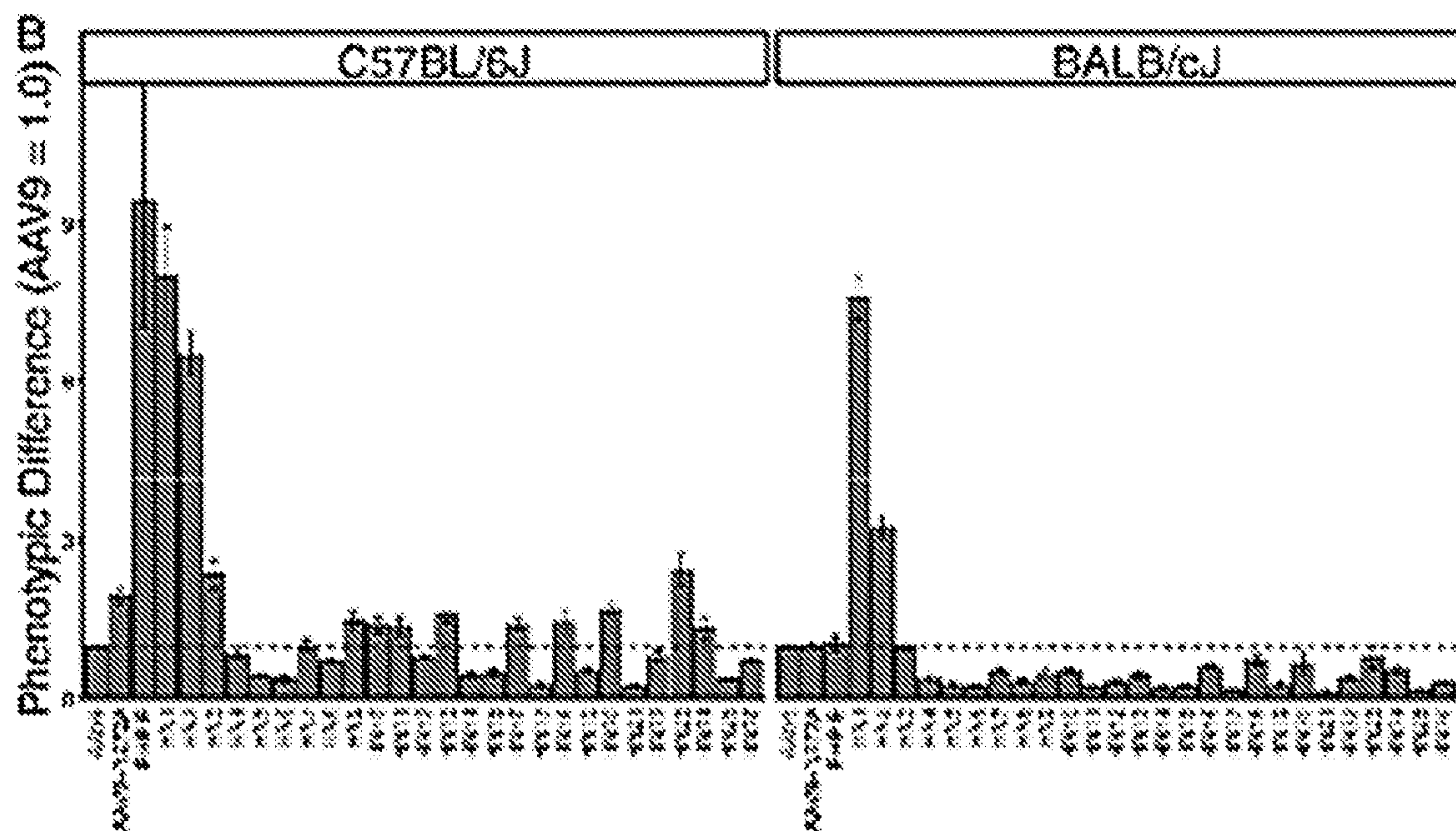
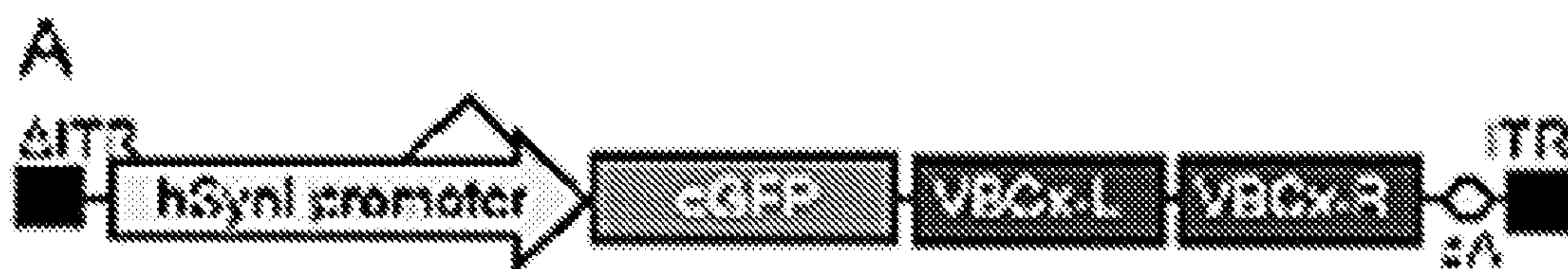


Fig. 13



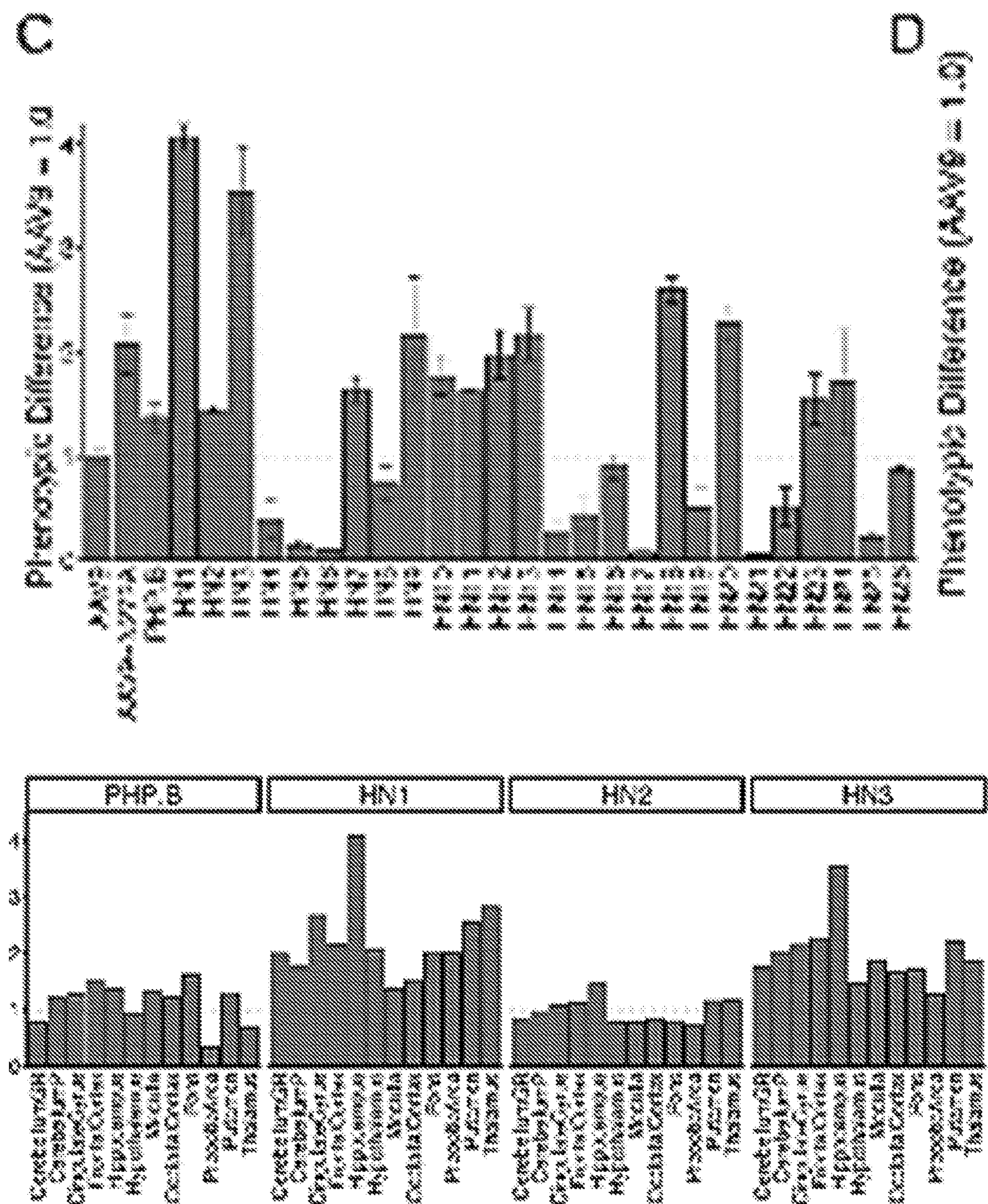
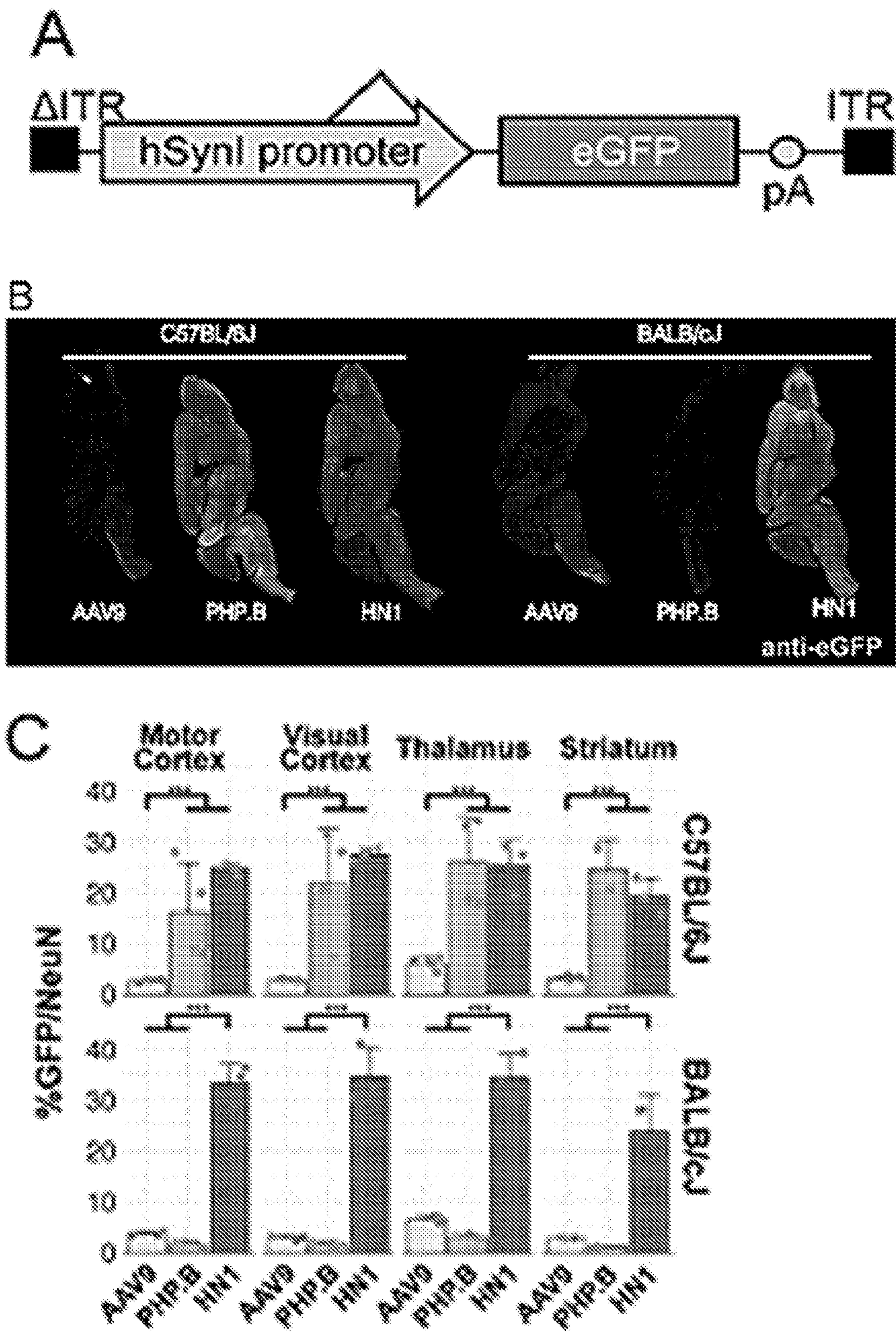


Fig. 14



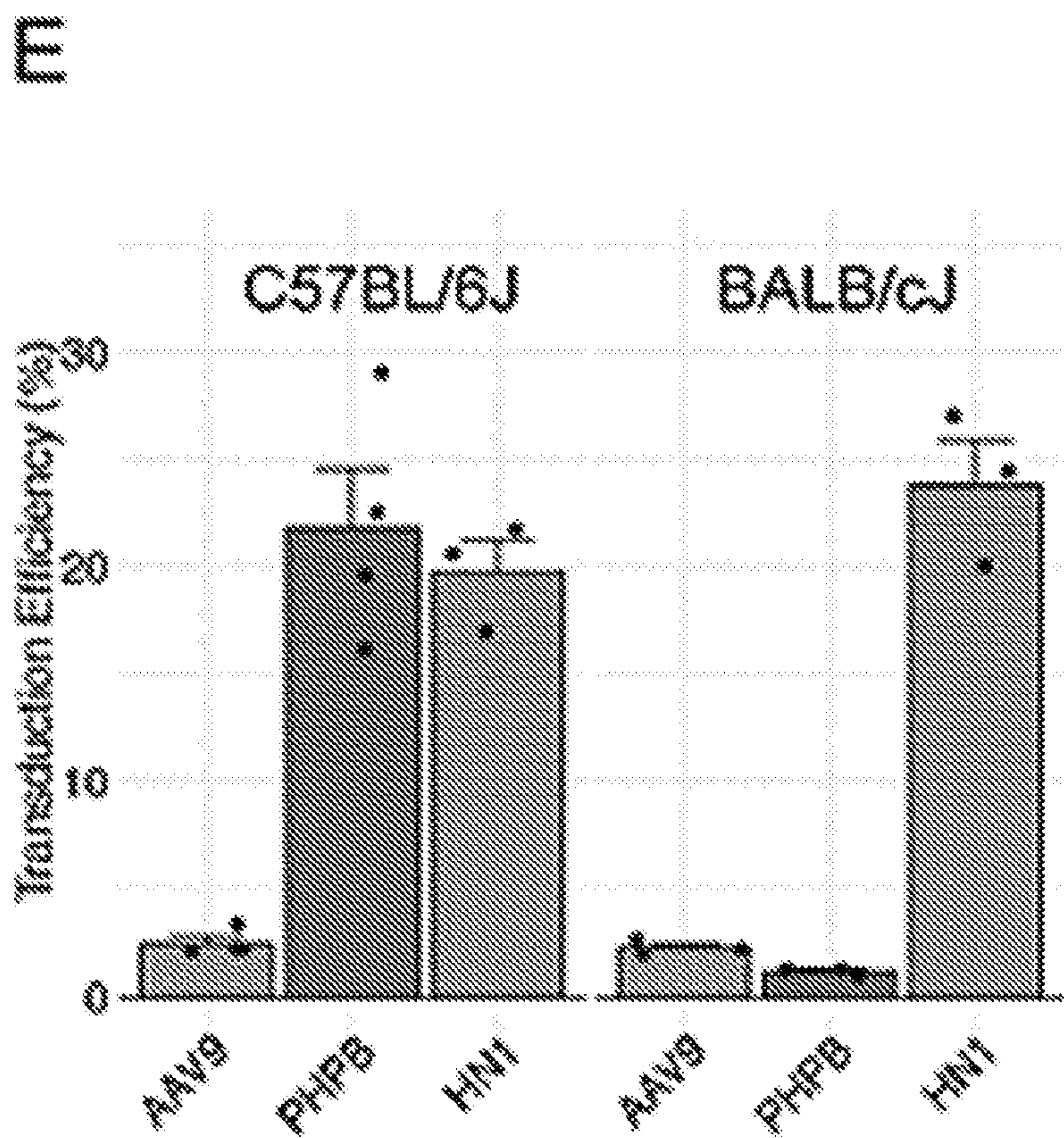
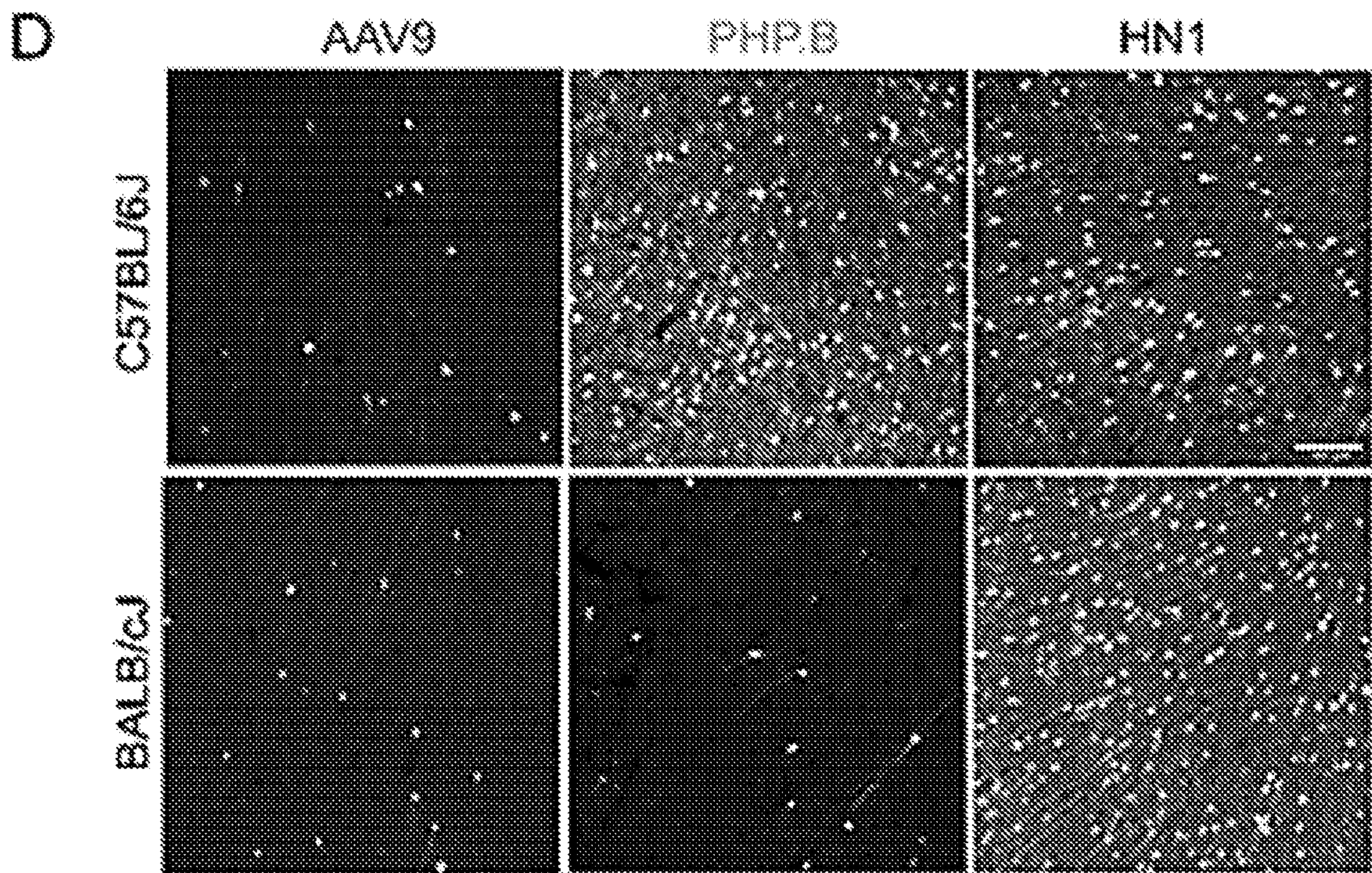


