Comprehensive mutagenesis maps the effect of all single codon mutations in the AAV2 *rep* gene on AAV production

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Abstract

Recombinant adeno-associated viruses (rAAVs) are the predominant gene therapy vector. Several rAAV vectored therapies have achieved regulatory approval, but production of sufficient rAAV quantities remains difficult. The AAV Rep proteins, which are essential for genome replication and packaging, represent a promising engineering target for improvement of rAAV production but remain underexplored. To gain a comprehensive understanding of the Rep proteins and their mutational landscape, we assayed the effects of all 39,297 possible single codon mutations to the AAV2 *rep* gene on AAV2 production. Most beneficial variants are not observed in nature, indicating that improved production may require synthetic mutations. Additionally, the effects of AAV2 *rep* mutations are largely consistent across capsid serotypes, suggesting that production benefits are capsid independent. Our results provide a detailed sequence-to-function map that enhances our understanding of Rep protein function and lays the groundwork for Rep engineering and enhancement of large scale gene therapy production.

Introduction

rAAVs are a popular tool for the delivery of gene therapies as wild-type AAV is not pathogenic. Wild-type AAV consists of a 4.7 kb single-stranded DNA genome, which is packaged into an approximately 26 nm icosahedral capsid[1,2]. The AAV genome is flanked on either end by 145 nucleotide inverted terminal repeats (ITRs), which serve as the origins of viral replication and are the only sequences required *in cis* for packaging of DNA into the capsid[3]. As such, the remainder of the AAV genome can be replaced with a gene of interest to generate rAAV vectors[4]. The wild-type AAV genome consists of two genes, *rep* and *cap*[1]. During rAAV production, these genes are supplied *in trans* to the ITR plasmid. The *cap* gene encodes three structural proteins, VP1, VP2, and VP3, which assemble in an approximately 1:1:10 ratio to form the 60-mer capsid[5,6]. Engineering of the *cap* gene has enabled targeting of AAV vectors to specific tissues and cell populations. The *rep* gene encodes four proteins, Rep78, Rep68, Rep52, and Rep40, which are generated through the use of two promoters, p5 and p19, and alternative splicing[7].

The larger Rep proteins, Rep78 and Rep68, are required for genome replication while the smaller Rep proteins, Rep52 and Rep40, facilitate genome packaging. Notably, Rep78 and Rep68 alone are each sufficient for the production of rAAV vector[8]. However, the presence of Rep52/40 enhances genome packaging and therefore rAAV titer[9,10]. The *rep* gene encodes three protein domains, an origin-binding domain (OBD), a helicase domain (HD), and a zinc-finger domain (ZFD, Figure 1A)[11–13]. All four Rep proteins contain the HD as well as a nuclear localization signal (NLS)[14]. Only Rep78 and Rep68 contain the OBD and only Rep78 and Rep52 contain the ZFD. Additionally, residues in the linker domain (LD) between the OBD and HD cooperate with residues at the N-terminus of the HD to facilitate oligomerization of the larger Rep proteins[15].

The OBD contains three separate DNA binding motifs that are important for genome replication[16,17]. Firstly, the OBD recognizes the Rep binding site (RBS), which consists of GCTC repeats present in the double stranded region of the ITR[18]. Unwinding of this double stranded DNA by the HD enables the OBD to interact with single stranded DNA at its second motif, the active site pocket. The OBD nicks the single stranded DNA at the terminal resolution site (*trs*), an essential step in viral genome replication[12,19]. Finally, the OBD contains a single stranded DNA hairpin binding site, which interacts with one of the ITR hairpins. However, DNA binding at this site is not required for *trs* nicking[20]. The OBD can also recognize GCTC repeats present in the p5 promoter; in the presence of a helper virus, such as adenovirus, the Rep proteins activate transcription from the endogenous promoters and in the absence of helper virus the Rep proteins repress transcription[21,22].