Fig. 15

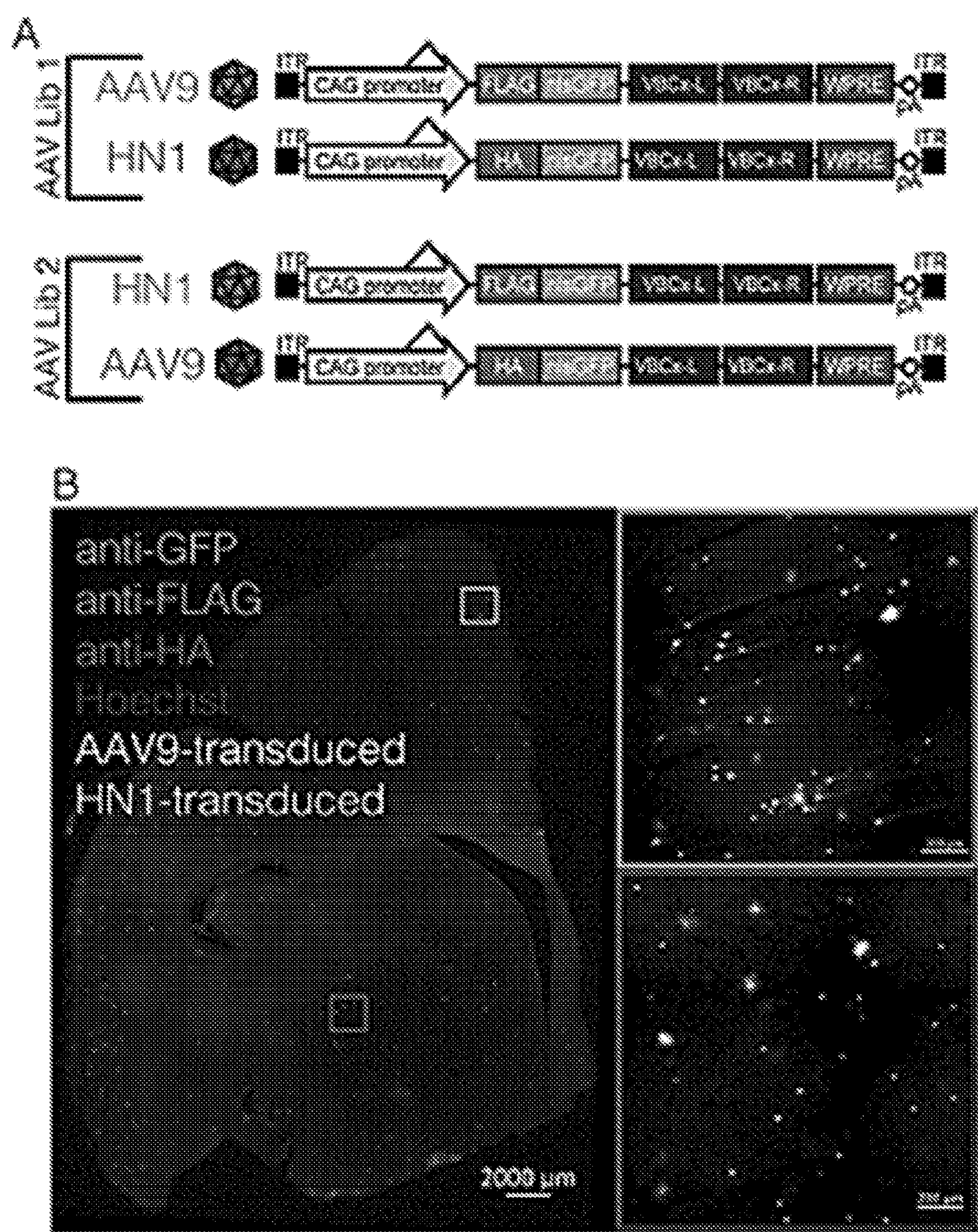
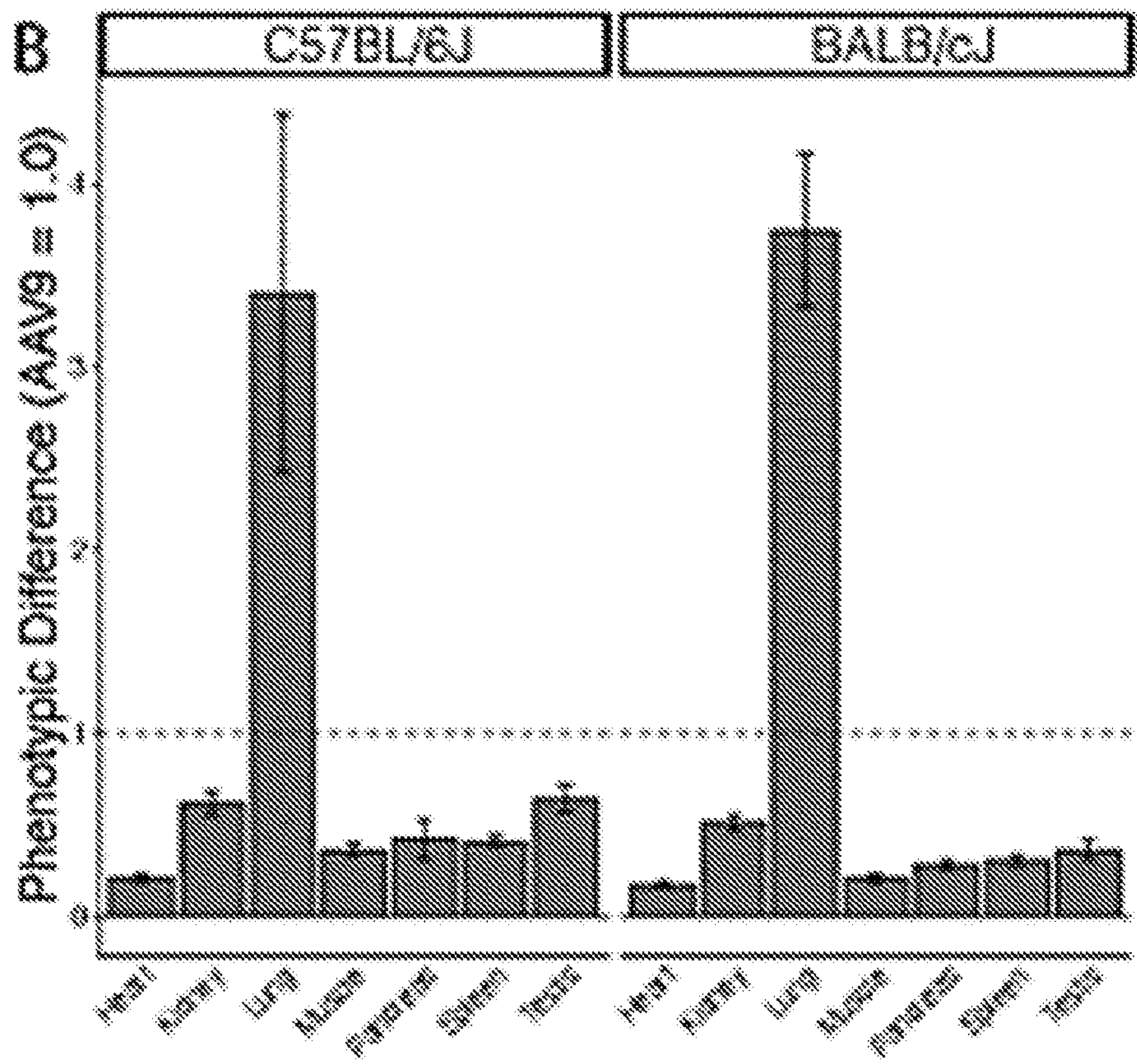
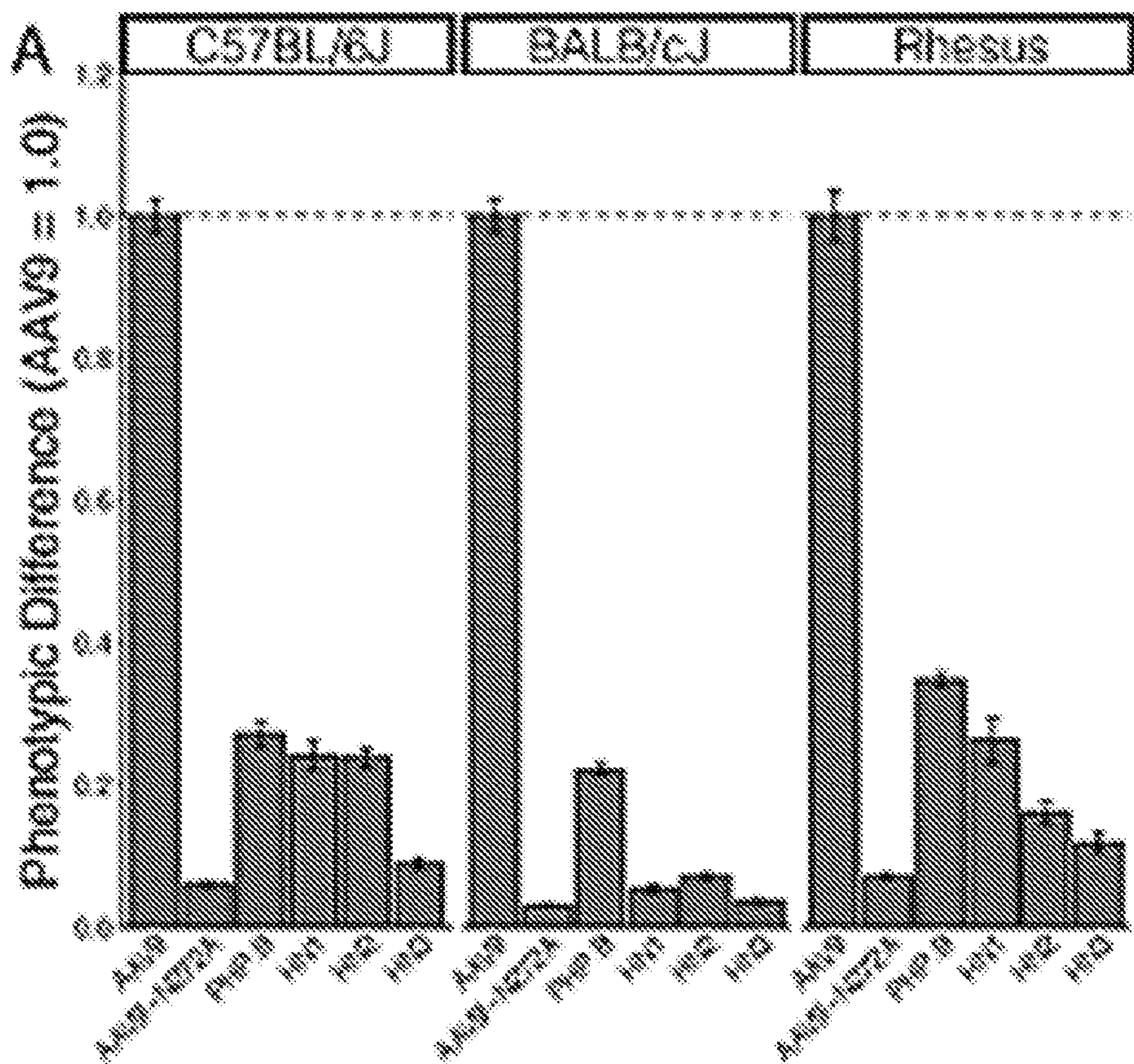


Fig. 16 Validation



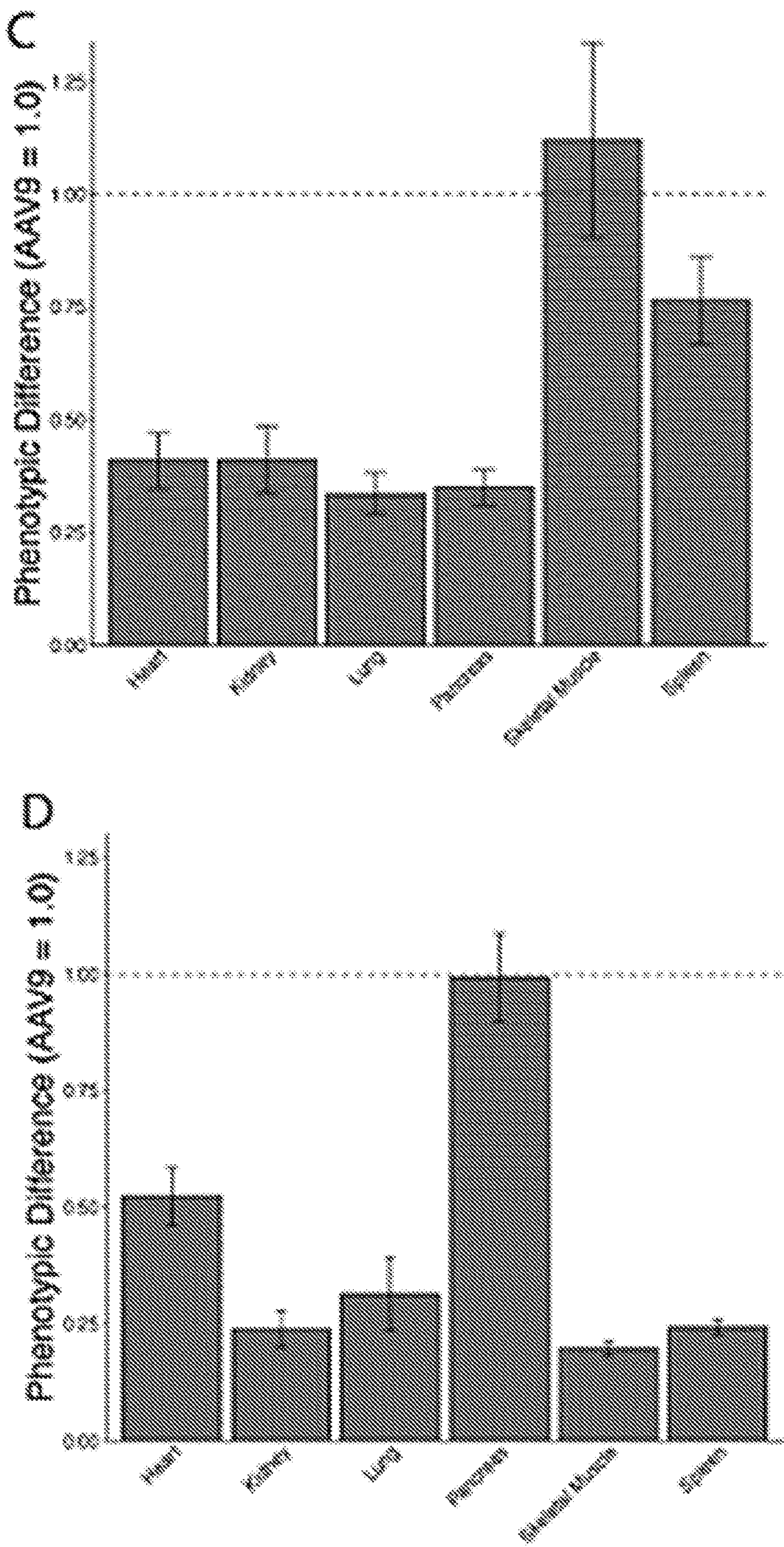
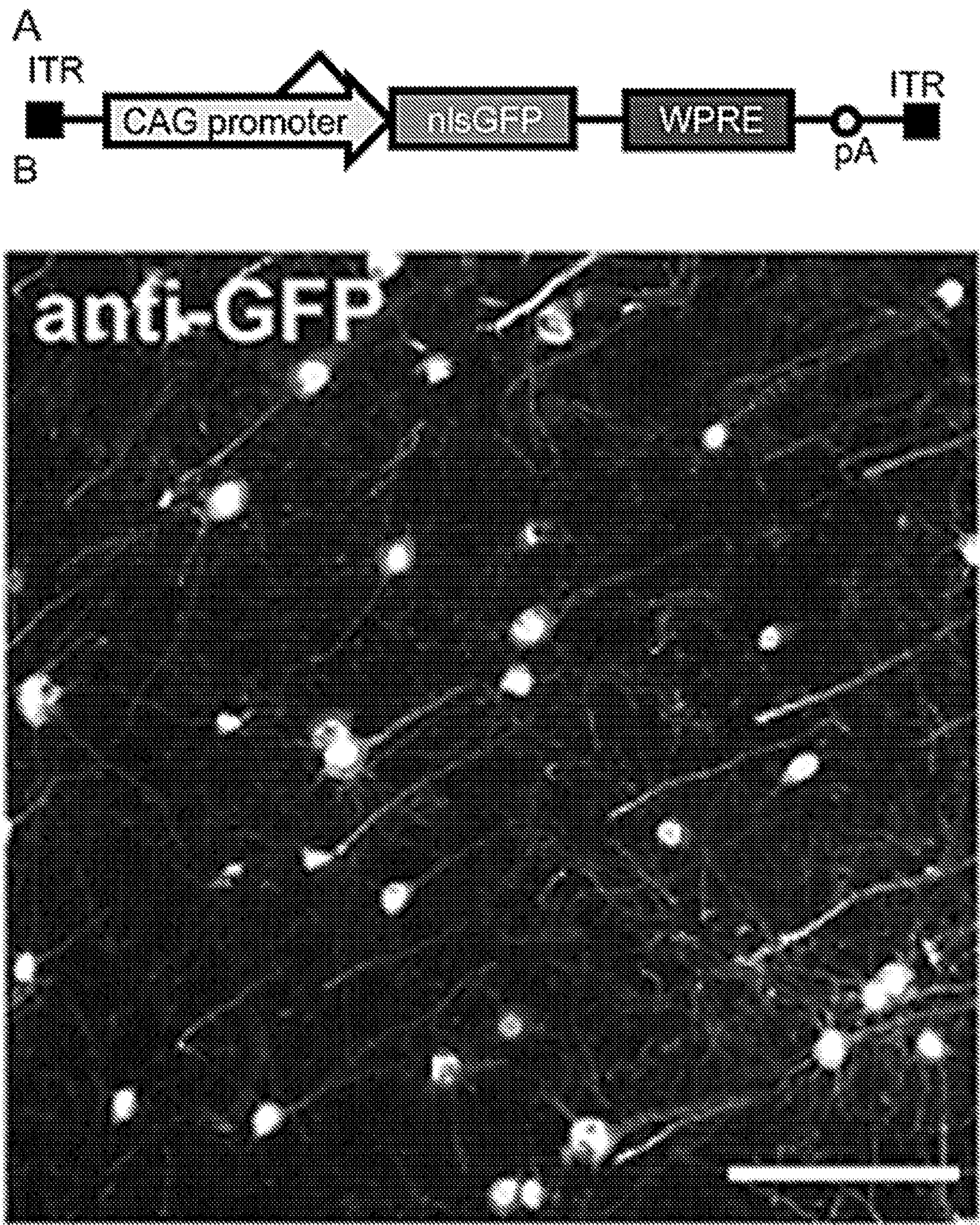
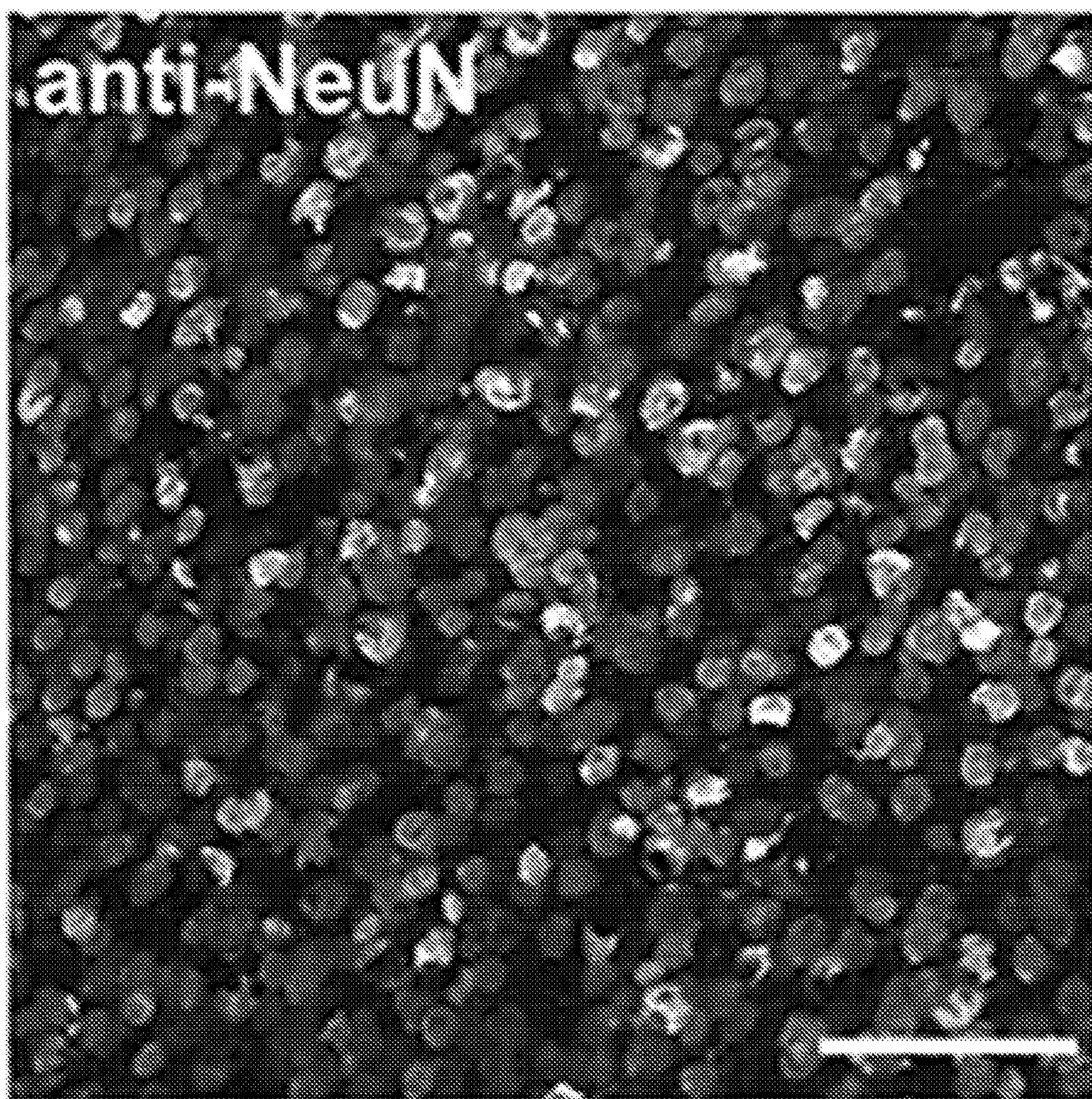


Fig. 17





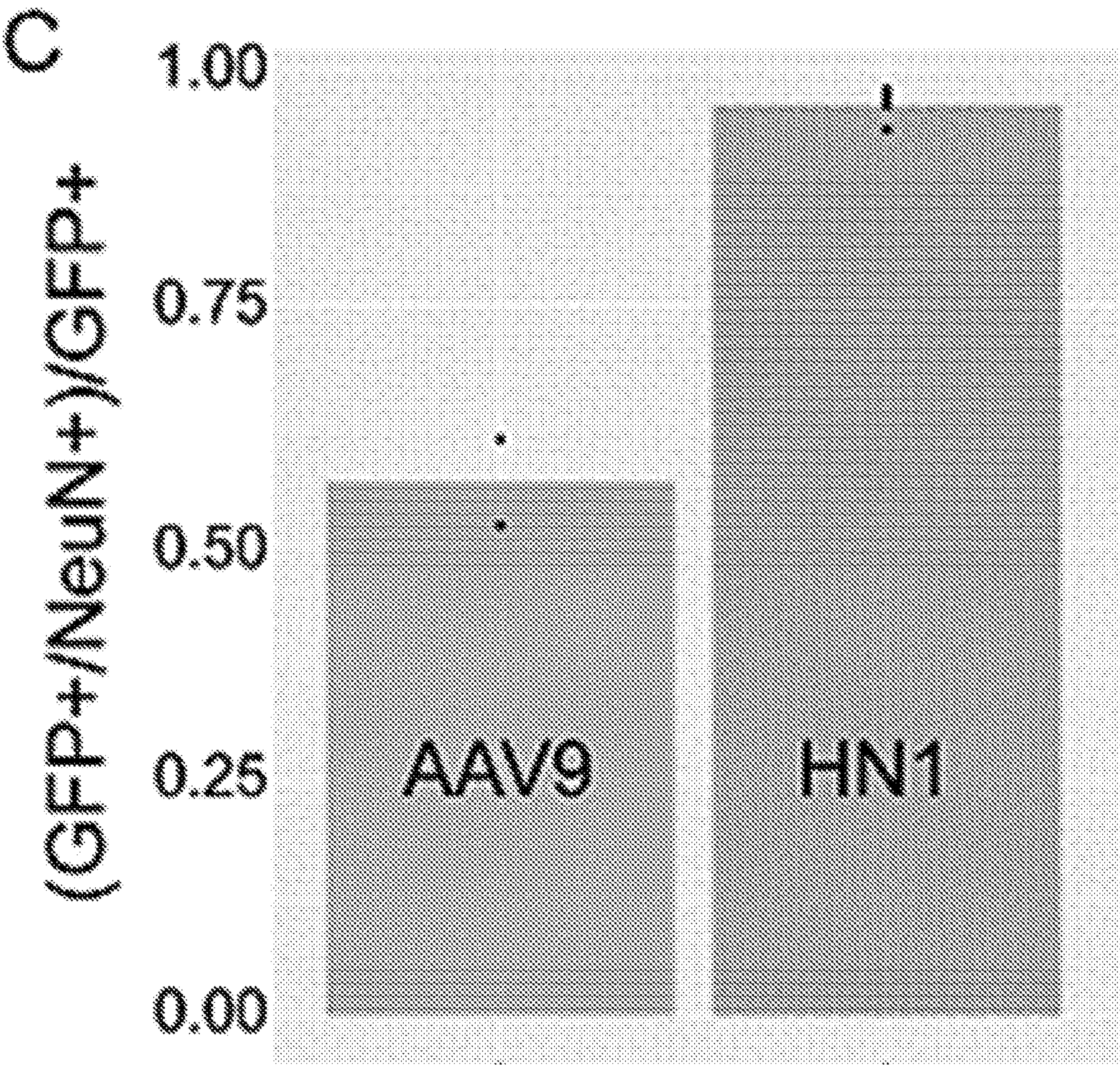


Fig. 18

METHODS FOR USING TRANSCRIPTION-DEPENDENT DIRECTED EVOLUTION OF AAV CAPSIDS

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0001] This invention was made with government support under Grant No. NS088399 awarded by the National Institutes of Health/National Institute of Neurological Disorders and Stroke. The government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] [0001.1] This application is the U.S. National Phase application of PCT/US2020/016273, filed on Jan. 31, 2020, which claims the benefit of U.S. Provisional Application No. 62/799,603 filed on Jan. 31, 2019, all of which are incorporated herein by reference in their entirety.

SEQUENCE LISTING

[0003] [0001.2] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Jan. 21, 2022, is named 60255-701-831-SL.txt and is 108,976 bytes in size.

TECHNICAL FIELD

[0004] This disclosure relates to viral vectors used in gene delivery. More specifically, this disclosure relates to a method for transcription-dependent directed evolution and adeno-associated virus ("AAV") vectors that are selected by using this method.

BACKGROUND

[0005] Recombinant adeno-associated virus ("AAV") vectors are among the most promising for in vivo gene delivery. The usefulness of AAV vectors has been expanded since a number of naturally occurring new serotypes and subtypes were isolated from human and non-human primate tissues. Gao et al., *J Virol* 78, 6381-6388 (2004) and Gao et al., *Proc Natl Acad Sci USA* 99, 11854-11859 (2002). Among the newly-identified AAV isolates, AAV serotype 8 (AAV8) and AAV serotype 9 (AAV9) have gained attention because AAV vectors derived from these two serotypes can transduce a variety of organs including the liver, heart, skeletal muscles and central nervous system with high efficiency following systemic administration. Ghosh et al., *Mol Ther* 15, 750-755 (2007); Pacak et al., *Circ Res* 99, 3-9 (2006); Inagaki et al., *Mol Ther* 14, 45-53 (2006); Zhu et al., *Circulation* 112, 2650-2659 (2005); Wang et al., *Nat Biotechnol* 23, 321-328 (2005); Nakai et al., *J Virol* 79, 214-224 (2005); and Foust et al., *Nature Biotechnol* 23, 321-328 (2009). This robust transduction by AAV8 and 9 vectors has been ascribed to strong tropism for these cell types, efficient cellular uptake of vectors, and/or rapid uncoating of virion shells in cells. Thomas et al., *J Virol* 78, 3110-3122 (2004). In addition, emergence of capsid-engineered AAV vectors with better performance has significantly broadened

the utility of AAV as a vector toolkit. Asokan et al., *Mol Ther* 20, 699-708 (2012).

[0006] A proof-of-concept using AAV-mediated gene therapy has been shown in many preclinical animal models of human diseases. Phase I/II clinical studies have shown promising results for the treatment for hemophilia B (Nathwani et al., *N Engl J Med* 71, 1994-2004 (2014)), lipoprotein lipase deficiency (Carpentier et al., *J Clin Endocrinol Metab* 97, 1635-1644 (2012)), Leber congenital amaurosis (Jacobson et al., *Arch Ophthalmol* 130, 9-24 (2012) and Pierce and Bennett, *Cold Spring Harb Perspect Med* 5, a017285 (2015)), among others (reviewed in Mingozzi and High, *Nat Rev Genet* 12, 341-355 (2011) and Wang et al., *Nat Rev Drug Discov* 18, 358-378 (2019)).

[0007] Despite this promise, human studies have also revealed unexpected issues and potential challenges in AAV-mediated gene therapy. Manno et al., *Nat Med* 12, 342-347 (2006). In addition, despite rapid progress in our understanding of AAV biology and capsid-phenotype relationships (Adachi et al., *Nat Commun* 5, 3075, (2014); Grimm et al., *Hum Gene Ther* 28, 1075-1086, (2017); and Ogden et al., *Science* 366, 1139-1143, (2019)), there remain many desirable properties for clinical AAV vectors that we cannot rationally design.

[0008] To this end, high throughput screening methods for identifying novel AAV capsids with such desirable phenotypes have been employed. In particular, the development of in vivo AAV library selection strategies have produced a variety of designer AAV variants capable of highly efficient transduction of previously refractory cell types (reviewed in Kotterman and Schaffer, *Nat Rev Genet* 15, 445-451 (2014) and Grimm et al., *Mol Ther* 23, 1819-1831 (2015)).

[0009] The earliest attempts at in vivo library selection (1st Generation) relied on recovery of vector genome DNA from dissected tissue. Theoretically, this strategy results in recovery of both effective AAV variants, as well as AAV variants that mediate some, but not all of the steps required for vector-mediated transgene expression (FIG. 1). Thus, screening a diverse library of synthetic AAV variants potentially leads to a high background recovery of AAV variants that are completely ineffective gene therapy vectors. Furthermore, targeting a specific cell type requires further processing, such as fluorescence-activated cell sorting or laser capture microdissection. Nonetheless, there have been several reports of successfully employing this technology. Excoffon et al., *Proc Natl Acad Sci U S A* 106, 3865-3870 (2009); Grimm et al., *J Virol* 82, 5887-5911 (2008); Lisowski et al., *Nature* 506, 382-386 (2014); and Dalkara et al., *Sci Transl Med* 5, 189ra176 (2013). However, a landmark study in 2016 by Deverman et al. showed that this process could be greatly improved upon by using a Cre-dependent selection strategy (2nd Generation). Deverman et al., *Nat Biotechnol* 34, 204-209 (2016). Cre-dependent library selection takes advantage of the selective ability of Cre recombinase to act on double-stranded DNA, but not single-stranded DNA, in order to invert vector genome DNA containing a primer binding sequence. Inversion of this sequence allows for direction-selective PCR to specifically amplify viral DNA delivered to cells by AAV variants that are able to undergo the late stage of transduction at which double stranded DNA is formed from single-stranded AAV genomes. In addition, the use of Cre driver lines facilitates selective expression of Cre recombinase in a cell type-specific manner, allowing for selection of novel AAV var-

iants that efficiently transduce. Indeed, the use of Cre-dependent selection allowed the authors to develop an AAV9 variant, AAV-PHP.B, that is capable of 40 times greater transduction than the parental AAV9 following systemic administration in C57BL/6J mice. Deverman et al., *Nat Biotechnol* 34, 204-209 (2016). Unfortunately, it has recently become clear that the enhancement exhibited by AAV-PHP.B in mice does not translate to the non-human primate context (Matsuzaki et al. 2018 and Hordeaux et al. 2019). Surprisingly, the enhancement does not even extend to all commonly used mouse strains (Matsuzaki et al. 2018 and Hordeaux et al. 2019). There is, therefore, a strong impetus to accelerate the development of clinically relevant AAV vectors by performing AAV library selection experiments in primate models. However, unlike the AAV variant selection in mice where a plethora of cell type-specific transgenic Cre driver lines are already established, Cre-dependent selection is not tractable in clinically relevant large animals, including non-human primates, because Cre transgenic animals are not readily available.

[0010] We therefore sought to develop a next-generation selection strategy (3rd Generation) with similar or better selective stringency as that provided by Cre-dependent selection, but without the need for Cre recombinase. In order to accomplish this goal, we developed the TRANscription-dependent Directed Evolution system, or TRADE. In the transcription-dependent selection, we express the AAV cap gene as a non-coding antisense mRNA driven by a cell type-specific enhancer-promoter. Recovery of this antisense transcript by RT-PCR allows for stringent recovery of AAV cap genes at the level of vector-mediated mRNA expression in a specific cell type without the use of Cre recombinase. Targeting of different cell types merely requires cloning of a different cell type-specific enhancer-promoter into the plasmid construct. Thus, TRADE is a highly flexible system that can be applied in a wide variety of contexts, including the non-human primate context for development of enhanced AAV vectors for clinical gene therapy. Note that the same principle can be used for expressing AAV cap gene in an sense orientation. However, the sense strand approach results in expression of immunogenic capsid proteins in target cells and is therefore less ideal than the antisense strand approach employed by the TRADE system.

SUMMARY

[0011] This disclosure provides a next-generation directed evolution strategy, termed TRANscription-dependent Directed Evolution (“TRADE”), that selects for AAV capsid transduction at the level of cell type-specific or ubiquitous mRNA expression. The method described herein provides the following advantages over Cre recombination-based AAV targeted evolution (“CREATE”), the most contemporary methods for AAV capsid directed evolution reported in the literature. Deverman et al., *Nat Biotech* 34, 204-209 (2016). First, the CREATE system requires Cre expression, which can be attained either by exogenously-delivered Cre expression or by the use of Cre-transgenic animals. In contrast, the TRADE system does not require Cre-transgenic animals; therefore, it can be applied to animals and cultured cells derived from any animal species and can be readily adapted to large animals, including non-human primates. Second, unlike the CREATE system, in which the cell-type specific selection is applied at the level of AAV viral gen-

ome conversion from single-stranded DNA to double-stranded DNA, TRADE allows for cell type-specific selection at the level of AAV genome transcription. Therefore, the TRADE system can provide greater selective pressure than the CREATE system. Third, multiple directed evolution schemes (e.g., neuron-specific, astrocyte-specific, oligodendrocyte-specific, and microglia-specific) can be integrated into one AAV capsid library and selection for AAV vectors targeting each cell type can be performed in a single animal. Fourth, any cell type-specific or tissue/organ-specific enhancers/promoters or ubiquitous enhancers/promoters can be readily used for AAV capsid directed evolution aimed at identification of cell type-specific or ubiquitous novel AAV capsids with enhanced potency. Fifth, the TRADE methodology is not limited to the genus Dependoparvovirus, including the common AAVs that have been used for gene delivery, but can also be applied more broadly to the family Parvoviridae, including in the genera Bocaparvoviruses and Erythroparvoviruses other than AAV (e.g., bocaviruses), and even more broadly to an DNA virus.

[0012] This disclosure also provides novel AAV capsid mutants. TRADE technology was used to identify novel AAV vectors that mediate neuronal transduction in the brain following intravenous administration. Application of TRADE in C57BL/6J mice and a rhesus macaque resulted in the identification of new AAV capsids that can transduce neurons more efficiently and more specifically than AAV9 in the mouse and non-human primate brain following intravenous administration. In addition, we identified a novel AAV capsid that can transduce an undefined cell population or populations, that reside in the lung and are potentially of neuronal origin, 5 to 18 times better than the AAV9.

[0013] The present disclosure also provides a method to prevent splicing of antisense mRNA of the AAV capsid gene. Antisense pre-mRNA transcribed from the AAV cap gene open reading frame (“ORF”) can be spliced making (a) truncated mRNA species. To our knowledge, this is a new discovery that has never previously been reported. Such splicing has the potential to hinder effective recovery of full-length antisense mRNA of the AAV cap ORF, which is essential for TRADE when a wide region of the cap ORF is mutagenized. This disclosure provides a novel strategy to prevent splicing of antisense mRNA of the cap gene.

[0014] The TRADE system described herein uses antisense mRNA to recover capsid sequence information, TRADE using sense strand mRNA (i.e., sense strand TRADE) is also feasible using the same principle. However, it should be noted that the sense strand TRADE approach results in expression of immunogenic capsid proteins in target cells and therefore is presumably less ideal than the antisense strand approach.

BRIEF DESCRIPTION OF THE FIGURES

[0015] FIG. 1 An overview of in vivo library selection strategies utilized for directed evolution of the AAV capsid. AAV vector-mediated transduction is a multi-step process that requires the virion to overcome extracellular barriers, bind receptors on the target cell, enter the cell via endocytosis, escape the endosome, traffic to the nucleus, uncoat, achieve a double-stranded DNA configuration, and finally undergo transcription/translation. The earliest strategies for in vivo library selection (1st Gen) recovered all vector genome DNA from a tissue sample. Theoretically, this strategy

would recover both effective AAV variants, as well as AAV variants that mediate some, but not all of the steps required for vector-mediated transgene expression. In addition, this strategy would also recover AAV vector genome DNA from AAV vector particles that do not enter cells and stay in the extracellular matrix. Thus, screening a diverse library of synthetic AAV variants would lead to a relatively high background recovery of AAV variants that are completely ineffective gene therapy vectors. Furthermore, focusing on a specific cell type requires further processing, such as fluorescence-activated cell sorting (FACS) or laser capture microdissection (LCM). The second generation of library selection (2nd Gen) substantially increased selection stringency by utilizing Cre-dependent recovery of only those AAV variants that are able to achieve the double-strand DNA stage of transduction. Furthermore, driving the expression of Cre with a cell type-specific enhancer-promoter allows for targeting of a specific cell type while retaining the benefits of processing bulk tissue samples. The third generation of library selection (3rd Gen) further builds on AAV directed evolution technology by employing transcription-dependent recovery of AAV variants that are able to mediate transgene mRNA expression from a cell type-specific enhancer-promoter, without the requirement of Cre expression.

[0016] FIG. 2 Principle of TRADE. (A) A map of the AAV vector genome in a TRADE configuration (AAV-TRADE). A cell type-specific enhancer-promoter is placed in an antisense orientation to drive AAV cap gene transcription expression as antisense mRNA. A polyadenylation signal (pA) derived from the simian virus 40 (SV40) genome is placed within the AAV genome intron in an antisense orientation to terminate antisense AAV cap gene mRNA transcription. The eGFP open-reading frame (ORF) can be placed as depicted to serve as a reporter or facilitate enrichment of transduced cells by FACS; however, such a marker gene is not strictly necessary for TRADE. A ubiquitous promoter such as the CAG promoter can be also used in TRADE in place of cell type-specific enhancers-promoters to identify AAV capsids that can transduce a variety of cell types. A cell type-specific enhancer-promoter can be placed upstream of the AAV cap gene ORF to drive expression of the AAV cap gene mRNA transcripts in a sense orientation (i.e., sense strand TRADE). However, this approach may not be ideal for TRADE because AAV capsid protein would be expressed in target cells, which may result in undesired biological consequences in the directed evolution process. (B) During AAV vector production in HEK293 cells, and in the presence of the adenoviral helper functions, the AAV2 viral p40 promoter drives cap gene expression (forward transcription) and cell type-specific transcripts are suppressed, leading to successful production of recombinant AAV vectors containing the AAV-TRADE vector genome. Following transduction of a specific cell type, the cell type-specific enhancer-promoter is activated, driving expression of eGFP and the antisense cap mRNA sequence, while the transcriptional activity of the p40 promoter remains inactive in transduced cells due to a lack of adenoviral helper functions. The entire cap gene ORF can be recovered by reverse transcription (RT)-PCR using antisense cap gene mRNA as a template that is expressed in a cell type-specific manner. We have observed that recombinant AAV vectors can be produced successfully at high levels even in the presence of antisense mRNA transcripts

expressed due to leaky expression from the human synapsin I gene (hSynI) enhancer-promoter in HEK293 cells. We have also observed that recombinant AAV vectors can be produced successfully at high titers even when we use the CAG promoter that drives expression of antisense AAV cap mRNA transcripts at high levels.

[0017] FIG. 3 Validation of the TRADE system targeting brain neurons. (A) A map of the AAV-PHP.B-hSynI-GFP-TRADE vector genome. (B) To verify the TRADE system, this AAV vector genome was packaged into the AAV-PHP.B capsid as a single-stranded DNA genome and the resulting AAV vector was injected into two 8-week-old C57BL/6J mice intravenously at a dose of 3×10^{11} vector genomes (vg) per mouse. Brain tissue was harvested 12 days post-injection. The brain tissue from one animal was fixed with 4% paraformaldehyde and used for immunofluorescence microscopy and the brain tissue from the other animal was unfixed and used for molecular analysis of AAV vector genome DNA and RNA. (C) Immunofluorescence microscopy image of brain sections stained with anti-GFP antibody confirmed expression of the cell type-specific enhancer-promoter-driven transcript. (D) hSynI enhancer-promoter-driven GFP expression was observed specifically in neurons (anti-HuC/D+). (E) RT-PCR was used to recover the full-length cap ORF sequence (RT+). RT-, a no reverse transcriptase control; Plas, a positive control obtained with DNA-PCR using a plasmid template containing the AAV-PHP.B-hSynI-GFP-TRADE vector genome sequence; NT, a no template PCR control. (F) Sanger sequencing of the RT-PCR product revealed expected splicing of the MVM intron in the antisense transcripts expressed by the hSynI enhancer-promoter (SEQ ID NO:190). The exon-exon junction is highlighted with gray. (G) Sanger sequencing confirmed the insertion of the PHP.B peptide (highlighted with gray) (SEQ ID NO:191).

[0018] FIG. 4 Splicing of the antisense mRNA of the AAV9 cap ORF. Two cell lines, HEK293 and Neuro2a, were transfected with plasmids containing the AAV9 cap ORF in the TRADE configuration, with or without a GFP reporter. They are indicated as “GFP TRADE” and “TRADE”, respectively, in the figure. Cells were harvested 3 days post-transfection, RNA was extracted, and RT-PCR was performed with a set of PCR primers that amplify the full cap ORF sequence. Instead of recovering the expected amplicon size of 2.4 kb as shown in the positive control (PC) lane, we consistently recovered amplicons of approximately 0.7 kb. Sanger sequencing of these RT-PCR products identified a truncation consistent with splicing of a 1.7 kb region of the AAV9 cap ORF indicated in FIG. 5. PC, a positive control using a plasmid template containing the AAV-PHP.B-hSynI-GFP-TRADE vector genome sequence; NC, a no template PCR control.

[0019] FIG. 5 An intron identified in antisense mRNA derived from the AAV9 cap gene (SEQ ID NO:192). When the AAV-PHP.B cap gene sequence was transcribed in an antisense orientation in HEK293 cells or Neuro2a cells under the control of the neuron-specific human synapsin I (hSynI) enhancer-promoter, a splicing event was identified with cryptic splice donor and splice acceptor sites (please refer to FIG. 6 as well). The underlined sequence indicates the intron found within the AAV9 cap ORF. This splicing event was not observed in mouse brain neurons. It should be noted that (1) although the hSynI enhancer-promoter has been used as a neuron-specific element, it has been shown to

drive leaky expression in HEK293 cells; and (2) the AAV9 cap ORF sequence used for the intron splicing experiment had the following silent mutations near the C-terminus: gaaccccgcccccattggcacGCgTtacCTGACTCGTAATCTGTAA (SEQ ID NO:1). The intron sequence is underlined, and the silent mutations that have been introduced into the intron to create an Mlul (ACGCGT) recognition site are indicated in uppercase.

[0020] FIG. 6 Cryptic splice donor (SD) and splice acceptor (SA) sites with the common features of exon-intron junctions present in the AAV cap ORFs in an antisense orientation. Nucleotide sequences of the cap genes derived from 122 naturally occurring AAV strains (serotypes and variants) are aligned using a multiple sequence alignment program (SEQ ID NO 223-316). The exon-intron junctions identified in the AAV9 cap ORF-derived antisense mRNA are indicated with solid lines. The dashed line in the splice acceptor region indicates putative splice acceptor sites in the AAV cap ORFs devoid of the splice acceptor AG/TC sequence at the position expected from the sequence conservation. The dashed line in the splice donor region indicates the splice donor site identified in the AAV3 cap ORF-derived antisense mRNA (please refer to FIG. 7). The GT/CA splice donor sites and the AG/TC splice acceptor motifs, followed by a stretch of T's, are the common features of exon-intron junctions and are very well-conserved across many AAV strains. The splice donor and acceptor sites identified in the AAV9 cap ORF shown in this figure have also been identified in the AAV1 cap ORF. For serotypes other than AAV1, 3, 5 and 9, splicing events in antisense mRNA of the AAV cap ORFs are currently under investigation. The highlighted variants are common AAV serotypes.

[0021] FIG. 7 Introns identified in antisense mRNA derived from the AAV3 cap gene. pAAV3-hnLSP-MCS-TRADE2 is a plasmid carrying the wild-type AAV3 cap ORF placed under a liver-specific enhancer-promoter with an MVM intron (hnLSP). The nucleotide sequence of the AAV3 cap ORF is the same as that of the naturally identified AAV3. HepG2 cells, a human hepatoma cell line, were transfected with plasmid pAAV3-hnLSP-MCS-TRADE2. Antisense mRNA derived from the AAV3 cap ORF was then analyzed by RT-PCR. Sequences of two truncated RT-PCR products were determined by Sanger sequencing following blunt-end TOPO cloning of the PCR products, which revealed introns found within the antisense AAV3 cap ORF (Panels A and B, SEQ ID NO:193). Intron sequences are in lowercase letters with underline. The most upstream splice donor site is found to be only 3 bp away from the splice donor site identified in the AAV9 cap ORF, which is indicated in a dashed line in FIG. 6. The most downstream splice acceptor site is found approximately 80 bp upstream of that of the AAV9 cap ORF. Please note that all the splice donor and acceptor sites identified in the AAV3 cap ORF have also been identified in the AAV1 cap ORF.

[0022] FIG. 8. Additional cryptic splice acceptor sites present in the AAV cap ORFs. (A and B) Nucleotide sequences of the cap genes derived from 122 naturally occurring AAV strains (serotypes and variants) are aligned using a multiple sequence alignment program (SEQ ID NO:317-420). The exon-intron junctions at the splice acceptor sites identified in the AAV3 cap ORF-derived antisense mRNA are indicated with solid thin lines. The dashed line in Panel A indicates alternative putative splice acceptor sites near the experimentally determined splice acceptor site. The AG/

TC splice acceptor sites, followed by a stretch of T's, are a common feature of exon-intron junctions at splice acceptor sites and are very well conserved across many AAV strains. The AAV3 cap ORF is highlighted. The splice acceptor sites identified in the AAV3 cap ORF shown in Panels A and B have also been identified in the AAV1 cap ORF. As for the AAV5 cap ORF, no splicing events have been observed at any sites in antisense mRNA transcription. For serotypes other than AAV1, 3, 5 and 9, splicing events in antisense mRNA of the AAV cap ORFs are currently under investigation.

[0023] FIG. 9 Additional potential splice donor sites present in the AAV cap ORFs. Nucleotide sequences of the cap genes derived from 122 naturally occurring AAV strains (serotypes and variants) are aligned using a multiple sequence alignment program (SEQ ID NO:421-461). The exon-intron junctions at the splice donor sites identified in the AAV3 cap ORF-derived antisense mRNA are indicated with a solid line. The GT/CA splice donor consensus sequence at this position is retained by only half of AAV strains. This splice donor site has been identified in the AAV1 cap ORF.

[0024] FIG. 10 Splice donor and splice acceptor sites identified in the AAV1 cap ORF. The nucleotide sequence of the AAV1 cap ORF is shown (SEQ ID NO:194). The AAV1 cap ORF was expressed by the hSynl enhancer-promoter in human embryonic kidney (HEK) 293 cells or Neuro2a cells in an antisense orientation. Antisense mRNA derived from the AAV1 cap ORF was then analyzed by RT-PCR. Sequences of RT-PCR products were determined by Sanger sequencing following blunt-end TOPO cloning of the PCR products, which revealed introns found within the AAV1 cap ORF. Exon-intron junctions identified in antisense AAV1 cap mRNA are indicated with AG/TC for the splice donor sites and GT/CA for the splice acceptor sites. AG/TC and GT/CA in uppercase are the consensus two nucleotides at the 5' end and the 3' end of an intron, respectively. Since the splicing occurs in antisense mRNA of the ORF, intron sequences are between CT (splice acceptor) and AC (splice donor) in various combinations in the above sequence. The detailed information about the observed combinations of the splice donors and acceptors is not shown. The two conserved nucleotides at exon-intron junctions (CT or AC) indicated in boldface are those that are highly conserved across different AAV serotypes. The two conserved nucleotides at exon-intron junctions (CT or AC) that are underlined are those that have also been identified in antisense AAV3 or AAV9 cap mRNA transcripts.