The HD plays a definitive role in all steps of AAV production while the ZFD is likely dispensable for production. The HD unwinds the double stranded ITR during genome replication and its activity has been shown to facilitate genome packaging[10,23]. There is also evidence that residues within the HD mediate capsid interactions. Mutations to the HD of Rep52/40, as well as mutations near the five-fold axis of symmetry in the capsid, reduced the interaction between the Rep proteins and capsid and resulted in lower rAAV titers[10,24]. The third Rep domain, the ZFD,

has not been shown to bind DNA but is involved in binding to various host cell proteins[13]. Previous work indicated that premature stop codons introduced into the ZFD-encoding region of *rep* do not affect rAAV titers, suggesting that the ZFD is not required for rAAV production[25].

As more rAAV vectored therapies are approved, manufacturing sufficient rAAV quantities to meet patient need is an increasingly important issue. Engineering the Rep proteins is a promising avenue for improving AAV production. The Rep proteins are involved in all steps of viral production, including regulation of VP expression, genome replication, and genome packaging. However, the Rep proteins are not a structural component of the final vector. As such, the Rep proteins can be designed to optimize viral production without affecting downstream processes, such as cell targeting, which are driven by the capsid. The sequence of the *rep* genes in naturally occurring serotypes is relatively well conserved (average of 78% amino acid sequence identity between AAV2 *rep* and AAV1-13 *rep*). While previous mutational studies of the Rep proteins have been conducted, these studies have focused primarily on identifying non-functional Rep variants to elucidate the location of key motifs[7,26–28]. As such, Rep engineering efforts may benefit from the exploration of additional sequence diversity.

Towards this end, we generated a library of all possible single codon mutations of the AAV2 *rep* gene, the *rep* gene most commonly used for AAV production. We assayed the effect of these mutations on AAV2 production and generated a detailed sequence-to-function map of the AAV2 *rep* gene.

Results

Comprehensive mutagenesis assays the effect of all single codon mutations in the AAV2 *rep* gene on AAV2 production

To better understand how mutations to *rep* affect AAV production, we generated two *rep* libraries. The first library, referred to as pCMV-Rep78/68, consisted of a cytomegalovirus (CMV) promoter followed by the *rep* open reading frame (ORF) with a M225G mutation introduced to prevent expression of the smaller Rep proteins. This library allowed us to assay the effect of mutating only Rep78 and Rep68. The second library, referred to as WT AAV2, was analogous to the sequence of the wild-type virus and contained the p5 promoter and *rep* and *cap* ORFs. Use of the endogenous promoters allowed us to capture the effect of *rep* mutations on Rep and VP expression. For each library, we generated all 39,297 possible single codon substitution and deletion variants spanning from the Rep78/68 start codon to the Rep78/52 stop codon (Figure 1A). All variants contained unique 20 bp barcodes at the 3' end of the *rep* gene (pCMV-Rep78/68 library) or *cap* gene (WT AAV2 library). To assess the diversity of the plasmid libraries, we amplified and sequenced the barcodes from each of the plasmid pools (Figure 1B, Figure S1A, and Table S1). We observed 100 and 99.9% of the expected amino acid variants in the pCMV-Rep78/68 and WT AAV2 libraries, respectively.

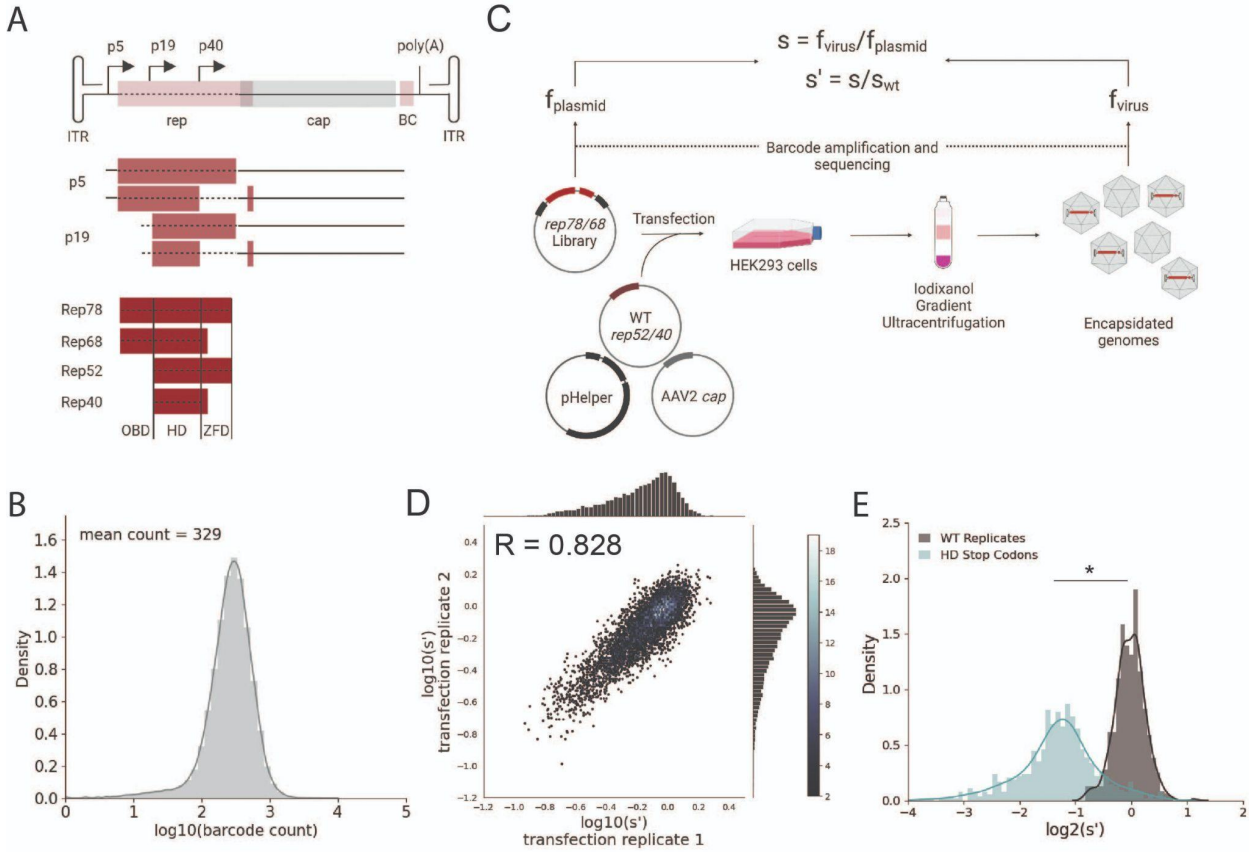

Figure 1. Comprehensive mutagenesis library design and production assay**.** (A) Organization of the AAV2 genome and Rep protein domains. Top: single-stranded DNA genome, middle: RNA transcripts, bottom: Rep proteins. Dotted lines indicate mutated regions. (B) Density plot of barcode counts in the pCMV-Rep78/68 plasmid library. (C) Overview of production assay for the pCMV-Rep78/68 library. (D) Amino acid level selection values from replicate transfections of the pCMV-Rep78/68 library. Pearson R correlation coefficient calculated after log transformation. (E) Density plot of wild-type (black) and premature stop codon (blue) controls for the pCMV-Rep78/68 library. *$p < 10^{-20}$ (Mann Whitney U test)

Next we transfected the plasmid libraries into HEK293T cells to assay the effect of all single codon mutations on production of genome-containing viral particles (Figure 1C). Selection values for each variant were calculated and normalized to internal wild-type controls as shown in Figure 1C. For each library, we performed transfections in duplicate and compared the selection values calculated from each replicate (Figure 1D and Figure S1B). For both libraries, selection values were well-correlated across replicates. After examining the correlation between biological replicates, we next looked at the distribution of selection values for the wild-type and premature stop codon controls (Figure 1E and Figure S1C). As expected, premature stop codons had a deleterious effect on AAV2 production.