[0025] FIG. 11 Splicing-suppressing mutagenesis of the AAV9 cap ORF. Silent mutations are introduced around the splice acceptor (SA) site and/or the splice donor (SD) site in the AAV9 cap ORF to suppress the splicing observed on the antisense mRNA transcripts. The spliced-out intron from the native sequence (SEQ ID NO:195, SEQ ID NO:196) is indicated with underlines. The AAV9NS1 genome (SEQ ID NO:197) has a set of mutations around the SA site while the AAV9NS2 genome (SEQ ID NO:198) has a set of mutations around the SD site. The AAV9NS3 genome has both sets of mutations. The numbers to the right indicate the nucleotide position relative to the first nucleotide of the AAV9 cap ORF.

[0026] FIG. 12 Mutations introduced around the splice donor and/or acceptor site(s) effectively suppress the splicing of antisense mRNA derived from the AAV9 cap ORF.

Neuro2a cells were transfected with plasmids containing the AAV9 cap ORF and various potentially splicing-suppressing mutations in the TRADE configuration (NS1-3). RNA was harvested 3 days post-transfection and RT-PCR was performed with a set of PCR primers that can recover the full cap ORF sequence. In stark contrast to results seen in FIG. 4, full-length amplicons were successfully recovered. NS1, the AAV9-TRADE vector genome with a codon-modified splice acceptor. NS2, the AAV9-TRADE vector genome with a codon-modified splice donor. NS3, the AAV9-TRADE vector genome with codon-modified splice acceptor and splice-donor. PC, a positive control using a plasmid template containing the AAV-PHP.B-hSynl-GFP-TRADE vector genome sequence; NC, a no template PCR control.

[0027] FIG. 13 Study design for application of TRADE to identify enhanced AAV variants for brain neuron transduction following systemic AAV vector injection. (A) A map of the AAV9-N272A-hSynl-GFP-TRADE-PepLib vector genome. The hSynl enhancer-promoter is utilized to drive expression specifically in neurons. The liver-detargeted AAV9-N272A cap (PCT/US2017/068050) serves as the platform for AAV library generation. A randomized 8 amino acid peptide encoded by (NNK)₈ and flanked by glycine-serine linkers (SEQ ID NO:2) was substituted for Q588 of the AAV9-N272A cap sequence (SEQ ID NO:222). (B) The plasmid library was used to produce an AAV library using a triple transfection protocol. The library was purified through PEG precipitation and two rounds of CsCl ultracentrifugation, then injected via tail vein at a dose of 3×10^{11} vg/mouse. Brain tissue was harvested 12 days post-injection. RNA was recovered using TRIzol and RT-PCR was used to recover a fragment of cap containing the peptide insertion, which was subsequently cloned back into the AAV vector plasmid backbone. This was repeated for 3 rounds of selection in C57BL/6J mice. In parallel, a single round of selection was performed in rhesus macaque using a dose of 2.7×10^{12} vg/kg.

[0028] FIG. 14 Validation of neuronal transduction of the 26 novel AAV capsids in mice and a nonhuman primate by AAV RNA Barcode-Seq. (A) A map of the double-stranded (ds) AAV-hSynl-GFP-BC vector. A pair of two 12 nucleotide-long DNA barcodes (VBCx-L and VBCx-R) are placed under the human synapsin I (hSynl) gene enhancer-promoter. These two virus barcodes (VBCs) can be expressed as transcripts specifically in cells where the hSynl enhancer-promoter is active (i.e., neurons). (B) Neuronal transduction of 26 novel AAV variants, HN1 to HN26, identified by TRADE (5 variants identified in mice and 21 variants identified in a non-human primate) and 3 control AAV capsids (AAV9, AAV9-N272A and AAV-PHP.B) in C57BL/6J and BALB/cJ mice. A DNA/RNA-barcoded dsAAV-hSynl-GFP-BC library (dsAAV-hSynl-GFP-BCLib) containing 26 novel AAV variants identified by TRADE (5 variants identified by TRADE in mice and 21 variants identified by TRADE in a non-human primate) and control AAV capsids (AAV9, AAV9-N272A and AAV-PHP.B) was injected intravenously into three adult male C57BL/6J mice and three adult male BALB/cJ mice at a dose of 5×10^{11} vg per mouse. Two weeks post-injection, various tissues were harvested and analyzed for transduction at AAV vector genome transcripts levels by AAV RNA Barcode-Seq. Transduction levels are expressed as phenotypic difference (PD) values relative to the reference control, AAV9. For the AAV capsid amino acid sequence information of the HN1 to HN26 var-

iants, please refer to Table 3. (C) Neuronal transduction of the 26 novel AAV variants and 3 control AAV capsids in the hippocampus of a rhesus macaque. The same DNA/RNA-barcoded AAV library was injected intravenously into one juvenile male rhesus macaque at a dose of 2×10^{13} vg/kg. Two weeks post-injection, various brain regions were harvested and analyzed for transduction by AAV RNA Barcode-Seq. (D) Relative neuronal transduction efficiencies of 3 TRADE variants, HN1, HN2 and HN3, and AAV-PHP.B were analyzed by AAV RNA Barcode-Seq in 12 different brain regions in the single rhesus macaque used for Panel C. In Panels B, C and D, dashed lines indicate the PD value of AAV9 (i.e., 1.0).

[0029] FIG. 15 Validation of enhanced neuronal transduction of AAV9-N272A-HN1 in mice using conventional eGFP reporter vectors and histological quantification. We produced AAV9, AAV-PHP.B, and AAV9-N272A-HN1 vectors containing self-complementary AAV genomes expressing eGFP under the control of the hSynl enhancer-promoter (dsAAV-hSynl-eGFP). Purified vectors were administered via the tail vein at a dose of 3×10^{11} vg/mouse into 8-week old male C57BL/6J or BALB/cJ mice ($n = 4$ mice / vector / mouse strain). Three weeks post-injection, mice were transcardially perfused with 4% paraformaldehyde and brain tissue was processed for immunohistochemistry. (A) A map of the self-complementary hSynl-eGFP vector genome. (B) Representative tilescan images of sagittal sections stained with anti-GFP antibody. (C) Quantification of neuronal transduction in (B) based on automated counts of cells expressing eGFP and NeuN in four brain regions. (D) Validation of the automated counting process in (B) and (C). Representative 20X confocal images from visual cortex are shown. Scale bar = 100 μ m. (E) Quantification of neuronal transduction in (D) based on hand counts of cells expressing eGFP and NeuN by a blinded observer. Error bars represent mean $\pm$ SEM. *** $p < 0.001$.

[0030] FIG. 16 Validation of enhanced AAV9-N272A-HN1 transduction relative to AAV9 in rhesus macaques using epitope-tagged eGFP reporter vectors. (A) AAV-CAG-nlsGFP vectors used for this study. We produced 4 AAV vectors: AAV9-CAG-FLAGnlsGFP-BCLib, AAV9-CAG-HAnlsGFP-BCLib, AAV9-N272A-HN1-CAG-FLAGnlsGFP-BCLib and AAV9-N272A-HN1-CAG-HAnlsGFP-BCLib. The nlsGFP (eGFP with the nuclear localization signal derived from the SV40 large T antigen) was tagged with either the FLAG tag or the HA tag at the N-terminus. Each vector was a DNA/RNA-barcoded library containing an approximately 1 to 1 mixture of 9 different DNA/RNA-barcoded viral clones; however, this feature was not used in this study. The two vectors in the top half depicted in Panel A were mixed at a ratio of 1:1 to make AAV Library 1 (AAVLib1) and the two vectors in the bottom half were mixed at a ratio of 1:1 to make AAV Library 2 (AAVLib2). In this experimental scheme, AAVLib1 and AAVLib2 each contain AAV9 and AAV9-N272A-HN1 vectors expressing epitope-tagged nlsGFP at a ratio of 1:1, but the capsid-epitope relationship is inverted in order to avoid potential antibody bias in downstream analyses. (B) Representative tile-scanned brain section from one animal receiving AAVLib. Each AAV library was administered intravenously into a juvenile rhesus macaque at a dose of 3×10^{13} vg/kg. Tissue was harvested 3-weeks post-injection, cut into 4 mm slabs, fixed in 4% paraformaldehyde, and processed

for immunohistochemical analysis with anti-GFP, anti-FLAG and anti-HA antibodies. eGFP expression indicates that a cell was transduced by either AAV9 or AAV9-N272A-HN1 or both. FLAG staining indicates that the AAV9 capsid mediated transduction, while HA staining indicates that AAV9-N272A-HN1 mediated transduction. Top-right inset, motor cortex; bottom-right inset, putamen. This experiment revealed that AAV9-N272A-HN1 transduced the brain cells better than AAV9 by several fold with strong neuronal tropism compared to AAV9. Therefore, as far as neuronal transduction is concerned, AAV9-N272A-HN1 mediates much higher neuronal transduction than AAV9.

[0031] FIG. 17 Biodistribution of AAV9-N272A-HN1 to major peripheral organs following systemic delivery in mice and rhesus macaques. We used AAV DNA Barcode-Seq to determine relative abundance of AAV vector genome DNAs in each peripheral organ, delivered by each AAV capsid contained in the dsAAV-hSynl-GFP-BCLib library (Panels A, B and C). As explained earlier, the dsAAV-hSynl-GFP-BCLib library contained 26 AAV variants identified by TRADE in mice and in a non-human primate together with the controls, AAV9, AAV9-N272A and AAV-PHP.B. DNA was extracted from various tissues following administration of the dsAAV-hSynl-GFP-BCLib library (see Table 3) and subjected to AAV DNA Barcode-Seq analysis. We also used AAV RNA Barcode-Seq to determine relative transduction efficiency compared to AAV9 in each peripheral organ of rhesus macaques intravenously injected with the ssAAV-CAG-nlsGFP-BCLib library depicted in FIG. 16A (Panel D). (A) Biodistribution of AAV9, AAV9-N272A, AAV-PHP.B, and TRADE variants to the liver, relative to AAV9, in C57BL/6J mice, BALB/cJ mice and rhesus macaques. (B) Biodistribution of AAV9-N272A-HN1 to major peripheral organs besides the liver in C57BL/6J mice and BALB/cJ mice (n = 3 mice / strain). (C) Biodistribution of AAV9-N272A-HN1 to major peripheral organs besides the liver in a rhesus macaque (n = 1) based on dsAAV-hSynl-GFP-BC analysis. For this experiment, AAV DNA Barcode-Seq analysis was performed on the samples collected from one rhesus macaque injected with the dsAAV-hSynl-GFP-BCLib library shown in FIG. 14D. (D) Biodistribution of AAV9-N272A-HN1 to major peripheral organs besides the liver in rhesus (n = 2) based on ssAAV-CAG-nlsGFP-BC analysis. For this experiment, AAV RNA Barcode-Seq analysis was performed on the samples collected from rhesus macaques injected with the ssAAV-GAG-nlsGFP-BCLib vectors shown in FIG. 16A. Error bars represent mean $\pm$ SEM. AAV9-N272A-HN1 capsid transduced peripheral organs to a lesser degree compared to AAV9 capsid.

[0032] FIG. 18 AAV9-N272A-HN1 is highly neurotropic following systemic administration in mice. AAV9 and AAV9-N272A-HN1 vectors expressing nlsGFP under the control of the strong, ubiquitous CAG promoter were injected intravenously into 8-week old male BALB/cJ mice at a dose of 3×10^{11} vg/mouse. Tissues were harvested 12 days post-injection and analyzed by immunostaining with anti-GFP and anti-NeuN antibodies. (A) A map of the single-stranded (ss) AAV-CAG-nlsGFP vector genomes used in this study. (B) Representative image from mouse cerebral cortex transduced with AAV9-N272A-HN1-CAG-nlsGFP. The vast majority of cells transduced with AAV9-N272A-HN1-CAG-nlsGFP are also positive for the neuronal marker NeuN. Scale bar= 100 μ m. (C) Neuronal specificity of AAV9 and AAV9-N272A-HN1 capsids. Quantifica-

tion of neuronal specificity was determined by dividing the number of double-positive cells (eGFP+/NeuN+) by the total number of GFP+ cells. AAV9-N272A-HN1 is highly specific to neurons (96%) compared to AAV9 (56%).

DETAILED DESCRIPTION

[0033] In some embodiments, the present disclosure provides a TRADE system that allows directed evolution of the AAV capsid using antisense mRNA of the cap ORF expressed in a cell type-specific or ubiquitous manner. Such a system does not require Cre-transgenic animals. Therefore, it can be applied to cell type-specific AAV capsid evolution in large animals, including non-human primates, for which Cre-transgenic strains are not readily available. Any cell type-specific or tissue/organ-specific enhancers/promoters or ubiquitous enhancers/promoters can be readily applied to the system with no requirement of transgenesis. The cell type-specific selection is given at the mRNA level. In certain embodiments, multiple directed evolution schemes may be combined into one directed evolution scheme. For example, selection of neuron-specific AAV capsids, astrocyte-specific AAV capsids, oligodendrocyte-specific AAV capsids and microglia-specific AAV capsids based on cell type-specific transgene mRNA expression can be performed simultaneously in a single animal.

[0034] In some embodiments, the present disclosure provides a sense strand TRADE system that allows directed evolution of the AAV capsid using mRNA of the cap ORF expressed in a cell type-specific or ubiquitous manner that is capable of expressing AAV capsid proteins in target cells. The sense strand TRADE has the same advantages of those antisense strand TRADE presented with data here in that it does not require Cre-transgenic animals, cell type-specific selection is given at the mRNA level, and it is capable of combining multiple directed evolution schemes into one directed evolution round done in a single animal. However, the possible disadvantage is that immunogenic AAV capsid proteins may be unavoidably expressed persistently in target cells, which may result in undesired consequences in the capsid selection process.

[0035] In some embodiments, the present disclosure also provides novel AAV capsids. In certain embodiments, these novel AAV capsids can transduce brain neurons several times better than AAV9 in C57BL/6J mice following intravenous injection. In certain embodiments, the novel AAV capsids transduced up to 8 times better than AAV9 in C57BL/6J mice following intravenous injection. The neuronal transduction levels may be greatly enhanced compared to AAV9 although they may not attain the levels obtained with AAV PHP.B. In certain embodiments, the novel AAV capsids may transduce brain neurons more efficiently than AAV PHP.B.

[0036] In some embodiments, this disclosure provides novel AAV capsids that can transduce brain neurons several times better than AAV9 following intravenous injection in BALB/cJ mice. In certain embodiments, the novel AAV capsids can transduce brain neurons up to 7 times better than AAV9 following intravenous injection in BALB/cJ mice. The transduction levels are much higher than AAV PHP.B.

[0037] In some embodiments, this disclosure provides novel AAV capsids that can transduce brain neurons several times better than AAV9 in rhesus macaques following intra-

venous injection. In certain embodiments, the novel AAV capsids can transduce brain neurons up to 4 times better than AAV9 in rhesus macaques following intravenous injection. These transduction levels are better than AAV PHP.B. [0038] In some embodiments, the disclosure provides AAV capsids that can transduce the pulmonary cells with neuronal cell marker expression several times better than AAV9. In certain embodiments, the AAV capsids can transduce such cells up to 17 times better than AAV9.

[0039] In some embodiments, the novel AAV capsids exhibit a liver-detargeting phenotype.

[0040] In some embodiments, the disclosure provides codon-modified AAV cap sequences that are not spliced when expressed in an antisense direction. We have observed that unmodified AAV cap ORFs are spliced when expressed in an antisense direction (e.g., AAV1, AAV3 and AAV9). In contrast, some of the codon-modified AAV cap ORFs described in this disclosure are not spliced. Based on the knowledge we have developed about the putative splice donor and acceptor sites, it has become possible to design such non-spliced versions of AAV cap ORFs. The use of such non-spliced cap ORFs may be used for directed evolution using the TRADE system when mutagenesis of the cap gene takes place over a wide region of the cap ORF.

[0041] 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 brain, heart, lung, skeletal muscle, liver, 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.

[0042] 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. Alternatively, 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.

[0043] Nucleotide sequences, such as polynucleotides, encoding proteins of the present disclosure are provided herein. The nucleotides of the present disclosure can be composed of either RNA or DNA. The disclosure also encompasses those polynucleotides that are complementary in sequence to the polynucleotides disclosed herein.

[0044] 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 one or more sequences that encode amino acid substitutions, deletions, additions, or insertions that do not eliminate the detectability

of the polypeptide encoded by the polynucleotides of the present disclosure.

[0045] 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.

[0046] Polynucleotide and polypeptide sequences of the current disclosure can also be defined in terms of particular identity and/or similarity with certain polynucleotides and polypeptides 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% identical 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.

[0047] Methods of producing AAV vectors as disclosed herein are well known in the art, including methods, for example, of 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. Virology* 72, 7024 (1998); WO1998/022607; and WO2005/072364.

[0048] 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.

[0049] 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.

[0050] 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”), a 2A peptide, 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 phosphoglycerate kinase (PGK) promoter and the β -actin promoter.

[0051] 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 homeostatic feedback loop that may maintain expression levels of the protein of interest at a physiological level.

[0052] In some embodiments of the AAV vectors disclosed herein, genome editing machinery may be used to genetically modify cellular genome DNA or mRNA transcripts at a site-specific manner. Komor et al., *Cell* 168, 20-36 (2017); and Katrekar et al., *Nature Methods* 16:239-242, 2019. For example, some embodiments of the AAV vectors may include a CRISPR-associated enzyme such as Cas9, a DNA base editor, an RNA editase and/or guide RNA (gRNA) to modify nucleic acid in cells in a site-specific manner. In addition, AAV vectors may contain a homology repair template (HDR) for genome editing.

[0053] 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.

[0054] As described herein, effective and long-term expression of therapeutic proteins of interest in brain, heart, lung, skeletal muscle, kidney, spleen, or pancreas can be achieved with non-invasive techniques, through peripheral administration of certain AAV vectors, such as a non-AAV9 vector with AAV9 sequences. Such peripheral administration may include any administration route that does not necessitate direct injection into brain, heart, lung, skeletal muscle, kidney, spleen, or pancreas. More particularly, peripheral administration may include systemic injections, such as intramuscular, intravascular (such as intrave-

nous,) intraperitoneal, intra-arterial, or subcutaneous injections. In some embodiments, peripheral administration also may include oral administration (see, e.g., WO96/40954), delivery using implants, (see, e.g., WO01/91803), or administration by instillation through the respiratory system, e.g., using sprays, aerosols or any other appropriate formulations.

[0055] In various embodiments, the desired doses of the AAV vectors may be 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.

[0056] 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.

[0057] 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 the same or a different peripheral administration route and/or the same vector or a distinct vector.

EXAMPLES

[0058] The following examples are for illustration only. In light of this disclosure, those of skill in the art will recognize that variations of these examples and other embodiments of the disclosed subject matter are enabled without undue experimentation.

[0059] We applied the TRADE system in both C57BL/6J mice and a rhesus macaque in order to identify novel AAV capsids that efficiently transduce brain neurons following systemic delivery. The TRADE system utilizes a plasmid construct containing an overlapping bicistronic AAV genome flanked by ITR sequences (FIG. 2A). In the sense direction, the AAV2 p40 promoter drives expression of the AAV cap gene to facilitate efficient production of viral particles (FIG. 2B). In the antisense direction, a cell type-specific enhancer-promoter (e.g. the human synapsin I (hSynI) enhancer-promoter) drives expression of transcripts encoding GFP and the antisense cap sequence (FIG. 2B), terminating at a polyadenylation signal (poly A) embedded in the intron present in the AAV2 genome. Utilizing the TRADE construct as a cloning backbone, we generated an AAV library based on the liver-detargeted AAV9-N272A (PCT/US2017/068050) cap gene platform that contained random

8-mer peptides with glycine-serine linkers (5'-GGGS; 3'-GGGGS) substituted at the position Q588 in the AAV9 capsid. In vivo selection in a specific cell type (e.g. neurons) was performed by recovering capsid sequences as antisense cap ORF mRNA from brain tissue by RT-PCR. This method ensures that recovered sequences are only derived from AAV variants that are capable of mediating RNA expression in infected cells of our interest. When the hSyn1 enhancer-promoter is used, only sequences of AAV capsids that are capable of transducing neurons can be retrieved, thus enabling neuron-specific selection of AAV capsids.

[0060] We first tested the ability of the TRADE system to recover the sequence of the AAV cap gene from cell type-specific antisense mRNA using an AAV-PHP.B-hSyn1-GFP-TRADE vector (FIG. 3). A hSyn1 enhancer-promoter-driven GFP expression cassette was incorporated in the AAV-PHP.B capsid gene-containing AAV vector genome in the TRADE configuration (FIG. 3A). This vector genome was packaged into the AAV-PHP.B capsid, and the resulting AAV vector was injected intravenously into two 8-week-old male C57BL/6J mice (FIG. 3B). Twelve days after injection, brain tissue was harvested. Tissue fixed with 4% paraformaldehyde was analyzed by immunofluorescence microscopy. Unfixed tissue was utilized for RNA extraction and RT-PCR analysis. We confirmed that eGFP was expressed only in neurons (FIGS. 3C and 3D), indicating that the antisense mRNA transcribed from the cap gene is expressed in a cell type-specific manner. We recovered antisense mRNA of the cap gene efficiently by RT-PCR (FIG. 3E). Sanger sequencing of a splice junction unique to the antisense mRNA confirmed that RT-PCR products were indeed derived from the hSyn1 enhancer-promoter-driven antisense mRNA (FIG. 3F). In addition, Sanger sequencing confirmed the sequence of the PHP.B peptide insertion (FIG. 3F). Together, these observations established the ability of the TRADE system to successfully recover the AAV cap sequence from the hSyn1 enhancer-promoter-driven antisense mRNA expressed in AAV vector-transduced brain neurons.