Annotation of the AAV2 *rep* sequence-to-function map

To better understand the sequence-to-function map of the AAV2 Rep proteins, we visualized the data from our production assay in multiple ways. First, we generated heatmaps containing the selection values for all single amino acid substitutions and deletions (Figure 2 and Figure S2). Additionally, we calculated the “mutability” of each residue by averaging the selection values at

each position across all substitutions. We mapped the mutability of each residue onto structures of the OBD in complex with the RBS (PDB: 4ZQ9, Figures 3A-B), the OBD in complex with single-stranded DNA from the ITR hairpin (PDB: 6XB8, Figure 3C), the OBD alone (PDB: 5D6X, Figure S4A), and the HD (PDB: 1S9H, Figure S4B)[18,29–31]. We observed that the OBD and ZFD were more tolerant of mutation than the HD. Interestingly, a stark boundary between amino acids D212 and A213 was observed; at residue A213 the larger Rep proteins become much less tolerant of mutation. This boundary is adjacent to LD residues P214-Y224, which were previously reported to be important for Rep78/68 oligomerization[15,30].

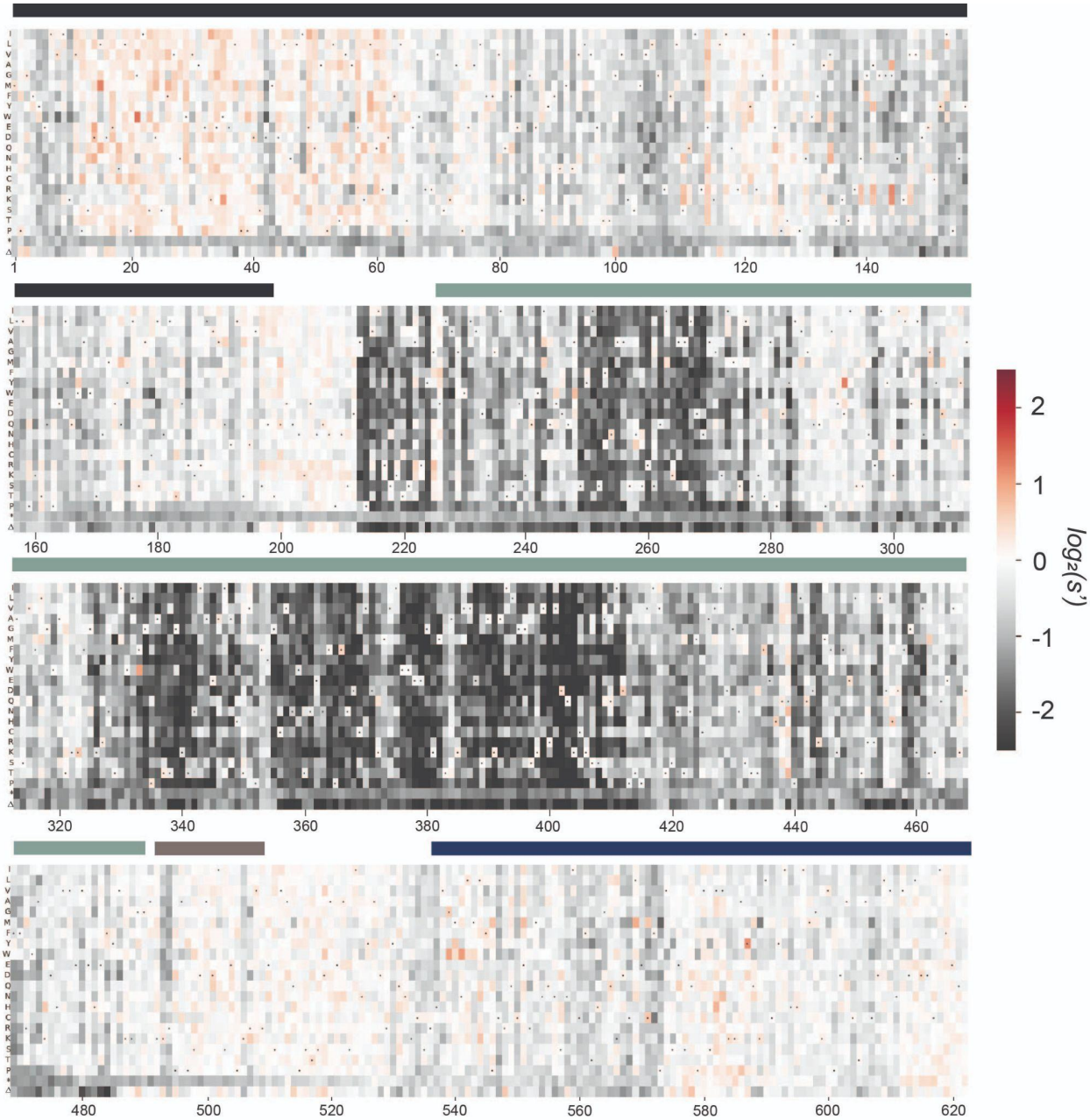

Figure 2. Effects of all single amino acid substitutions and deletions in the Rep78/68 proteins on AAV2 production. Amino acid level selection values from the pCMV-Rep78/68 production assay were calculated as in Figure 1C by summing barcode counts for synonymous mutations. Colored bars indicate protein domains. Black: OBD, light blue: HD, gray: NLS, and navy blue: ZFD. Black dots indicate wild type amino acid identity.

When looking across all library members, the selection values determined for the pCMV-Rep78/68 and WT AAV2 libraries were well-correlated (Figure S3A). However, as expected, introduction of a methionine upstream of the Rep52/40 start codon was deleterious in the WT AAV2 format but not in the pCMV-Rep78/68 format, where Rep52/40 were expressed *in trans*.

The majority of beneficial substitutions clustered in the OBD. In particular substitutions between residues V11 and D62 of the OBD enhanced AAV2 production relative to wild-type. These residues are involved in recognition of the ITR hairpin (Figure 3C)[30]. The majority of RBS interacting residues, however, are intolerant of mutation (Figure 3B). Interestingly, there are a handful of RBS interacting residues that are somewhat mutable, including S110 and N139, which form contacts with the phosphate backbone, and A141, which forms contacts with bases in the RBS. Mutation of S110, N139, and A141, as well as G144 and V147, to positively charged residues had a beneficial effect on AAV2 production. Residues N139, A141, G144, and V147 are part of the loop that connects -strand 4 to α-helix E[18]. The sequence of this loop is highly variable across serotypes[30]. Notably, the asparagine at position 139 is positively charged in AAV5 (K139). However, no serotypes contain positively charged amino acids at positions 141, 144, or 147. Our data indicate that substitution of DNA-interacting residues in the OBD can enhance AAV production and identifies beneficial substitutions not observed in nature.