[0061] With the successful establishment of the TRADE system, we performed two AAV capsid directed evolution experiments; one used 8-week-old male C57BL/6J mice and the other used one 8-month-old male rhesus macaque. We produced an AAV9-N272A-hSyn1-GFP-TRADE-Lib library composed of AAV9-derived mutant capsids that have a GGGS(N₈)GGGGS (SEQ ID NO:2) peptide insertion at the position of Q588 where N₈ represents a random 8-mer peptide encoded by (NNK)₈. For the peptide insertion, Q588 was substituted with each peptide sequence. The diversity of the AAV library was at least 10⁷. In the mouse directed evolution experiment, we infused the AAV library via the tail vein at a dose of 3 x 10¹¹ vector genomes (vg) per mouse. For the second round of selection, we injected the AAV library at a dose of 1x10¹², 1x10¹¹, 1x10¹⁰, or 1x10⁹ vector genomes (vg) using two mice. For the third round of selection, we injected the AAV library at a dose of 1x10¹¹ vg using two mice. We harvested brain tissues twelve days after injection, and separated them into three regions, i.e., the cerebrum, the cerebellum and the brain stem. Only the cerebrum samples were used for the directed evolution experiments. We extracted total RNA from the cerebrum, reverse-transcribed the RNA using an oligo dT primer, and amplified the peptide region including the flanking regions by a pair of the cap gene-specific PCR

primers. The RT-PCR products were then used to create the next AAV9-N272A-hSyn1-TRADE-Lib plasmid library, which was subsequently used to produce the next AAV9-N272A-hSyn1-TRADE-Lib virus library. For the second and third round selection, we packaged an AAV9-N272A-hSyn1-TRADE-Lib genome that was devoid of the GFP ORF. In the non-human primate directed evolution experiment, we infused the AAV9-N272A-hSyn1-GFP-TRADE-Lib library via the saphenous vein at a dose of 2.0 x 10¹² vg per kg. Twelve days post-injection, the whole brain was harvested and sliced using a brain matrix, treated with RNA-later (Thermo Fisher Scientific), and stored frozen. Total RNA was then extracted from the following brain regions: frontal cortex, occipital cortex, cerebellum (Purkinje and granular layers), medulla, pons, frontal cortex, hypothalamus, thalamus, cingulate gyrus, caudate nucleus, putamen, hippocampus, and preoptic area. We retrieved the peptide sequences by RT-PCR in the same manner as described above except that we performed nested PCR to obtain PCR products sufficient for the downstream Illumina and Sanger sequencing procedures. For some samples, we cloned the first PCR products directly into a plasmid backbone without performing nested PCR for Sanger sequencing. Following three rounds of selection in mice (Table 1) and one round of selection in non-human primate, we identified a number of potentially transduction-enhancing peptides inserted into the AAV9 capsids (Table 2). We then generated a barcoded AAV library and utilized DNA/RNA Barcode-Seq technology, previously developed in the Nakai lab (Adachi et al. *Nat Commun* 5, 3075 (2014); and PCT/US2017/068050), to compare the transduction efficiency, tropism/biodistribution, and pharmacokinetics of 26 selected novel AAV variants (Table 3) following intravenous administration in two commonly used mouse lines (C57BL/6J and BALB/cJ) and one rhesus macaque. As a result, we have found: (1) Some of the novel variants identified by TRADE technology, in particular AAV9-N272A-TTNLAKNS (HN1) and AAV9-N272A-QQNGTRPS (HN2), performed up to 8 times better than AAV9 in the brain of C57BL/6J mice (FIGS. 14B and 14C). For HN_x designation, please refer to Table 3. (2) As previously reported by Hordeaux et al. (Hordeaux et al. 2018), AAV-PHP.B transduced the brain of BALB/cJ mice only at a level comparable to or lower than that of AAV9 (FIGS. 14B and 14C), demonstrating a mouse strain dependency for AAV-PHP.B's robust neurotropic enhancement. (3) In contrast, AAV9-N272A-TTNLAKNS (HN1) and AAV9-N272A-QQNGTRPS (HN2) retained robust neuronal transduction in BALB/cJ mice showing up to 7 times better transduction than AAV9 (FIG. 14B). (4) In a rhesus macaque, many of the novel AAV mutants showed enhanced neuronal transduction, up to 4-fold greater than AAV9 in certain brain regions, while AAV-PHP.B transduced non-human primate brain similarly to or lower than AAV9. In particular, AAV9-N272A-TTNLAKNS (HN1) transduced the non-human primate brain best in multiple brain regions (FIGS. 14C and 14D). (5) All of the AAV9-N272A-derived variants including HN1, HN2 and HN3 showed varying degrees of liver-detracting properties in mice and rhesus macaques (FIG. 17A). (6) AAV9-N272A-TTNLAKNS (HN1) and AAV9-N272A-QQNGTRPS (HN2) can transduce cells with the hSyn1 enhancer-promoter transcriptional activity in the lung up to 17 times better than AAV9 in mice (FIG. 17B, Tables 4 and 6). (7) AAV9-N272A-TTNLAKNS (HN1)

exhibits vector genome dissemination to peripheral organs to a lesser degree compared to AAV9 (FIGS. 17C and 17D). The AAV Barcode-Seq data are summarized in Tables 4 to 9. Representative data presented in Tables 4 to 9 are also shown in a graph format in FIG. 14 and FIG. 17.

TABLE 1

Peptide sequences identified by the hSynl-TRADE system using an AAV9-N272N-GGGS(Ns)GGGS library targeting mouse brain neurons.					
1st round		2nd round		3rd round	
ADKPPGLS	SEQ ID NO:3	APTNEAHP	SEQ ID NO:97	AGAAAYTPA (2)	SEQ ID NO:150
AGEDGSSR	SEQ ID NO:4	AQTNLAAG	SEQ ID NO:98	APSVSREK (2)	SEQ ID NO:151
ALGTATQR	SEQ ID NO:5	ASLPNLGQ	SEQ ID NO:99	DYMHKTGL	SEQ ID NO:152
ALNTALVE	SEQ ID NO:6	DYMHNTGL	SEQ ID NO:100	EEDAQLLI (2)	SEQ ID NO:14
AMVRLTHN	SEQ ID NO:7	DYMHTTGL	SEQ ID NO:101	ENKSAPLP	SEQ ID NO:18
ASRDPSAT	SEQ ID NO:8	ERNAWHAG	SEQ ID NO:102	GDYTVQRP	SEQ ID NO:107
DANDARQR	SEQ ID NO:9	ETQATPMP	SEQ ID NO:103	GGMNETTR	SEQ ID NO:153
DLARMAAA	SEQ ID NO:10	EWEDSARS	SEQ ID NO:104	GGSAFVTG	SEQ ID NO:154
DQGSITAH	SEQ ID NO:11	FTGDTDTL	SEQ ID NO:105	GGSPLAHP	SEQ ID NO:21
DRTPGVNV	SEQ ID NO:12	FTNRTSTT	SEQ ID NO:106	GNSHTGSS	SEQ ID NO:155
DTDTLSPG	SEQ ID NO:13	GDYTVQRP	SEQ ID NO:107	GPQEGSER (2)	SEQ ID NO:109
EEDAQLLI	SEQ ID NO:14	GGLRTDYG	SEQ ID NO:108	GQRGLPIA	SEQ ID NO:27
EKLNDWPT	SEQ ID NO:15	GGSPLAHP	SEQ ID NO:21	GSNHTQSL	SEQ ID NO:110
ELNSARQV	SEQ ID NO:16	GKQPVQPY	SEQ ID NO:24	HQVTSSGA (4)	SEQ ID NO:33
ELQSFAGL	SEQ ID NO:17	GPQEGSER	SEQ ID NO:109	LEQQRGAS	SEQ ID NO:113
ENKSAPLP	SEQ ID NO:18	GSNHTQSL	SEQ ID NO:110	LERNRDS	SEQ ID NO:39
ERTAVKGN	SEQ ID NO:19	GTPQTTKE	SEQ ID NO:29	LLVTARSH (3)	SEQ ID NO:44
GGIQTWT	SEQ ID NO:20	HDRDTRQA	SEQ ID NO:111	MESQRANS (2)	SEQ ID NO:117
GGSPLAHP	SEQ ID NO:21	LDQNRRPQ	SEQ ID NO:112	MSGQGYQA (2)	SEQ ID NO:50
GGTAAQGV	SEQ ID NO:22	LEQQRGAS	SEQ ID NO:113	NSARTQLS	SEQ ID NO:156
GKMASGSL	SEQ ID NO:23	LERNRDS	SEQ ID NO:39	PLTILNRH	SEQ ID NO:157
GKQPVQPY	SEQ ID NO:24	LGGNAQGL	SEQ ID NO:114	QGTRTNPP	SEQ ID NO:158
GNPHTGST	SEQ ID NO:25	LLVTTRSH	SEQ ID NO:115	QQNGTRPS (4)	SEQ ID NO:128
GPTLGGSG	SEQ ID NO:26	LVTNTTR	SEQ ID NO:116	QSGDSALN (3)	SEQ ID NO:67
GQRGLPIA	SEQ ID NO:27	MESQRANS	SEQ ID NO:117	QSSAMPRN (2)	SEQ ID NO:159
GREPRRLH	SEQ ID NO:28	MISQTLMA	SEQ ID NO:118	SATISLQV	SEQ ID NO:136
GTPQTTKE	SEQ ID NO:29	MMSQSLRA	SEQ ID NO:119	SHNSQPVA	SEQ ID NO:160
GVTERPNR	SEQ ID NO:30	NNVQSALN	SEQ ID NO:120	SHTNLRDT	SEQ ID NO:137
HLGDNLAR	SEQ ID NO:31	NSARTQLS	SEQ ID NO:121	SSGYLTAN	SEQ ID NO:139
HPGSGAGP	SEQ ID NO:32	PQWNRTP	SEQ ID NO:122	TAQGAAFR (4)	SEQ ID NO:161
HQVTSSGA	SEQ ID NO:33	PRFNSSL	SEQ ID NO:123	TPGLNNAR	SEQ ID NO:162
HVGSQMHA	SEQ ID NO:34	PRPTWGT	SEQ ID NO:60	TSLGTPEA	SEQ ID NO:163
IG*TVPMQ	SEQ ID NO:35	PVDGGRHL	SEQ ID NO:124	TTNLAKNS (6)	SEQ ID NO:164
KFTRDGPY	SEQ ID NO:36	PWFNKSSL	SEQ ID NO:125	WQGEQKR (4)	SEQ ID NO:146
KGPAEQGH	SEQ ID NO:37	QDMNSQRS	SEQ ID NO:126	WSPDAVEG	SEQ ID NO:165
LAHSPLRW	SEQ ID NO:38	QGASNSQL	SEQ ID NO:127	WSQDAVKG (2)	SEQ ID NO:148
LERNRDS	SEQ ID NO:39	QQNGTRPS	SEQ ID NO:128	WTGGGSGT (3)	SEQ ID NO:149
LEHTSLT	SEQ ID NO:40	QRSAYPTS	SEQ ID NO:129	WTGGRHL	SEQ ID NO:166
LHDGKYST	SEQ ID NO:41	QRTPSITP	SEQ ID NO:130		
LKATGRGK	SEQ ID NO:42	QWMKEQAG	SEQ ID NO:131		
LLPGSADG	SEQ ID NO:43	RDGRHPSE	SEQ ID NO:132		
LLVTARSH	SEQ ID NO:44	RGTVTVEQ	SEQ ID NO:133		
LPEVEPTN	SEQ ID NO:45	RPANHSTA	SEQ ID NO:134		
LPWENSSQ	SEQ ID NO:46	RQGDADTL	SEQ ID NO:135		
LQRNSDAN	SEQ ID NO:47	SATISLQV	SEQ ID NO:136		
LQSAPRAT	SEQ ID NO:48	SHTNLRDT	SEQ ID NO:137		
MLGSQVPT	SEQ ID NO:49	SRMGETPQ	SEQ ID NO:138		
MSGQGYQA	SEQ ID NO:50	SSGYLTAN	SEQ ID NO:139		
NPGRDFRD	SEQ ID NO:51	SSWSQGP	SEQ ID NO:79		
NQPSDYVS	SEQ ID NO:52	TGNSPEQA	SEQ ID NO:140		
NSVGSADK	SEQ ID NO:53	THSQGRLA	SEQ ID NO:141		
NVQRTQRG	SEQ ID NO:54	TPIVGSNV	SEQ ID NO:142		
PAQLNGPR	SEQ ID NO:55	TPPKSPSM	SEQ ID NO:143		
PERERLPR	SEQ ID NO:56	TRMDERSP	SEQ ID NO:144		
PGNGSHTM	SEQ ID NO:57	TTATTSIT	SEQ ID NO:145		
PIPGTPQP	SEQ ID NO:58	WQGEQKR	SEQ ID NO:146		
PMSVPASN	SEQ ID NO:59	WNDRSGER	SEQ ID NO:147		
PRPTWGT	SEQ ID NO:60	WSQDAVKG	SEQ ID NO:148		
PRTNRGPE	SEQ ID NO:61	WTGGGSGT	SEQ ID NO:149		

TABLE 1-continued		
Peptide sequences identified by the hSyn1-TRADE system using an AAV9-N272N-GGGS(N ₈)GGGGS library targeting mouse brain neurons.		
1st round	2nd round	3rd round
PVANPTTA	SEQ ID NO:62	
PVLGGPPK	SEQ ID NO:63	
QGSRQGSS	SEQ ID NO:64	
QMAETPIS	SEQ ID NO:65	
QMLGIGRS	SEQ ID NO:66	
QSGDSALN	SEQ ID NO:67	
RAGLTSSE	SEQ ID NO:68	
RLDNTGVG	SEQ ID NO:69	
RMPGKPYS	SEQ ID NO:70	
RVAGASQP	SEQ ID NO:71	
RVESSQLE	SEQ ID NO:72	
SARTGASE	SEQ ID NO:73	
SERNRASM	SEQ ID NO:74	
SIDVRMAA	SEQ ID NO:75	
SRDGHILR	SEQ ID NO:76	
SRQWLPG	SEQ ID NO:77	
SSRGYTST	SEQ ID NO:78	
SSWSQGP	SEQ ID NO:79	
SVAESGRE	SEQ ID NO:80	
TALTANTQ	SEQ ID NO:81	
TESSVGNL	SEQ ID NO:82	
TGREGANL	SEQ ID NO:83	
TLSEPPKK	SEQ ID NO:84	
TNAVSGKS	SEQ ID NO:85	
TRAPTIHL	SEQ ID NO:86	
TRESTDRG	SEQ ID NO:87	
TVAAAPNL	SEQ ID NO:88	
TYHNNTPR	SEQ ID NO:89	
VSNSTRTS	SEQ ID NO:90	
VTLQIDTK	SEQ ID NO:91	
WMSRPGPT	SEQ ID NO:92	
WPYRGLTQ	SEQ ID NO:93	
WRRQGSRA	SEQ ID NO:94	
YAQRFAKM	SEQ ID NO:95	
YNSPRQTV	SEQ ID NO:96	

The table lists peptide insertions on AAV9-N272A after each of three rounds of selection. The numbers in parentheses indicate the frequency of each peptide among a total of 69 peptides identified after the three round of selection. Peptides with no number were found only once. The sequences of the peptide region were determined by Sanger sequencing. Actual peptide sequences were randomized octapeptides flanked by glycine-serine linkers such that position Q588 was substituted with GGGS(N₈)GGGGS.

For example,

“-TNHQSAGGGSTTNLAKNSGGGGSAQAQTG-” for TTNLAKNS and

“-TNHQSAGGGSQQNGTRPSGGGGSAQAQTG-” for QQNGTRPS.

TABLE 2	
Peptide sequences identified by the hSyn1-TRADE system using an AAV9-N272A-GGGS(N ₈)GGGGS library targeting rhesus macaque brain neurons	
1st round	
AVAGDRLL	SEQ ID NO:167
DLLTRSVS	SEQ ID NO:168
EWKTQLAL	SEQ ID NO:169
GNINWPH	SEQ ID NO:170
GSPAASSW	SEQ ID NO:171
KHSLTLES	SEQ ID NO:172
KPVSTDTF	SEQ ID NO:173
LDRSGSTG	SEQ ID NO:174
LGAQNHVV	SEQ ID NO:175
LMATDYGP	SEQ ID NO:176
LRATDYGP	SEQ ID NO:177
MERTEPLG	SEQ ID NO:178
NDGLRLHL	SEQ ID NO:179
NLSAHSOA	SEQ ID NO:180
NLSAHSOD	SEQ ID NO:181
RALDLVTR	SEQ ID NO:182
SAGMARN	SEQ ID NO:183

TABLE 2-continued	
Peptide sequences identified by the hSyn1-TRADE system using an AAV9-N272A-GGGS(N ₈)GGGGS library targeting rhesus macaque brain neurons	
1st round	
SGQRVGSA	SEQ ID NO:184
SGQRVGSD	SEQ ID NO:185
TAQGAAFR	SEQ ID NO:161
TGRPEQPK	SEQ ID NO:186
THSPIKLP	SEQ ID NO:187
TQFSQAQR	SEQ ID NO:188
VGDSANLR	SEQ ID NO:189

The sequences of the peptide region were determined either by Illumina sequencing or Sanger sequencing. Actual peptide sequences were randomized octapeptides flanked by glycine-serine linkers such that position Q588 was substituted with GGGS(N₈)GGGGS. These peptides were recovered from frontal cortex, occipital cortex, hypothalamus and thalamus.

TABLE 3

A list of the 29 AAV capsids contained in the DNA/RNA-barcoded dsAAV-hSynl-GFP-BCLib library used for phenotype determination of each AAV strain			
AAV strain (AAV capsid)	Abbrevia- tion	Number of viral clones in the AAV library	Note
AAV9	AAV9	15	Reference
AAV9-N272A	AAV9-N272A	5	Reference
AAV-PHP.B	AAV-PHP.B	2	Reference
AAV9-N272A-TTNLAKNS (peptide insertion site SEQ ID NO:164)	HN1	2	TRADE variant (C57BL/6J)
AAV9-N272A-QQNGTRPS (peptide insertion site SEQ ID NO:128)	HN2	2	TRADE variant (C57BL/6J)
AAV9-N272A-SGQRVGSD (peptide insertion site SEQ ID NO:185)	HN3	2	TRADE variant (rhesus macaque)
AAV9-N272A-AVAGDRLL (peptide insertion site SEQ ID NO:167)	HN4	2	TRADE variant (rhesus macaque)
AAV9-N272A-DLLTRSVS (peptide insertion site SEQ ID NO:168)	HN5	2	TRADE variant (rhesus macaque)
AAV9-N272A-EWKTQLAL (peptide insertion site SEQ ID NO:169)	HN6	2	TRADE variant (rhesus macaque)
AAV9-N272A-GNINVVPH (peptide insertion site SEQ ID NO:170)	HN7	2	TRADE variant (rhesus macaque)
AAV9-N272A-GSPAASSW (peptide insertion site SEQ ID NO:171)	HN8	2	TRADE variant (rhesus macaque)
AAV9-N272A-KHSLTLES (peptide insertion site SEQ ID NO:172)	HN9	2	TRADE variant (rhesus macaque)
AAV9-N272A-KPVSTDTF (peptide insertion site SEQ ID NO:173)	HN10	2	TRADE variant (rhesus macaque)
AAV9-N272A-LDRSGSTG (peptide insertion site SEQ ID NO:174)	HN11	2	TRADE variant (rhesus macaque)
AAV9-N272A-LGAQNHVV (peptide insertion site SEQ ID NO:175)	HN12	2	TRADE variant (rhesus macaque)
AAV9-N272A-LRATDYGP (peptide insertion site SEQ ID NO:177)	HN13	2	TRADE variant (rhesus macaque)
AAV9-N272A-	HN14	2	TRADE variant

TABLE 3-continued

A list of the 29 AAV capsids contained in the DNA/RNA-barcoded dsAAV-hSynl-GFP-BCLib library used for phenotype determination of each AAV strain			
AAV strain (AAV capsid)	Abbrevia- tion	Number of viral clones in the AAV library	Note
MERTEPLG (peptide insertion site SEQ ID NO:178)			(rhesus macaque)
AAV9-N272A-NDGLRLHL (peptide insertion site SEQ ID NO:179)	HN15	2	TRADE variant (rhesus macaque)
AAV9-N272A-NLSAHSHD (peptide insertion site SEQ ID NO:181)	HN16	2	TRADE variant (rhesus macaque)
AAV9-N272A-RALDLVTR (peptide insertion site SEQ ID NO:182)	HN17	2	TRADE variant (rhesus macaque)
AAV9-N272A-SAGMARN (peptide insertion site SEQ ID NO:183)	HN18	2	TRADE variant (rhesus macaque)
AAV9-N272A-TAQGAAGR (peptide insertion site SEQ ID NO:161)	HN19	2	TRADE variant (rhesus macaque)
AAV9-N272A-TGRPEQPK (peptide insertion site SEQ ID NO:186)	HN20	2	TRADE variant (rhesus macaque)
AAV9-N272A-THSPIKLP (peptide insertion site SEQ ID NO:187)	HN21	2	TRADE variant (rhesus macaque)
AAV9-N272A-TQFSQAQR (peptide insertion site SEQ ID NO:188)	HN22	2	TRADE variant (rhesus macaque)
AAV9-N272A-VGDSANLR (peptide insertion site SEQ ID NO:189)	HN23	2	TRADE variant (rhesus macaque)
AAV9-N272A-HQVTSSGA (peptide insertion site SEQ ID NO:33)	HN24	2	TRADE variant (mouse)
AAV9-N272A-LLVTARSH (peptide insertion site SEQ ID NO:44)	HN25	2	TRADE variant (mouse)
AAV9-N272A-VVQGEQKR (peptide insertion site SEQ ID NO:146)	HN26	2	TRADE variant (mouse)
The novel AAV9-hSynl-TRADE-derived capsid variants were selected from those identified following three rounds of selection in mice (Table 1) and one round of selection in a rhesus macaque (Table 2). Each recovered AAV variant was assigned an abbreviation, HN _x . A DNA/RNA-barcoded dsAAV-hSynl-GFP-BCLib library containing was constructed such that each AAV variant packaged a unique dsAAV-hSynl-GFP-BC viral genome expressing AAV variant-specific RNA barcodes. The number of unique AAV barcode clones for each variant, including critical reference variants, is presented in this table.			

TABLE 4

Brain neuronal transduction efficiency and biodistribution of the TRADE-identified AAV variants in C57BL/6J mice following intravenous administration										
	Brain (RNA)	Lung (RNA)	Heart (DNA)	Kidney (DNA)	Liver (DNA)	Lung (DNA)	Muscle (DNA)	Pancreas (DNA)	Spleen (DNA)	Testis (DNA)
AAV9	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
AAV9-N272A	1.93	0.22	0.82	2.11	0.06	0.51	0.17	0.25	4.49	1.42
AAV-PHP.B	9.42	0.64	0.92	2.35	0.27	0.81	0.36	0.62	2.69	1.45

TABLE 4-continued

Brain neuronal transduction efficiency and biodistribution of the TRADE-identified AAV variants in C57BL/6J mice following intravenous administration										
	Brain (RNA)	Lung (RNA)	Heart (DNA)	Kidney (DNA)	Liver (DNA)	Lung (DNA)	Muscle (DNA)	Pancreas (DNA)	Spleen (DNA)	Testis (DNA)
HN1	8.01	2.74	0.21	0.62	0.24	3.40	0.36	0.42	0.41	0.64
HN2	6.52	1.19	0.36	0.56	0.24	2.55	0.32	0.33	0.66	0.91
HN3	2.35	0.41	0.95	1.29	0.09	0.45	0.20	0.25	2.57	1.45
HN4	0.87	0.15	0.65	2.96	0.02	0.48	0.11	0.21	4.02	1.22
HN5	0.46	0.10	0.57	2.66	0.01	0.47	0.09	0.21	3.90	1.10
HN6	0.37	0.22	0.64	3.13	0.01	0.52	0.08	0.27	4.27	1.22
HN7	1.03	0.21	0.40	0.54	0.25	0.17	0.23	0.10	0.75	0.43
HN8	0.74	0.11	0.61	2.61	0.02	0.45	0.09	0.16	3.27	1.06
HN9	1.47	0.28	0.62	1.49	0.08	0.30	0.15	0.16	2.41	0.82
HN10	1.40	0.18	0.64	1.48	0.04	0.24	0.12	0.16	2.64	0.97
HN11	1.38	0.21	0.73	1.37	0.05	0.29	0.15	0.17	3.17	1.12
HN12	0.80	0.17	0.26	0.42	0.24	0.16	0.20	0.06	0.49	0.21
HN13	1.59	0.28	0.77	1.17	0.10	0.28	0.19	0.18	2.28	0.93
HN14	0.45	0.05	0.47	1.31	0.01	0.20	0.07	0.14	1.86	0.65
HN15	0.50	0.21	0.68	3.48	0.01	0.58	0.10	0.24	4.70	1.30
HN16	1.43	0.24	0.58	1.28	0.02	0.32	0.11	0.22	3.70	1.07
HN17	0.29	0.07	0.50	2.80	0.01	0.46	0.08	0.19	3.73	1.05
HN18	1.46	0.12	0.68	1.78	0.11	0.28	0.17	0.14	2.10	0.92
HN19	0.56	0.10	0.57	3.07	0.01	0.52	0.09	0.18	3.98	1.16
HN20	1.68	0.35	0.90	1.10	0.18	0.29	0.24	0.18	1.89	0.89
HN21	0.26	0.08	0.50	2.53	0.01	0.46	0.06	0.14	3.44	1.01
HN22	0.82	0.06	0.51	2.55	0.02	0.42	0.10	0.19	3.32	1.05
HN23	2.45	0.26	0.72	1.02	0.05	0.30	0.15	0.21	2.25	1.12
HN24	1.33	0.22	0.63	1.31	0.05	0.28	0.14	0.15	2.83	0.90
HN25	0.37	0.12	0.64	3.25	0.02	0.53	0.09	0.22	4.35	1.23
HN26	0.73	0.14	0.63	2.53	0.06	0.40	0.12	0.17	2.86	1.07

A DNA/RNA-barcoded dsAAV-hSynl-GFP-BC library (dsAAV-hSynl-GFP-BCLib) containing 26 novel AAV variants identified by TRADE and control AAV capsids was injected intravenously into 3 C57BL/6J mice at a dose of 5 x 10¹¹ vg per mouse (for the library, see Table 3). Two weeks post-injection, various tissues were harvested and analyzed for brain transduction by AAV RNA Barcode-Seq and biodistribution to peripheral organs by AAV DNA Barcode-Seq. All the values are normalized with those of AAV9 (AAV9=1.0).