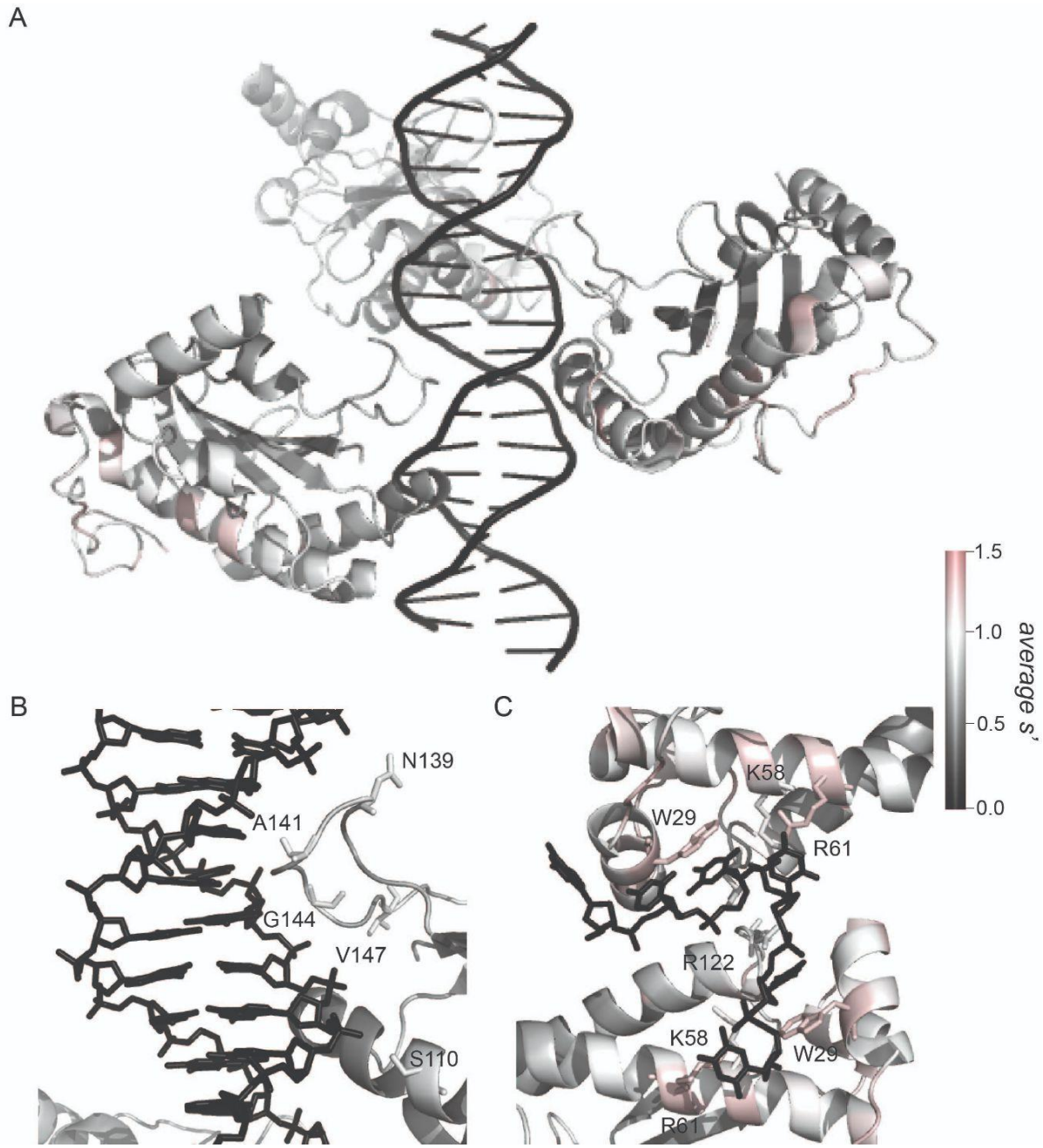

Figure 3. Beneficial substitutions cluster in ITR-hairpin-interacting residues. (A) Average selection values for all substitutions at each position mapped onto the structure of the OBD in complex with the AAVS1 RBS (PDB 4ZQ9). (B) Close up view of OBD-RBS interactions. Residues where substitutions to positively charged residues are enriched are shown as sticks. (C) Average selection values mapped onto the structure of the OBD in complex with the single stranded ITR hairpin (PDB 6XB8). DNA interacting residues are shown as sticks.

A clear pattern in the location of mutable residues in the OBD active site can also be observed (Figure S4A). This active site is responsible for cleaving single stranded DNA and is formed by the OBD beta sheet and Y156, the active site nucleophile[17]. Residues with side chains directed towards the active site are less mutable than adjacent residues with their side chains directed

away from the active site. It is more difficult to discern positional trends in mutability within the HD as it is less tolerant of mutation than the OBD (Figure S4B).

Generation of all single codon variants enables interrogation of nucleotide-level effects
As our libraries contain all possible single codon mutations, we were able to search for variants with nucleotide-level effects on production by comparing differences in selection values between synonymous codon variants (Figures S5-6). In general there was good agreement in the selection values for synonymous variants. Comparison of selection values between synonymous codons that do and do not introduce premature stop codons into alternate reading frames allowed us to search for possible frameshifted ORFs. However, no evidence of frameshifted ORFs was observed (Figures S7A-B).

We did observe an interesting pattern at amino acid Y283 (*rep* nucleotides 847-849). Variants with a c.849C>G mutation had lower selection values than synonymous variants without a c.849C>G ($p < 10^{-8}$, Mann-Whitney U-test). The deleterious effect of this mutation can also be observed by plotting the average selection value at each nucleotide position for each of the four bases (Figures S7C-D). The negative effect of a c.849C>G mutation was consistent across the pCMV-Rep78/68 and WT AAV2 library formats and was also observed in experiments with alternate *cap* genes. In each library, there were fifteen codon variants with a c.849C>G mutation, thirteen of which had synonymous codons without a c.849C>G mutation for comparison. Our results indicate that the nucleotide sequence of the *rep* gene is highly optimized.