TABLE 5

Pharmacokinetic profiles of TRADE-identified AAV variants in C57BL/6J mice following intravenous administration								
	1 m	10 m	30 m	1 h	4 h	8 h	24 h	72 h
AAV9	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
AAV9-N272A	1.16	1.17	1.28	1.34	1.77	2.02	3.14	0.13
AAV-PHP.B	1.38	1.42	1.44	1.62	1.88	2.15	3.14	0.39
HN1	0.87	0.47	0.29	0.29	0.27	0.31	0.37	0.02
HN2	0.79	0.60	0.49	0.55	0.59	0.66	0.91	0.03
HN3	1.12	1.15	1.21	1.32	1.65	1.88	3.05	0.04
HN4	1.32	1.50	1.43	1.52	2.01	2.37	3.78	0.03
HN5	1.14	1.30	1.37	1.42	1.84	2.03	3.37	0.02
HN6	1.21	1.34	1.43	1.60	1.99	2.38	3.90	0.02
HN7	0.92	0.88	0.93	0.93	1.09	1.21	1.22	0.03
HN8	1.20	1.29	1.30	1.44	1.83	2.10	3.42	0.03
HN9	0.94	0.91	0.97	0.99	1.26	1.36	1.97	0.04
HN10	0.98	0.98	1.00	1.06	1.31	1.36	1.89	0.02
HN11	1.00	1.04	1.04	1.14	1.39	1.48	2.11	0.02
HN12	0.93	0.93	0.84	0.79	0.71	0.61	0.62	0.01
HN13	0.95	0.90	0.95	0.95	1.20	1.28	1.60	0.03
HN14	0.94	0.95	1.00	1.08	1.41	1.58	2.56	0.01
HN15	1.39	1.56	1.67	1.66	2.20	2.76	4.26	0.03
HN16	0.98	1.00	1.04	1.15	1.46	1.63	2.77	0.02
HN17	1.32	1.28	1.27	1.31	1.94	2.13	4.03	0.04
HN18	1.10	1.06	0.96	0.93	0.82	0.84	1.27	0.01
HN19	1.39	1.39	1.51	1.49	2.04	2.50	4.09	0.03
HN20	1.15	1.09	1.19	1.14	1.41	1.70	1.97	0.06
HN21	1.25	1.19	1.34	1.38	1.90	1.99	3.30	0.02
HN22	1.16	1.24	1.32	1.35	1.74	2.13	3.74	0.02
HN23	1.03	1.02	1.04	1.14	1.43	1.64	2.56	0.03
HN24	0.99	1.01	1.05	1.16	1.45	1.58	2.38	0.03
HN25	1.29	1.40	1.44	1.49	1.93	2.49	3.74	0.03
HN26	1.21	1.19	1.29	1.30	1.74	2.03	3.09	0.03

TABLE 5-continued

Pharmacokinetic profiles of TRADE-identified AAV variants in C57BL/6J mice following intravenous administration								
	1 m	10 m	30 m	1 h	4 h	8 h	24 h	72 h
AAV DNA Barcode-Seq analysis was performed on the blood samples obtained from the mice injected with 1×10^{13} vg/kg of the DNA/RNA-barcoded dsAAV-hSynl-GFP-BCLib library (see Table 3, n=2) All the values are normalized with those of AAV9 (AAV9=1.0). All the values are normalized to AAV9 (AAV9=1.0).								

TABLE 6

Brain neuronal transduction efficiency and biodistribution of the TRADE-identified AAV variants in BALB/cJ mice following intravenous administration.										
	Brain (RNA)	Lung (RNA)	Heart (DNA)	Kidney (DNA)	Liver (DNA)	Lung (DNA)	Muscle (DNA)	Pancreas (DNA)	Spleen (DNA)	Testis (DNA)
AAV9	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
AAV9-N272A	1.00	0.30	0.45	1.23	0.03	0.64	0.29	1.23	2.75	0.17
AAV-PHP.B	1.06	0.44	0.56	1.30	0.22	0.67	0.40	1.12	1.69	0.30
HN1	7.59	17.84	0.18	0.52	0.05	3.75	0.21	0.28	0.31	0.36
HN2	3.26	4.71	0.31	0.52	0.07	3.13	0.25	0.39	0.69	0.44
HN3	1.00	0.35	0.51	1.04	0.04	0.41	0.20	0.60	1.38	0.14
HN4	0.41	0.41	0.29	1.58	0.01	0.44	0.16	1.18	2.50	0.09
HN5	0.28	0.15	0.25	1.34	0.00	0.38	0.13	1.05	2.25	0.07
HN6	0.25	0.06	0.30	1.60	0.01	0.45	0.17	1.17	2.69	0.07
HN7	0.54	0.21	0.27	0.65	0.12	0.22	0.16	0.32	0.75	0.11
HN8	0.34	0.03	0.28	1.53	0.01	0.38	0.14	0.94	2.10	0.07
HN9	0.49	0.19	0.28	1.18	0.02	0.22	0.11	0.45	1.08	0.07
HN10	0.54	0.19	0.33	1.25	0.01	0.30	0.14	0.50	1.82	0.08
HN11	0.23	0.12	0.23	1.15	0.03	0.17	0.09	0.62	0.95	0.05
HN12	0.34	0.13	0.19	0.98	0.03	0.12	0.07	0.23	0.48	0.04
HN13	0.43	0.25	0.36	1.25	0.03	0.25	0.15	0.41	1.01	0.09
HN14	0.22	0.08	0.20	0.93	0.02	0.14	0.08	0.38	0.68	0.04
HN15	0.25	0.26	0.33	1.80	0.01	0.46	0.19	1.39	3.03	0.10
HN16	0.62	0.25	0.32	0.93	0.01	0.41	0.16	0.71	1.55	0.10
HN17	0.18	0.12	0.22	1.40	0.00	0.37	0.13	0.96	2.29	0.07
HN18	0.75	0.16	0.40	1.46	0.04	0.29	0.15	0.59	1.36	0.08
HN19	0.28	0.10	0.28	1.57	0.01	0.44	0.15	1.14	2.51	0.08
HN20	0.69	0.11	0.42	0.56	0.08	0.31	0.13	0.53	0.85	0.12
HN21	0.14	0.15	0.25	1.38	0.00	0.34	0.11	0.91	2.34	0.08
HN22	0.41	0.09	0.22	1.33	0.01	0.36	0.12	1.01	2.09	0.07
HN23	0.79	0.32	0.33	0.99	0.02	0.36	0.17	0.43	1.24	0.10
HN24	0.56	0.25	0.34	1.17	0.02	0.34	0.14	0.53	1.33	0.08
HN25	0.19	0.02	0.30	1.65	0.01	0.49	0.17	1.15	2.68	0.09
HN26	0.31	0.11	0.33	1.52	0.02	0.35	0.17	0.84	1.77	0.09

A DNA/RNA-barcoded dsAAV-hSynl-GFP-BC library (dsAAV-hSynl-GFP-BCLib) containing 26 novel AAV variants identified by TRADE and control AAV capsids was injected intravenously into 3 BALB/cJ mice at a dose of 5×10^{11} vg per mouse (for the library, see Table 3). Two weeks post-injection, various tissues were harvested and analyzed for brain transduction by AAV RNA Barcode-Seq and biodistribution to peripheral organs by AAV DNA Barcode-Seq. All the values are normalized with those of AAV9 (AAV9=1.0).

TABLE 7

Transduction efficiency of hSynl-TRADE-derived AAV variants in various brain regions of one rhesus macaque following intravenous administration as determined by AAV hSynl-RNA Barcode-Seq analysis												
	Cerebel- lu (Granu- lar layer)	Cerebel- lum (Pur- kinje)	Cingula- teGyrus	Frontal Cortex	Hippo- campus	Hypo- thala- mus	Medulla	Occipi- tal Cortex	Pons	Preoptic Area	Put- men	Thala- mus
AAV9	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
AAV9-N272A	1.28	1.63	1.76	1.75	2.08	1.00	1.45	1.51	1.45	0.66	1.99	1.19
AAV-PHPB	0.78	1.21	1.27	1.50	1.39	0.94	1.33	1.23	1.62	0.35	1.29	0.69
HN1	1.99	1.78	2.68	2.18	4.07	2.05	1.39	1.54	1.99	1.99	2.54	2.84
HN2	0.81	0.96	1.06	1.13	1.45	0.78	0.77	0.84	0.79	0.72	1.15	1.16
HN3	1.75	2.03	2.14	2.28	3.54	1.48	1.87	1.67	1.71	1.28	2.20	1.87
HN4	0.27	0.61	0.70	0.68	0.37	0.39	0.58	0.54	0.27	0.30	0.49	0.62
HN5	0.15	0.27	0.35	0.22	0.15	0.01	0.27	0.17	0.27	0.22	0.31	0.15
HN6	0.06	0.28	0.36	0.12	0.10	0.27	0.04	0.12	0.12	0.01	0.26	0.10
HN7	0.96	1.40	1.44	1.45	1.64	1.13	1.48	1.18	1.01	0.87	1.39	1.29
HN8	0.38	0.61	0.59	0.67	0.73	0.42	0.56	0.42	0.45	0.35	0.57	0.50
HN9	1.17	1.45	1.91	1.66	2.15	0.92	1.65	1.30	1.27	1.10	1.96	1.48
HN10	1.08	1.24	1.40	1.45	1.78	0.87	1.33	1.16	1.11	0.76	1.50	1.00
HN11	0.96	1.22	1.37	1.42	1.62	1.00	1.28	1.15	1.12	0.65	1.41	1.19
HN12	1.04	1.43	1.64	1.70	1.98	0.97	1.49	1.26	1.09	0.73	1.74	1.44
HN13	1.77	1.74	1.86	1.82	2.17	1.20	2.38	1.54	1.97	1.36	2.14	1.77
HN14	0.13	0.45	0.27	0.26	0.28	0.14	0.30	0.32	0.19	0.04	0.23	0.17
HN15	0.38	0.43	0.19	0.46	0.44	0.04	0.28	0.19	0.23	0.63	0.36	0.09
HN16	0.57	0.65	0.79	0.82	0.89	0.35	0.75	0.77	0.64	0.18	0.72	0.54
HN17	0.05	0.18	0.24	0.14	0.08	0.28	0.16	0.11	0.07	0.01	0.19	0.03
HN18	1.21	1.23	1.62	1.70	2.60	1.17	1.47	1.15	1.09	0.69	1.46	1.13
HN19	0.24	0.24	0.21	0.59	0.50	0.13	0.14	0.26	0.26	0.01	0.31	0.17
HN20	1.08	1.42	1.60	1.81	2.28	1.45	1.51	1.22	1.41	1.32	2.27	1.34
HN21	0.19	0.11	0.05	0.15	0.04	0.49	0.27	0.19	0.08	0.01	0.10	0.14
HN22	0.27	0.17	0.48	0.59	0.49	0.24	0.24	0.27	0.19	0.01	0.23	0.12
HN23	0.60	1.01	1.21	1.11	1.55	0.61	1.23	0.92	0.95	0.49	1.25	0.76
HN24	0.99	1.18	1.19	1.33	1.71	0.70	1.17	1.06	1.04	0.57	1.39	1.21
HN25	0.13	0.14	0.06	0.32	0.21	0.12	0.23	0.24	0.07	0.01	0.28	0.08
HN26	0.35	0.52	0.42	0.60	0.88	0.40	0.50	0.44	0.36	0.27	0.61	0.28

AAV RNA Barcode-Seq analysis was performed on RNAs extracted from various brain regions of one rhesus macaque (n=1) intravenously injected with 2.0×10^{13} vg/kg of a DNA/RNA-barcoded dsAAV-hSynl-GFP-BCLib library that expresses RNA barcodes under the control of the hSynl enhancer-promoter. All the values are normalized with those of AAV9 (AAV9=1.0).

TABLE 8

Pharmacokinetic profiles of hSynl-TRADE-derived AAV variants in rhesus macaque following intravenous administration.								
	1 m	10 m	30 m	1 h	4 h	8 h	24 h	72 h
AAV9	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
AAV9-N272A	0.95	0.96	1.06	1.05	1.24	1.79	2.64	1.18
AAV-PHP.B	1.00	1.10	1.16	1.11	1.27	1.99	2.94	1.69
HN1	0.78	0.66	0.71	0.65	0.67	0.88	0.65	0.30
HN2	0.69	0.72	0.69	0.62	0.75	0.92	1.24	0.25
HN3	0.85	0.86	0.96	0.77	1.12	1.70	2.60	0.60
HN4	1.07	1.07	1.15	1.15	1.30	1.88	3.39	2.15
HN5	0.99	0.98	1.14	1.06	1.19	1.62	2.83	1.39
HN6	1.13	1.09	1.18	1.09	1.32	1.97	3.32	1.90
HN7	0.75	0.78	0.81	0.79	0.95	1.33	1.78	0.27
HN8	1.05	1.06	1.02	1.07	1.27	1.82	3.02	1.67
HN9	0.83	0.76	0.87	0.78	0.92	1.36	1.91	0.29
HN10	0.84	0.87	0.90	0.87	1.14	1.32	2.32	0.28
HN11	0.88	0.89	0.90	0.86	1.20	1.55	2.42	0.61
HN12	0.81	0.80	0.86	0.80	0.99	1.41	1.90	0.27
HN13	0.76	0.71	0.82	0.76	0.90	1.31	1.86	0.29
HN14	0.76	0.75	0.83	0.78	0.99	1.40	2.21	0.18
HN15	1.31	1.07	1.27	1.23	1.36	2.37	4.46	2.03
HN16	0.76	0.80	0.91	0.84	0.98	1.41	1.90	0.26
HN17	1.02	1.07	1.21	1.00	1.29	1.88	2.91	1.84
HN18	0.88	0.88	0.96	0.90	1.10	1.57	2.44	0.58
HN19	1.08	1.04	1.15	1.14	1.26	2.04	3.69	2.05
HN20	0.89	0.82	0.88	0.81	0.91	1.62	2.28	0.39
HN21	1.00	1.00	1.11	0.98	1.20	1.54	2.41	2.10
HN22	0.96	0.97	1.09	1.03	1.24	1.74	2.90	1.82
HN23	0.76	0.76	0.86	0.80	0.97	1.50	2.14	0.35
HN24	0.93	1.00	0.96	1.00	1.41	1.48	2.31	1.41
HN25	1.05	1.16	1.08	1.19	1.18	2.00	3.52	1.54
HN26	1.03	0.98	1.08	1.07	1.18	1.77	2.72	1.56

AAV DNA Barcode-Seq analysis was performed on the blood samples obtained from a single rhesus macaque injected with 2 x 10¹³ vg/kg of the DNA/RNA-barcoded dsAAV-hSynl-GFP-BCLib library (the same animal as in Table 7). All the values are normalized with those of AAV9 (AAV9=1.0).

TABLE 9

Biodistribution of hSynl-TRADE-derived AAV variants to peripheral tissues of a rhesus macaque following intravenous administration as determined by AAV DNA Barcode-Seq analysis											
	Liver	Heart	Lung	Kidney	Pancreas	Spleen	Gastoc-nemius muscle	oleus muscleS	Intestine	Bone marrow	Smooth muscle (stomach
AAV9	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
AAV9-N272A	0.07	0.20	0.79	1.29	2.91	4.67	0.26	0.50	0.64	0.06	0.82
AAV-PHP.B	0.35	0.42	1.09	1.22	2.55	2.14	0.54	0.77	0.40	0.43	0.80
HN1	0.26	0.41	0.34	0.41	0.35	0.76	1.04	1.12	0.24	0.33	0.33
HN2	0.16	0.17	0.35	0.32	0.51	0.71	0.32	0.52	0.11	0.07	0.30
HN3	0.12	0.21	0.78	0.91	2.19	2.48	0.25	0.58	0.18	0.05	0.63
HN4	0.02	0.08	0.78	1.12	2.25	3.42	0.17	0.41	0.15	0.03	0.39
HN5	0.01	0.05	0.73	0.98	2.24	2.85	0.10	0.30	0.07	0.01	0.37
HN6	0.01	0.05	0.92	1.11	2.15	3.50	0.11	0.40	0.12	0.01	0.30
HN7	0.27	0.19	0.36	0.43	0.47	0.51	0.18	0.23	0.16	0.12	0.37
HN8	0.04	0.08	0.71	0.98	2.28	2.57	0.12	0.38	0.11	0.02	0.44
HN9	0.12	0.13	0.32	0.36	0.71	0.79	0.13	0.31	0.11	0.04	0.29
HN10	0.08	0.13	0.63	0.63	1.39	2.53	0.16	0.38	0.11	0.02	0.28
HN11	0.06	0.16	0.63	0.74	1.62	1.68	0.18	0.44	0.16	0.03	0.47
HN12	0.19	0.11	0.28	0.38	0.56	0.61	0.10	0.19	0.09	0.05	0.30
HN13	0.18	0.23	0.58	0.44	0.88	1.12	0.21	0.38	0.18	0.06	0.49
HN14	0.00	0.04	0.30	0.49	0.59	0.83	0.06	0.28	0.05	0.01	0.34
HN15	0.01	0.06	1.01	1.43	2.43	3.80	0.13	0.46	0.10	0.02	0.69
HN16	0.02	0.07	0.37	1.00	0.70	0.87	0.08	0.17	0.06	0.02	0.28
HN17	0.01	0.04	0.85	1.01	2.38	3.25	0.08	0.29	0.10	0.01	0.32
HN18	0.15	0.13	0.36	0.52	0.90	1.17	0.13	0.31	0.09	0.01	0.33
HN19	0.06	0.08	0.93	1.14	2.98	3.41	0.15	0.42	0.10	0.02	0.36
HN20	0.36	0.22	0.34	0.50	0.88	0.96	0.21	0.48	0.14	0.05	0.31
HN21	0.02	0.05	0.74	1.03	2.17	3.08	0.12	0.33	0.06	0.01	0.57
HN22	0.06	0.07	0.72	0.89	1.73	2.68	0.14	0.31	0.09	0.02	0.25
HN23	0.04	0.08	0.61	0.28	0.74	0.71	0.09	0.30	0.10	0.01	0.24

TABLE 9-continued

Biodistribution of hSynl-TRADE-derived AAV variants to peripheral tissues of a rhesus macaque following intravenous administration as determined by AAV DNA Barcode-Seq analysis											
	Liver	Heart	Lung	Kidney	Pancreas	Spleen	Gastoc-nemius muscle	oleus muscleS	Intestine	Bone marrow	Smooth muscle (stomach)
HN24	0.06	0.14	0.66	0.74	1.99	1.98	0.18	0.43	0.13	0.03	0.50
HN25	0.06	0.07	0.90	1.21	2.76	3.28	0.22	0.43	0.10	0.03	0.56
HN26	0.17	0.10	0.64	0.90	1.84	2.38	0.12	0.35	0.12	0.02	0.56

AAV DNA Barcode-Seq analysis was performed on DNA extracted from various peripheral tissues of one rhesus macaque (n=1, the same animal as presented in Table 7) intravenously injected with 2 x 10¹³ vg/kg of a DNA/RNA-barcoded dsAAV-hSynl-GFP-BCLib library. All values are normalized to AAV9 (AAV9=1.0).

TABLE 9

Splice donor and splice acceptor sites identified in antisense AAV cap ORF transcripts.			
SEQ ID	AAV serotype	SD or SA	Exon-intron junction sequence (Introns are underlined)
SEQ ID NO:199	AAV1	SD	1009-CTTACCAGCA-1018
SEQ ID NO:199	AAV3	SD	1006-CTTACCAGCA-1015
SEQ ID NO:200	AAV1	SD	1228-TTTACCTTCA-1237
SEQ ID NO:201	AAV3	SD	1237-TATACCTTCG-1246
SEQ ID NO:202	AAV1	SD	1331-ATTACCTGAA-1340
SEQ ID NO:203	AAV1	SD	1434-GCTACCTGGA-1443
SEQ ID NO:204	AAV1	SD	1502-TTTACCTGGA-1510
SEQ ID NO:205	AAV1	SD	1803-ATTACCTGGC-1812
SEQ ID NO:206	AAV3	SD	1803-CTTACCTGGC-1812
SEQ ID NO:207	AAV1	SD	1835-TGTACCTGCA-1844
SEQ ID NO:208	AAV1	SD	2189-GTTACCTTAC-2198
SEQ ID NO:209	AAV9	SD	2189-GATACCTGAC-2198
SEQ ID NO:210	AAV1	SD	2194-CTTACCCGTC-2203
SEQ ID NO:211	AAV3	SD	2194-CTCACACGAA-2203
SEQ ID NO:212	AAV1	SA	305-AGCGTCTGCA-314
SEQ ID NO:213	AAV1	SA	414-GGCTCCTGGA-423
SEQ ID NO:213	AAV3	SA	414-GGCTCCTGGA-423
SEQ ID NO:214	AAV1	SA	495-GCCCCGTAAA-504
SEQ ID NO:214	AAV9	SA	495-GCCCCGTAAA-504
SEQ ID NO:215	AAV3	SA	1133-TCACCCTGAA-1142
SEQ ID NO:216	AAV1	SA	1181-ACTGCCTGGA-1190
SEQ ID NO:202	AAV1	SA	1331-ATTACCTGAA-1340
SEQ ID NO:217	AAV3	SA	1328-ACTACCTGAA-1337
SEQ ID NO:218	AAV1	SA	1464-CGTTTCTAAA-1473
SEQ ID NO:219	AAV1	SA	1653-AAACACTGCA-1662
SEQ ID NO:220	AAV1	SA	2054-GGGAGCTGCA-2063
SEQ ID NO:463	AAV3	SA	2054-GGGAGCTACA-2063

Ten nucleotides around exon-intron junctions identified in antisense AAV cap mRNA are presented with the junction at the center. Letters with underlines represent intron sequences. Letters with no underline represent

TABLE 9-continued

Splice donor and splice acceptor sites identified in antisense AAV cap ORF transcripts.			
SEQ ID	AAV serotype	SD or SA	Exon-intron junction sequence (Introns are underlined)

exon sequences. Numbers indicate nucleotide positions of the AAV cap ORF. SD, splice donor; SA, splice acceptor. Please note that SEQ ID NO: 199 of AAV1 and SEQ ID NO: 199 of AAV3 are corresponding to each other in sequence alignment. Likewise, SEQ ID NO: 213 of AAV1 and SEQ ID NO: 213 of AAV3 are corresponding to each other in sequence alignment.

[0062] In the course of the experiment, when the AAV9 cap gene ORF was expressed in an antisense orientation in HEK293 cells or Neuro2a cells, the majority of the antisense AAV9 cap gene mRNA-derived RT-PCR products were truncated by approximately 1.7 kb (FIG. 4), although this was not the case with the RNA recovered from the AAV-PHPB-hSynl-GFP-TRADE-transduced mouse brain tissue (FIG. 3). Sequencing of the truncated RT-PCR products revealed that a 1694 bp-long region was missing within the AAV9 cap ORF (FIG. 5). Without being bound by any particular theory, it appears that the truncation results from a splicing event, based on the observation that we could identify splice donor and acceptor sites in the PCR products that have the common features of exon-intron junctions. Intriguingly, a sequence alignment study revealed that the cryptic splice donor and acceptor sites with the common features of exon-intron junctions can also be identified in many naturally occurring AAV serotypes at the regions corresponding to the splice donor and acceptor sites identified in the AAV9 cap gene and they are highly conserved (FIG. 6). This indicates that splicing could potentially take place in the cap ORF-derived antisense mRNA of not only AAV9 but also many other AAV strains. To date, we have found that splicing occurs on the AAV3 cap ORF-derived antisense mRNA when it is expressed under the control of a human liver-specific promoter (LSP) in HepG2 cells. Although full characterization has not yet been completed, a preliminary RT-PCR using an antisense mRNA-specific RT primer yielded truncated RT-PCR products in addition to the full-length, non-spliced product. The sequencing analysis of two truncated RT-PCR products revealed that there were multiple splicing events on the antisense mRNA (FIG. 7). A sequencing alignment study has identified additional potential splice donor and acceptor sites (FIG. 8 and FIG. 9). We also found splicing events in the antisense mRNA derived from the AAV1 cap ORF when antisense mRNA was transcribed by the hSynl enhancer-promoter in HEK293 cells or Neuro2a cells (FIG. 10). Many of the identified splice donor sites (GT/CA) and splice acceptor sites (AG/TC) are highly conserved across different serotypes, indicating the possibi-

lity that these sites are also utilized as splicing donor and acceptor sites in the AAV serotypes that have yet to be investigated. Indeed, we have found that splicing of antisense mRNA transcripts of the AAV1, AAV3 and AAV9 cap ORFs uses several common splice donor and acceptor sites (FIG. 10). To date, we have not yet observed splicing of antisense mRNA transcripts of the AAV5 cap ORF. For serotypes other than AAV1, 3, 5 and 9, splicing events in antisense mRNA of the AAV cap ORFs have not yet been investigated.

[0063] Potential splicing of the cap ORF-derived antisense mRNA is scientifically intriguing, but may hinder the TRADE system when the full-length cap ORF sequence needs to be recovered from antisense mRNA. To overcome this potential issue, we introduced silent mutations that presumably disrupt the conserved sequences at exon-intron junctions and branching points. To demonstrate proof of principle of this approach, we introduced silent mutations into the AAV9 cap ORF contained in the plasmid, pAAV9-N272A-PHPB-hSyn1-GFP-TRADE, that disrupt the splice acceptor (SA) consensus sequence (pAAV9NS1 construct), the splice donor (SD) consensus sequence (pAAV9NS2 construct), and both the splice acceptor and donor consensus sequences (pAAV9NS3 construct). Please note that NS stands for “non-spliced.” The method we use to disrupt these consensus sequences is described below.

[0064] We codon-optimize the AAV cap ORF sequence for human cell expression.

[0065] To identify potential splice donor and acceptor sites on antisense mRNA derived from the cap ORFs, we develop and use our proprietary database of potential splice donor and acceptor sites on antisense mRNA based on our experimental and bioinformatics observations (i.e., FIGS. 5, 6, 7, 8, 9 and 10).

[0066] We destroy the GT (splice donor) and / or AG (splice acceptor) consensus sequence by changing at least one nucleotide using the codon-optimized sequence. If the codon-optimized sequence is not applicable, we use an alternative nucleotide(s) that can destroy the consensus sequence.

[0067] We remove a stretch of T’s upstream of the splice acceptor sites by introducing silence mutations based on the

codon-optimized sequence. If the codon-optimized sequence is not sufficient to destroy a stretch of T’s, we use alternative nucleotides.

[0068] We also avoid G at the exon termini as much as possible.

[0069] Using several programs that can predict branching points (e.g., Human Splicing Finder (Desmet, Hamroun et al. 2009)), we identify potential branching points and replace them with the codon-optimized sequence. If the degree of nucleotide changes attainable by this method is not sufficient, we introduce alternative nucleotides to disrupt potential branching points.

[0070] With this method, we have created AAV9NS1 (SA, destroyed), AAV9NS2 (SD, destroyed) and AAV9NS3 (both SD and SA, destroyed) cap ORFs (FIG. 9). We expressed these ORFs in an antisense orientation under the control of the hSyn1 enhancer-promoter in Neuro2a cells by transient plasmid transfection, and analyzed the antisense transcripts by RT-PCR. This experiment revealed that the splicing could be effectively suppressed in all of the NS1, NS2 and NS3 cap ORFs (FIG. 10). It should be noted that even if splicing takes place on the cap ORF-derived antisense mRNA, it would still be possible to recover the relatively small peptide insertion region of the cap ORF by RT-PCR from pre-mRNA.

[0071] The TRADE method described herein uses antisense mRNA for viral protein evolution to establish the proof-of-principle and to show successful reduction of the method to practice. The TRADE system can also utilize mRNA in a sense orientation as long as the viruses can be produced and potential expression of viral proteins in target cells during the directed evolution procedure does not hinder successful evolution of novel capsids.

[0072] Additional information related to nucleic acid splicing and AAV may be found in Desmet et al., *Nucleic Acids Res* 37, e67 (2009); Matsuzaki et al., *Neurosci Lett* 665, 182-188 (2018); and Hordeaux et al., *Mol Ther* 26, 664-668 (2018).

[0073] All references cited in this disclosure are incorporated by reference in their entirety.

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Ala Ser Arg Asp	Pro Ser Ala Thr
1	5
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Asp Ala Asn Asp	Ala Arg Gln Arg
1	5
<210> SEQ ID NO 10	
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<212> TYPE: PRT	
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Asp Leu Ala Arg Met Ala Ala Ala
1 5

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<210> SEQ ID NO 11
<211> LENGTH: 8
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<213> ORGANISM: Artificial Sequence
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<220> FEATURE:
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<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 11

Asp Gln Gly Ser Ile Thr Ala His
1 5

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<210> SEQ ID NO 12
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 12

Asp Arg Thr Pro Gly Val Asn Val
1 5

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<211> LENGTH: 8
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
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<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 13

Asp Thr Asp Thr Leu Ser Pro Gly
1 5

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<210> SEQ ID NO 14
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<220> FEATURE:
<221> NAME/KEY: PEPTIDE
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<400> SEQUENCE: 14

Glu Glu Asp Ala Gln Leu Leu Ile
1 5

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<210> SEQ ID NO 15
<211> LENGTH: 8
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<212> TYPE: PRT	
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<220> FEATURE:	
<223> OTHER INFORMATION: peptide	
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Glu Lys Leu Asn Asp Trp Pro Thr	
1 5	
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<400> SEQUENCE: 16	
Glu Leu Asn Ser Ala Arg Gln Val	
1 5	
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Glu Leu Gln Ser Phe Ala Gly Leu	
1 5	
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Glu Asn Lys Ser Ala Pro Leu Pro	
1 5	
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<212> TYPE: PRT	
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<220> FEATURE:	
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<221> NAME/KEY: PEPTIDE	
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Glu Arg Thr Ala Val Lys Gly Asn	
1	5
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Gly Gly Ile Gln Thr Val Val Thr	
1	5
<210> SEQ ID NO 21	
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Gly Gly Ser Pro Leu Ala His Pro	
1	5
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<220> FEATURE:	
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<400> SEQUENCE: 22	
Gly Gly Thr Ala Ala Gln Gly Val	
1	5
<210> SEQ ID NO 23	
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<220> FEATURE:	
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<400> SEQUENCE: 23	

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Gly Lys Met Ala Ser Gly Ser Leu
1 5

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<210> SEQ ID NO 24
<211> LENGTH: 8
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<220> FEATURE:
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<400> SEQUENCE: 24

Gly Lys Gln Pro Val Gln Pro Tyr
1 5

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<210> SEQ ID NO 25
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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<220> FEATURE:
<221> NAME/KEY: PEPTIDE
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<400> SEQUENCE: 25

Gly Asn Pro His Thr Gly Ser Thr
1 5

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<210> SEQ ID NO 26
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 26

Gly Pro Thr Leu Gly Gly Ser Gly
1 5

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<210> SEQ ID NO 27
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 27

Gly Gln Arg Gly Leu Pro Ile Ala
1 5

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<210> SEQ ID NO 28
<211> LENGTH: 8
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<210> SEQ ID NO 32
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
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<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1) .. (8)
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<400> SEQUENCE: 32

His Pro Gly Ser Gly Ala Gly Pro
1 5

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<210> SEQ ID NO 33
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<220> FEATURE:
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<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 33

His Gln Val Thr Ser Ser Gly Ala
1 5

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<210> SEQ ID NO 34
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 34

His Val Gly Ser Gln Met His Ala
1 5

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<210> SEQ ID NO 35
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
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<220> FEATURE:
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<222> LOCATION: (3)..(3)
<223> OTHER INFORMATION: any amino acid
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<400> SEQUENCE: 35

Ile Gly Xaa Thr Val Pro Met Gln
1 5

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<210> SEQ ID NO 36
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
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<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)

<400> SEQUENCE: 36

Lys Phe Thr Arg Asp Gly Pro Tyr
1 5

<210> SEQ ID NO 37
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)

<400> SEQUENCE: 37

Lys Gly Pro Ala Glu Gln Gly His
1 5

<210> SEQ ID NO 38
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)

<400> SEQUENCE: 38

Leu Ala His Ser Pro Arg Leu Trp
1 5

<210> SEQ ID NO 39
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)

<400> SEQUENCE: 39

Leu Glu Arg Asn Arg Asp Ser Asp
1 5

<210> SEQ ID NO 40
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)

<400> SEQUENCE: 40

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Leu Glu Thr His Thr Ser Leu Thr
1 5

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<210> SEQ ID NO 41
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 41

Leu His Asp Gly Lys Tyr Ser Thr
1 5

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<210> SEQ ID NO 42
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 42

Leu Lys Ala Thr Gly Arg Gly Lys
1 5

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<210> SEQ ID NO 43
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 43

Leu Leu Pro Gly Ser Ala Asp Gly
1 5

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<210> SEQ ID NO 44
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 44

Leu Leu Val Thr Ala Arg Ser His
1 5

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<210> SEQ ID NO 45
<211> LENGTH: 8
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<210> SEQ ID NO 49
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
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<221> NAME/KEY:  PEPTIDE
<222> LOCATION:  (1) .. (8)
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<400> SEQUENCE: 49

Met Leu Gly Ser Gln Val Pro Thr
1 5

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<210> SEQ ID NO 50
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 50

Met Ser Gly Gln Gly Tyr Gln Ala
1 5

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<210> SEQ ID NO 51
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 51

Asn Pro Gly Arg Asp Phe Arg Asp
1 5

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<210> SEQ ID NO 52
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 52

Asn Gln Pro Ser Asp Tyr Val Ser
1 5

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<210> SEQ ID NO 53
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
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<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 53

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<210> SEQ ID NO 58
<211> LENGTH: 8
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<210> SEQ ID NO 62
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
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<221> NAME/KEY:  PEPTIDE
<222> LOCATION:  (1) .. (8)
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<400> SEQUENCE: 62

Pro Val Ala Asn Pro Thr Thr Ala
1 5

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<210> SEQ ID NO 63
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 63

Pro Val Leu Gly Gly Pro Pro Lys
1 5

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<210> SEQ ID NO 64
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 64

Gln Gly Ser Arg Gln Gly Ser Ser
1 5

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<210> SEQ ID NO 65
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 65

Gln Met Ala Glu Thr Pro Ile Ser
1 5

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<210> SEQ ID NO 66
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
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<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 66

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Gln Met Leu Gly Ile Gly Arg Ser	
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<210> SEQ ID NO 67	
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<212> TYPE: PRT	
<213> ORGANISM: Artificial Sequence	
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<400> SEQUENCE: 67	
Gln Ser Gly Asp Ser Ala Leu Asn	
1	5
<210> SEQ ID NO 68	
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<212> TYPE: PRT	
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<220> FEATURE:	
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Arg Ala Gly Leu Thr Ser Ser Glu	
1	5
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<212> TYPE: PRT	
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<220> FEATURE:	
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<220> FEATURE:	
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<400> SEQUENCE: 69	
Arg Leu Asp Asn Thr Gly Val Gly	
1	5
<210> SEQ ID NO 70	
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<212> TYPE: PRT	
<213> ORGANISM: Artificial Sequence	
<220> FEATURE:	
<223> OTHER INFORMATION: peptide	
<220> FEATURE:	
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<222> LOCATION: (1)..(8)	
<400> SEQUENCE: 70	
Arg Met Pro Gly Lys Pro Tyr Ser	
1	5
<210> SEQ ID NO 71	
<211> LENGTH: 8	

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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 71

Arg Val Ala Gly Ala Ser Gln Pro
1 5

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<210> SEQ ID NO 72
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 72

Arg Val Glu Ser Ser Gln Leu Glu
1 5

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<210> SEQ ID NO 73
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 73

Ser Ala Arg Thr Gly Ala Ser Glu
1 5

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<210> SEQ ID NO 74
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 74

Ser Glu Arg Asn Arg Ala Ser Met
1 5

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<210> SEQ ID NO 75
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
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<221> NAME/KEY: PEPTIDE	
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<400> SEQUENCE: 75	
Ser Ile Asp Val Arg Met Ala Ala	
1 5	
<210> SEQ ID NO 76	
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<212> TYPE: PRT	
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<400> SEQUENCE: 76	
Ser Arg Asp Gly His Ile Leu Arg	
1 5	
<210> SEQ ID NO 77	
<211> LENGTH: 8	
<212> TYPE: PRT	
<213> ORGANISM: Artificial Sequence	
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<220> FEATURE:	
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<222> LOCATION: (1)..(8)	
<400> SEQUENCE: 77	
Ser Arg Gln Val Val Leu Pro Gly	
1 5	
<210> SEQ ID NO 78	
<211> LENGTH: 8	
<212> TYPE: PRT	
<213> ORGANISM: Artificial Sequence	
<220> FEATURE:	
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<220> FEATURE:	
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<400> SEQUENCE: 78	
Ser Ser Arg Gly Tyr Thr Ser Thr	
1 5	
<210> SEQ ID NO 79	
<211> LENGTH: 8	
<212> TYPE: PRT	
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<220> FEATURE:	
<221> NAME/KEY: PEPTIDE	
<222> LOCATION: (1)..(8)	
<400> SEQUENCE: 79	

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<210> SEQ ID NO 84
<211> LENGTH: 8
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<212> TYPE: PRT	
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<220> FEATURE:	
<223> OTHER INFORMATION: peptide	
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<222> LOCATION: (1)..(8)	
<400> SEQUENCE: 84	
Thr Leu Ser Glu Pro Pro Lys Lys	
1 5	
<210> SEQ ID NO 85	
<211> LENGTH: 8	
<212> TYPE: PRT	
<213> ORGANISM: Artificial Sequence	
<220> FEATURE:	
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<221> NAME/KEY: PEPTIDE	
<222> LOCATION: (1)..(8)	
<400> SEQUENCE: 85	
Thr Asn Ala Val Ser Gly Lys Ser	
1 5	
<210> SEQ ID NO 86	
<211> LENGTH: 8	
<212> TYPE: PRT	
<213> ORGANISM: Artificial Sequence	
<220> FEATURE:	
<223> OTHER INFORMATION: peptide	
<220> FEATURE:	
<221> NAME/KEY: PEPTIDE	
<222> LOCATION: (1)..(8)	
<400> SEQUENCE: 86	
Thr Arg Ala Pro Thr Ile His Leu	
1 5	
<210> SEQ ID NO 87	
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<212> TYPE: PRT	
<213> ORGANISM: Artificial Sequence	
<220> FEATURE:	
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<220> FEATURE:	
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<222> LOCATION: (1)..(8)	
<400> SEQUENCE: 87	
Thr Arg Glu Ser Thr Asp Arg Gly	
1 5	
<210> SEQ ID NO 88	
<211> LENGTH: 8	
<212> TYPE: PRT	
<213> ORGANISM: Artificial Sequence	
<220> FEATURE:	
<223> OTHER INFORMATION: peptide	
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<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1) .. (8)
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<400> SEQUENCE: 88

Thr Val Ala Ala Ala Pro Asn Leu
1 5

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<210> SEQ ID NO 89
<211> LENGTH: 8
<212> TYPE: PRT
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<400> SEQUENCE: 89

Thr Tyr His Asn Asn Thr Pro Arg
1 5

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<210> SEQ ID NO 90
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<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 90

Val Ser Asn Ser Thr Arg Thr Ser
1 5

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<210> SEQ ID NO 91
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 91

Val Thr Leu Gln Ile Asp Thr Lys
1 5

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<400> SEQUENCE: 92

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<210> SEQ ID NO 97
<211> LENGTH: 8
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Ala Pro Thr Asn Phe Ala His Pro	
1 5	
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Ala Gln Thr Asn Leu Ala Ala Gly	
1 5	
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Ala Ser Leu Pro Asn Leu Gly Gln	
1 5	
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Asp Tyr Met His Asn Thr Gly Leu	
1 5	
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<400> SEQUENCE: 101

Asp Tyr Met His Thr Thr Gly Leu
1 5

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<400> SEQUENCE: 102

Glu Arg Asn Ala Trp His Ala Gly
1 5

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<220> FEATURE:
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<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 103

Glu Thr Gln Ala Thr Pro Met Pro
1 5

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<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 104

Glu Trp Glu Asp Ser Ala Arg Ser
1 5

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<400> SEQUENCE: 105

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Gly Ser Asn His Thr Gln Ser Leu	
1 5	
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His Asp Arg Asp Thr Arg Gln Ala	
1 5	
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Leu Asp Gln Asn Arg Arg Pro Gln	
1 5	
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Leu Glu Gln Gln Arg Gly Ala Ser	
1 5	
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Leu Leu Val Thr Thr Arg Ser His	
1	5
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Leu Val Thr Asn Thr Thr Arg	
1	5
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<220> FEATURE:	
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<400> SEQUENCE: 117	
Met Glu Ser Gln Arg Ala Asn Ser	
1	5
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Met Ile Ser Gln Thr Leu Met Ala
1 5

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<212> TYPE: PRT
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<400> SEQUENCE: 119

Met Met Ser Gln Ser Leu Arg Ala
1 5

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<210> SEQ ID NO 120
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<212> TYPE: PRT
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<400> SEQUENCE: 120

Asn Asn Val Gln Ser Ala Leu Asn
1 5

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<211> LENGTH: 8
<212> TYPE: PRT
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<400> SEQUENCE: 121

Asn Ser Ala Arg Thr Gln Leu Ser
1 5

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<212> TYPE: PRT
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<220> FEATURE:
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<221> NAME/KEY: PEPTIDE
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<400> SEQUENCE: 122

Pro Gln Trp Asn Arg Thr Pro Leu
1 5

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<210> SEQ ID NO 123
<211> LENGTH: 8
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<210> SEQ ID NO 127
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
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<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)

<400> SEQUENCE: 127

Gln Gly Ala Ser Asn Ser Gln Leu
1 5

<210> SEQ ID NO 128
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
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<222> LOCATION: (1)..(8)

<400> SEQUENCE: 128

Gln Gln Asn Gly Thr Arg Pro Ser
1 5

<210> SEQ ID NO 129
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<220> FEATURE:
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<222> LOCATION: (1)..(8)

<400> SEQUENCE: 129

Gln Arg Ser Ala Tyr Pro Thr Ser
1 5

<210> SEQ ID NO 130
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
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<222> LOCATION: (1)..(8)

<400> SEQUENCE: 130

Gln Arg Thr Pro Ser Ile Thr Pro
1 5

<210> SEQ ID NO 131
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)

<400> SEQUENCE: 131

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<210> SEQ ID NO 136
<211> LENGTH: 8
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<212> TYPE: PRT	
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<220> FEATURE:	
<223> OTHER INFORMATION: peptide	
<220> FEATURE:	
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Ser Ala Thr Ile Ser Leu Gln Val	
1 5	
<210> SEQ ID NO 137	
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<212> TYPE: PRT	
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<222> LOCATION: (1)..(8)	
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Ser His Thr Asn Leu Arg Asp Thr	
1 5	
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<212> TYPE: PRT	
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<220> FEATURE:	
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<400> SEQUENCE: 138	
Ser Arg Met Gly Glu Thr Pro Gln	
1 5	
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<212> TYPE: PRT	
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<220> FEATURE:	
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<400> SEQUENCE: 139	
Ser Ser Gly Tyr Leu Thr Ala Asn	
1 5	
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<211> LENGTH: 8	
<212> TYPE: PRT	
<213> ORGANISM: Artificial Sequence	
<220> FEATURE:	
<223> OTHER INFORMATION: peptide	
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<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)

<400> SEQUENCE: 140

Thr Gly Asn Ser Pro Glu Gln Ala
1 5

<210> SEQ ID NO 141
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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 141

Thr His Ser Gln Gly Arg Leu Ala
1 5

<210> SEQ ID NO 142
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<220> FEATURE:
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<222> LOCATION: (1)..(8)

<400> SEQUENCE: 142

Thr Pro Ile Val Gly Ser Asn Val
1 5

<210> SEQ ID NO 143
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<220> FEATURE:
<221> NAME/KEY: PEPTIDE
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<400> SEQUENCE: 143

Thr Pro Pro Lys Ser Pro Ser Met
1 5

<210> SEQ ID NO 144
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
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<222> LOCATION: (1)..(8)

<400> SEQUENCE: 144

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<210> SEQ ID NO 149
<211> LENGTH: 8
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<210> SEQ ID NO 153
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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
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<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)

<400> SEQUENCE: 153

Gly Gly Met Asn Glu Thr Thr Arg
1 5

<210> SEQ ID NO 154
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)

<400> SEQUENCE: 154

Gly Gly Ser Ala Phe Val Thr Gly
1 5

<210> SEQ ID NO 155
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)

<400> SEQUENCE: 155

Gly Asn Ser His Thr Gly Ser Ser
1 5

<210> SEQ ID NO 156
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
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<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)

<400> SEQUENCE: 156

Asn Ser Ala Arg Thr Gln Leu Ser
1 5

<210> SEQ ID NO 157
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
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<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)

<400> SEQUENCE: 157

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<210> SEQ ID NO 162
<211> LENGTH: 8
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<212> TYPE: PRT
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<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
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<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 162

Thr Pro Gly Leu Asn Asn Ala Arg
1 5

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<210> SEQ ID NO 163
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<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 163

Thr Ser Leu Gly Thr Pro Glu Ala
1 5

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<210> SEQ ID NO 164
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 164

Thr Thr Asn Leu Ala Lys Asn Ser
1 5

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<210> SEQ ID NO 165
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 165

Trp Ser Pro Asp Ala Val Glu Gly
1 5

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<210> SEQ ID NO 166
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
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<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(7)

<400> SEQUENCE: 166

Trp Thr Gly Gly Arg His Leu
1 5

<210> SEQ ID NO 167
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
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<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)

<400> SEQUENCE: 167

Ala Val Ala Gly Asp Arg Leu Leu
1 5

<210> SEQ ID NO 168
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
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<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)

<400> SEQUENCE: 168

Asp Leu Leu Thr Arg Ser Val Ser
1 5

<210> SEQ ID NO 169
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
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<222> LOCATION: (1)..(8)

<400> SEQUENCE: 169

Glu Trp Lys Thr Gln Leu Ala Leu
1 5

<210> SEQ ID NO 170
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
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<222> LOCATION: (1)..(8)

<400> SEQUENCE: 170

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Gly Asn Ile Asn Val Val Pro His
1 5

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<210> SEQ ID NO 171
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<212> TYPE: PRT
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<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 171

Gly Ser Pro Ala Ala Ser Ser Trp
1 5

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<210> SEQ ID NO 172
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<212> TYPE: PRT
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<220> FEATURE:
<223> OTHER INFORMATION: peptide
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<221> NAME/KEY: PEPTIDE
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<400> SEQUENCE: 172

Lys His Ser Leu Thr Leu Glu Ser
1 5

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<210> SEQ ID NO 173
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
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<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 173

Lys Pro Val Ser Thr Asp Thr Phe
1 5

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<210> SEQ ID NO 174
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 174

Leu Asp Arg Ser Gly Ser Thr Gly
1 5

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<210> SEQ ID NO 175
<211> LENGTH: 8
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<210> SEQ ID NO 179
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
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<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1) .. (8)
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<400> SEQUENCE: 179

Asn Asp Gly Leu Arg Leu His Leu
1 5

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<210> SEQ ID NO 180
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
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<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 180

Asn Leu Ser Ala His Ser His Ala
1 5

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<210> SEQ ID NO 181
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
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<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 181

Asn Leu Ser Ala His Ser His Asp
1 5

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<210> SEQ ID NO 182
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 182

Arg Ala Leu Asp Leu Val Thr Arg
1 5

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<210> SEQ ID NO 183
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
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<400> SEQUENCE: 183

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Ser Ala Gly Met Ala Arg Asn Ser
1 5

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<210> SEQ ID NO 184
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 184

Ser Gly Gln Arg Val Gly Ser Ala
1 5

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<210> SEQ ID NO 185
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 185

Ser Gly Gln Arg Val Gly Ser Asp
1 5

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<210> SEQ ID NO 186
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 186

Thr Gly Arg Pro Glu Gln Pro Lys
1 5

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<210> SEQ ID NO 187
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: peptide
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)..(8)
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<400> SEQUENCE: 187

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1 5

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<210> SEQ ID NO 188
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aaggggggagc cgggtcaacgc agcagacgcg gcggccctcg agcacgacaa ggcctacgac 240		
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			20					25					30			
Lys	Ala	Asn	Gln	Gln	His	Gln	Asp	Asn	Ala	Arg	Gly	Leu	Val	Leu	Pro	
		35					40					45				
Gly	Tyr	Lys	Tyr	Leu	Gly	Pro	Gly	Asn	Gly	Leu	Asp	Lys	Gly	Glu	Pro	
	50					55					60					
Val	Asn	Ala	Ala	Asp	Ala	Ala	Ala	Leu	Glu	His	Asp	Lys	Ala	Tyr	Asp	
65					70					75				80		
Gln	Gln	Leu	Lys	Ala	Gly	Asp	Asn	Pro	Tyr	Leu	Lys	Tyr	Asn	His	Ala	
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Asn	Leu	Gly	Arg	Ala	Val	Phe	Gln	Ala	Lys	Lys	Arg	Leu	Leu	Glu	Pro	
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	130					135					140					
Pro	Val	Glu	Gln	Ser	Pro	Gln	Glu	Pro	Asp	Ser	Ser	Ala	Gly	Ile	Gly	
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Lys	Ser	Gly	Ala	Gln	Pro	Ala	Lys	Lys	Arg	Leu	Asn	Phe	Gly	Gln	Thr	
			165						170					175		
Gly	Asp	Thr	Glu	Ser	Val	Pro	Asp	Pro	Gln	Pro	Ile	Gly	Glu	Pro	Pro	
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Ala	Ala	Pro	Ser	Gly	Val	Gly	Ser	Leu	Thr	Met	Ala	Ser	Gly	Gly	Gly	
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Ala	Pro	Val	Ala	Asp	Asn	Asn	Glu	Gly	Ala	Asp	Gly	Val	Gly	Ser	Ser	
	210					215					220					
Ser	Gly	Asn	Trp	His	Cys	Asp	Ser	Gln	Trp	Leu	Gly	Asp	Arg	Val	Ile	
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Ala	Tyr	Phe	Gly	Tyr	Ser	Thr	Pro	Trp	Gly	Tyr	Phe	Asp	Phe	Asn	Arg	
		275					280					285				
Phe	His	Cys	His	Phe	Ser	Pro	Arg	Asp	Trp	Gln	Arg	Leu	Ile	Asn	Asn	
	290					295				300						
Asn	Trp	Gly	Phe	Arg	Pro	Lys	Arg	Leu	Asn	Phe	Lys	Leu	Phe	Asn	Ile	
305					310				315						320	
Gln	Val	Lys	Glu	Val	Thr	Asp	Asn	Asn	Gly	Val	Lys	Thr	Ile	Ala	Asn	
				325					330					335		
Asn	Leu	Thr	Ser	Thr	Val	Gln	Val	Phe	Thr	Asp	Ser	Asp	Tyr	Gln	Leu	
			340					345					350			
Pro	Tyr	Val	Leu	Gly	Ser	Ala	His	Glu	Gly	Cys	Leu	Pro	Pro	Phe	Pro	
		355				360						365				
Ala	Asp	Val	Phe	Met	Ile	Pro	Gln	Tyr	Gly	Tyr	Leu	Thr	Leu	Asn	Asp	
		370				375					380					
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385					390					395					400	
Pro	Ser	Gln	Met	Leu	Arg	Thr	Gly	Asn	Asn	Phe	Gln	Phe	Ser	Tyr	Glu	
			405					410						415		
Phe	Glu	Asn	Val	Pro	Phe	His	Ser	Ser	Tyr	Ala	His	Ser	Gln	Ser	Leu	
			420					425					430			
Asp	Arg	Leu	Met	Asn	Pro	Leu	Ile	Asp	Gln	Tyr	Leu	Tyr	Tyr	Leu	Ser	
		435					440					445				
Lys	Thr	Ile	Asn	Gly	Ser	Gly	Gln	Asn	Gln	Gln	Thr	Leu	Lys	Phe	Ser	
	450					455					460					
Val	Ala	Gly	Pro	Ser	Asn	Met	Ala	Val	Gln	Gly	Arg	Asn	Tyr	Ile	Pro	
465					470					475					480	
Gly	Pro	Ser	Tyr	Arg	Gln	Gln	Arg	Val	Ser	Thr	Thr	Val	Thr	Gln	Asn	
				485					490					495		
Asn	Asn	Ser	Glu	Phe	Ala	Trp	Pro	Gly	Ala	Ser	Ser	Trp	Ala	Leu	Asn	
			500					505					510			
Gly	Arg	Asn	Ser	Leu	Met	Asn	Pro	Gly	Pro	Ala	Met	Ala	Ser	His	Lys	
		515					520					525				
Glu	Gly	Glu	Asp	Arg	Phe	Phe	Pro	Leu	Ser	Gly	Ser	Leu	Ile	Phe	Gly	
	530					535					540					
Lys	Gln	Gly	Thr	Gly	Arg	Asp	Asn	Val	Asp	Ala	Asp	Lys	Val	Met	Ile	
545					550					555					560	
Thr	Asn	Glu	Glu	Glu	Ile	Lys	Thr	Thr	Asn	Pro	Val	Ala	Thr	Glu	Ser	
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Tyr	Gly	Gln	Val	Ala	Thr	Asn	His	Gln	Ser	Ala	Gln	Ala	Gln	Ala	Gln	
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Thr	Gly	Trp	Val	Gln	Asn	Gln	Gly	Ile	Leu	Pro	Gly	Met	Val	Trp	Gln	
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	610					615					620						
Thr	Asp	Gly	Asn	Phe	His	Pro	Ser	Pro	Leu	Met	Gly	Gly	Phe	Gly	Met		
625					630					635					640		
Lys	His	Pro	Pro	Pro	Gln	Ile	Leu	Ile	Lys	Asn	Thr	Pro	Val	Pro	Ala		
				645					650					655			
Asp	Pro	Pro	Thr	Ala	Phe	Asn	Lys	Asp	Lys	Leu	Asn	Ser	Phe	Ile	Thr		
			660					665					670				
Gln	Tyr	Ser	Thr	Gly	Gln	Val	Ser	Val	Glu	Ile	Glu	Trp	Glu	Leu	Gln		
		675					680					685					
Lys	Glu	Asn	Ser	Lys	Arg	Trp	Asn	Pro	Glu	Ile	Gln	Tyr	Thr	Ser	Asn		
	690					695					700						
Tyr	Tyr	Lys	Ser	Asn	Asn	Val	Glu	Phe	Ala	Val	Asn	Thr	Glu	Gly	Val		
705					710					715					720		
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			20					25					30				
Lys	Ala	Asn	Gln	Gln	His	Gln	Asp	Asn	Ala	Arg	Gly	Leu	Val	Leu	Pro		
		35					40					45					
Gly	Tyr	Lys	Tyr	Leu	Gly	Pro	Gly	Asn	Gly	Leu	Asp	Lys	Gly	Glu	Pro		
	50					55					60						
Val	Asn	Ala	Ala	Asp	Ala	Ala	Ala	Leu	Glu	His	Asp	Lys	Ala	Tyr	Asp		
65					70					75					80		
Gln	Gln	Leu	Lys	Ala	Gly	Asp	Asn	Pro	Tyr	Leu	Lys	Tyr	Asn	His	Ala		
			85						90					95			
Asp	Ala	Glu	Phe	Gln	Glu	Arg	Leu	Lys	Glu	Asp	Thr	Ser	Phe	Gly	Gly		
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Asn	Leu	Gly	Arg	Ala	Val	Phe	Gln	Ala	Lys	Lys	Arg	Leu	Leu	Glu	Pro		
		115					120					125					
Leu	Gly	Leu	Val	Glu	Glu	Ala	Ala	Lys	Thr	Ala	Pro	Gly	Lys	Lys	Arg		
	130						135				140						
Pro	Val	Glu	Gln	Ser	Pro	Gln	Glu	Pro	Asp	Ser	Ser	Ala	Gly	Ile	Gly		
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Lys	Ser	Gly	Ala	Gln	Pro	Ala	Lys	Lys	Arg	Leu	Asn	Phe	Gly	Gln	Thr		
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Gly	Asp	Thr	Glu	Ser	Val	Pro	Asp	Pro	Gln	Pro	Ile	Gly	Glu	Pro	Pro		
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		195					200					205					
Ala	Pro	Val	Ala	Asp	Asn	Asn	Glu	Gly	Ala	Asp	Gly	Val	Gly	Ser	Ser		
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Ser	Gly	Asn	Trp	His	Cys	Asp	Ser	Gln	Trp	Leu	Gly	Asp	Arg	Val	Ile		
225					230					235					240		
Thr	Thr	Ser	Thr	Arg	Thr	Trp	Ala	Leu	Pro	Thr	Tyr	Asn	Asn	His	Leu		
				245					250					255			
Tyr	Lys	Gln	Ile	Ser	Asn	Ser	Thr	Ser	Gly	Gly	Ser	Ser	Asn	Asp	Ala		
			260					265					270				
Ala	Tyr	Phe	Gly	Tyr	Ser	Thr	Pro	Trp	Gly	Tyr	Phe	Asp	Phe	Asn	Arg		
		275					280					285					
Phe	His	Cys	His	Phe	Ser	Pro	Arg	Asp	Trp	Gln	Arg	Leu	Ile	Asn	Asn		
	290					295					300						
Asn	Trp	Gly	Phe	Arg	Pro	Lys	Arg	Leu	Asn	Phe	Lys	Leu	Phe	Asn	Ile		
305					310					315					320		
Gln	Val	Lys	Glu	Val	Thr	Asp	Asn	Asn	Gly	Val	Lys	Thr	Ile	Ala	Asn		
				325					330					335			
Asn	Leu	Thr	Ser	Thr	Val	Gln	Val	Phe	Thr	Asp	Ser	Asp	Tyr	Gln	Leu		
			340					345					350				
Pro	Tyr	Val	Leu	Gly	Ser	Ala	His	Glu	Gly	Cys	Leu	Pro	Pro	Phe	Pro		
		355					360					365					
Ala	Asp	Val	Phe	Met	Ile	Pro	Gln	Tyr	Gly	Tyr	Leu	Thr	Leu	Asn	Asp		
	370					375					380						
Gly	Ser	Gln	Ala	Val	Gly	Arg	Ser	Ser	Phe	Tyr	Cys	Leu	Glu	Tyr	Phe		
385					390					395					400		
Pro	Ser	Gln	Met	Leu	Arg	Thr	Gly	Asn	Asn	Phe	Gln	Phe	Ser	Tyr	Glu		
			405						410					415			
Phe	Glu	Asn	Val	Pro	Phe	His	Ser	Ser	Tyr	Ala	His	Ser	Gln	Ser	Leu		
			420					425					430				
Asp	Arg	Leu	Met	Asn	Pro	Leu	Ile	Asp	Gln	Tyr	Leu	Tyr	Tyr	Leu	Ser		
		435					440					445					
Lys	Thr	Ile	Asn	Gly	Ser	Gly	Gln	Asn	Gln	Gln	Thr	Leu	Lys	Phe	Ser		
	450					455					460						
Val	Ala	Gly	Pro	Ser	Asn	Met	Ala	Val	Gln	Gly	Arg	Asn	Tyr	Ile	Pro		
465					470					475					480		
Gly	Pro	Ser	Tyr	Arg	Gln	Gln	Arg	Val	Ser	Thr	Thr	Val	Thr	Gln	Asn		
			485					490						495			
Asn	Asn	Ser	Glu	Phe	Ala	Trp	Pro	Gly	Ala	Ser	Ser	Trp	Ala	Leu	Asn		
		500					505					510					
Gly	Arg	Asn	Ser	Leu	Met	Asn	Pro	Gly	Pro	Ala	Met	Ala	Ser	His	Lys		
	515						520					525					
Glu	Gly	Glu	Asp	Arg	Phe	Phe	Pro	Leu	Ser	Gly	Ser	Leu	Ile	Phe	Gly		
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Lys	Gln	Gly	Thr	Gly	Arg	Asp	Asn	Val	Asp	Ala	Asp	Lys	Val	Met	Ile
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Thr	Asn	Glu	Glu	Glu	Ile	Lys	Thr	Thr	Asn	Pro	Val	Ala	Thr	Glu	Ser
				565					570					575	
Tyr	Gly	Gln	Val	Ala	Thr	Asn	His	Gln	Ser	Ala	Gln	Ala	Gln	Ala	Gln
			580					585					590		
Thr	Gly	Trp	Val	Gln	Asn	Gln	Gly	Ile	Leu	Pro	Gly	Met	Val	Trp	Gln
		595					600					605			
Asp	Arg	Asp	Val	Tyr	Leu	Gln	Gly	Pro	Ile	Trp	Ala	Lys	Ile	Pro	His
	610					615					620				
Thr	Asp	Gly	Asn	Phe	His	Pro	Ser	Pro	Leu	Met	Gly	Gly	Phe	Gly	Met
625				630						635					640
Lys	His	Pro	Pro	Pro	Gln	Ile	Leu	Ile	Lys	Asn	Thr	Pro	Val	Pro	Ala
				645					650					655	
Asp	Pro	Pro	Thr	Ala	Phe	Asn	Lys	Asp	Lys	Leu	Asn	Ser	Phe	Ile	Thr
			660					665					670		
Gln	Tyr	Ser	Thr	Gly	Gln	Val	Ser	Val	Glu	Ile	Glu	Trp	Glu	Leu	Gln
		675					680					685			
Lys	Glu	Asn	Ser	Lys	Arg	Trp	Asn	Pro	Glu	Ile	Gln	Tyr	Thr	Ser	Asn
	690					695					700				
Tyr	Tyr	Lys	Ser	Asn	Asn	Val	Glu	Phe	Ala	Val	Asn	Thr	Glu	Gly	Val
705				710					715						720
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<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: adeno-associated virus

<400> SEQUENCE: 223

gcaagaaagg ccagcagccc gccagaaaga gactcaattt c 41

<210> SEQ ID NO 224
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: adeno-associated virus

<400> SEQUENCE: 224

gcaagaaagg ccagcagccc gccggaaaga gactcaattt c 41

<210> SEQ ID NO 225
<211> LENGTH: 41
<212> TYPE: DNA
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<400> SEQUENCE: 225

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<210> SEQ ID NO 226
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<212> TYPE: DNA	
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<212> TYPE: DNA	
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<212> TYPE: DNA	
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<212> TYPE: DNA	
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<212> TYPE: DNA	
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<212> TYPE: DNA	
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gaaaggcggg ccagcagcct gcaagaaaaa gatgaatttt	40
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<212> TYPE: DNA	
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<212> TYPE: DNA	
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<210> SEQ ID NO 245	
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<212> TYPE: DNA	
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<212> TYPE: DNA	
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<210> SEQ ID NO 252	
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<400> SEQUENCE: 252	
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<212> TYPE: DNA	
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<212> TYPE: DNA	
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<212> TYPE: DNA	
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<210> SEQ ID NO 261	
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<212> TYPE: DNA	
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<212> TYPE: DNA	
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<210> SEQ ID NO 266	
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<212> TYPE: DNA	
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<212> TYPE: DNA	
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<212> TYPE: DNA	
<213> ORGANISM: adeno-associated virus	
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<211> LENGTH: 55	
<212> TYPE: DNA	
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<210> SEQ ID NO 273	
<211> LENGTH: 55	
<212> TYPE: DNA	
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<212> TYPE: DNA	
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<212> TYPE: DNA	
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cacttattct gagcctcgcc ccatcggcac ccgttacctc acccgtaatc tgtaa	55	
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<210> SEQ ID NO 286		
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cgtgtactct gaaccccgcc ccattggcac ccgttacctc acccgtaatc tgtaa	55	
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<212> TYPE: DNA		
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gtagagccgt cacctcagcg ttcccccgac tcctccacgg gcatcg	106	
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gtagaacgt cacctcagcg ttcccccgac tcctcccacg ggcacg	107	

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gtagagcagt cgccacaaga gccagactcc cctcgggca tcg	103
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<212> TYPE: DNA		
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1-19. (canceled)

20. A nucleic acid comprising:
A Parvoviridae genome flanked by ITR sequences, wherein the Parvoviridae genome comprises a Parvoviridae intron, a Parvoviridae cap gene, and a first polyadenylation signal in a first orientation;
A first promoter in the first orientation that drives expression of the Parvoviridae cap gene in the presence of adenoviral helper functions; and
A second promoter in the first orientation that drives expression of the Parvoviridae cap gene in the absence of adenoviral helper functions.

21. The nucleic acid of claim 20, wherein the second promoter is a cell type-specific promoter.

22. The nucleic acid of claim 20, wherein the second promoter is a ubiquitous promoter.

23. The nucleic acid of claim 20, wherein the Parvoviridae cap gene is a wild-type AAV cap gene.

24. The nucleic acid of claim 23, wherein the AAV cap gene sequences is the AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV13 or other natural AAV isolate cap gene sequence.

25. The nucleic acid of claim 20, wherein the Parvoviridae cap gene is an engineered AAV cap gene.

26. The nucleic acid of claim 20, wherein the Parvoviridae cap gene is one of a library of diverse AAV cap genes.

27. A nucleic acid library comprising a plurality of nucleic acids of claim 20, wherein the nucleic acids comprise a plurality of unique Parvoviridae cap gene sequences.

28. The nucleic acid library of claim **27**, wherein the nucleic acid library comprises greater than about 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , 10^7 or 10^8 unique AAV cap gene sequences.

29. The nucleic acid of claim **20**, further comprising a gene of interest.

30. A method for identifying an AAV vector with a cap gene sequence that has increased ability to transduce cells from a tissue of interest when compared to at least one other AAV vector with a different cap gene sequence, the method comprising:

Preparing a first-round AAV TRADE vector library by introducing the nucleic acid library of claim **27** into an AAV packaging cell line and recovering the first round AAV TRADE vector library from the packaging cell line;
Injecting one or more animals with the first-round AAV TRADE vector library;

Recovering cap gene sequences of AAV vectors that are enriched in cells of the tissue of interest in the animals;

Preparing a second-round AAV TRADE nucleic acid library comprising recovered cap gene sequences of the enriched AAV vectors and introducing this library into an AAV packaging cell line and recovering the second round AAV TRADE vector library from the packaging cell line;

Performing a second round of enrichment by injecting one or more animals with the second-round AAV TRADE vector library and recovering cap gene sequence that are enriched in cells of the tissue of interest in the animals; and

Identifying enriched AAV cap gene sequences after the first-round enrichment, after the second-round enrichment, and after any subsequent rounds of enrichment.

31-38. (canceled)

39. An AAV cap ORF sequence comprising one or more following mutations in the exon-intron junctions at splicing donor sites:

AAV1 VP1 cap ORF 1009-CTTAC(junction)CAGCA-1018* (SEQ ID NO:199)

AAV3 VP1 cap ORF 1006-CTTAC(junction)CAGCA-1015* (SEQ ID NO:199)

AAV1 VP1 cap ORF 1228-TTTAC(junction)CTTCA-1237 (SEQ ID NO:200)

AAV3 VP1 cap ORF 1237-TATAC(junction)CTTCG-1246 (SEQ ID NO:201)

AAV1 VP1 cap ORF 1331-ATTAC(junction)CTGAA-1340 (SEQ ID NO:202)

AAV1 VP1 cap ORF 1434-GCTAC(junction)CTGGA-1443 (SEQ ID NO:203)

AAV1 VP1 cap ORF 1502-TTTAC(junction)CTGGA-1510 (SEQ ID NO:204)

AAV1 VP1 cap ORF 1803-ATTAC(junction)CTGGC-1812 (SEQ ID NO:205)

AAV3 VP1 cap ORF 1803-CTTAC(junction)CTGGC-1812 (SEQ ID NO:206)

AAV1 VP1 cap ORF 1835-TGTAC(junction)CTGCA-1844 (SEQ ID NO:207)

AAV1 VP1 cap ORF 2189-GTTAC(junction)CTTAC-2198 (SEQ ID NO:208)

AAV9 VP1 cap ORF 2189-GATAC(junction)CTGAC-2198 (SEQ ID NO:209)

AAV1 VP1 cap ORF 2194-CTTAC(junction)CCGTC-2203 (SEQ ID NO:210)

AAV3 VP1 cap ORF 2194-CTCAC(junction)ACGAA-2203 (SEQ ID NO:211).

40. (canceled)

41. (canceled)

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