Comprehensive mutagenesis identifies beneficial variants not observed in nature
We observed that mutations to amino acids found in other naturally occurring serotypes were better tolerated than mutations to amino acids that are not found in nature (Figure 4A). Conversely, a majority of the variants with selection values greater than that of wild-type are not observed in nature (Figure 4B). Only 245/2351 (6.17%) of variants with s' > 1 and only 4/115 (3.48%) of variants with s' > 1.5 are observed in AAV serotypes 1-13. These data emphasize the power of our comprehensive approach to identify novel functional sequence diversity.

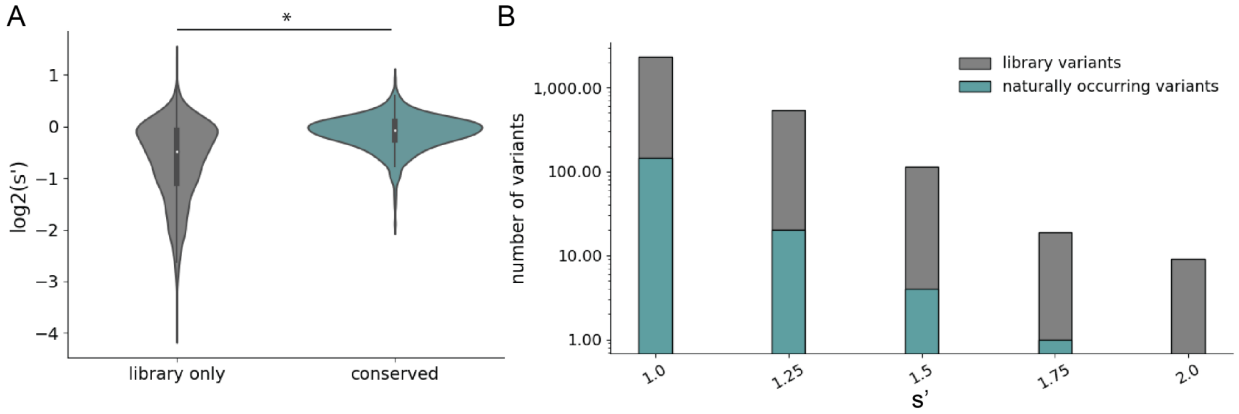

Figure 4. Comparison of comprehensive mutagenesis measurements to variation in nature. (A) Distribution of selection values for conserved variants (blue) and variants found only in the library (gray). (B) Total number of variants and number of conserved variants with s' greater than wild-type (s' > 1). *$p < 10^{-20}$ (Mann Whitney U test)

Validation of multiplexed production assay results

To validate the results of our multiplexed production assay we selected fourteen variants, cloned them individually into the pCMV-Rep78/68 format, and determined their effect on production by measuring DNase-resistant particle (DRP) titers (Figure 5A). We included H92A and K340H, mutations to the OBD and HD active sites, respectively, as controls[11,30]. Twelve variants with mutations in the OBD, LD, and HD and selection values greater than wild-type were also assayed. Finally, we included two deleterious variants identified in our multiplexed assay, N139G and A213V. The DRP titers determined from individual transfections are well-correlated with the selection values determined from the multiplexed production assay (Figure 5B). We performed similar validation for a panel of WT AAV2 format variants (Figure S3B). Several single codon variants, including N139R, A222R, and Q439T, showed 50-100% improvements in titer over wild-type.

To enable multiplexed analysis of *rep* variants in our library assay, the mutant *rep* genes themselves are packaged into the AAV capsids. Relatively small amounts of *rep* library plasmids are also used in transfection to reduce the frequency at which multiple *rep* variant plasmids enter the same cell. These procedures maintain a genotype-phenotype linkage and allow us to identify the *rep* sequences that enable production of genome-containing particles. We sought to confirm that the effects observed in the library production assay would be conserved when the *rep* variants were supplied *in trans* to the ITR genome, as in the traditional triple plasmid transfection method used for rAAV production. To this end, we selected a small subset of *rep* variants and cloned them into an AAV2 pRepCap plasmid without ITRs. Here, we assayed variants with single codon mutations in each of the Rep domains.

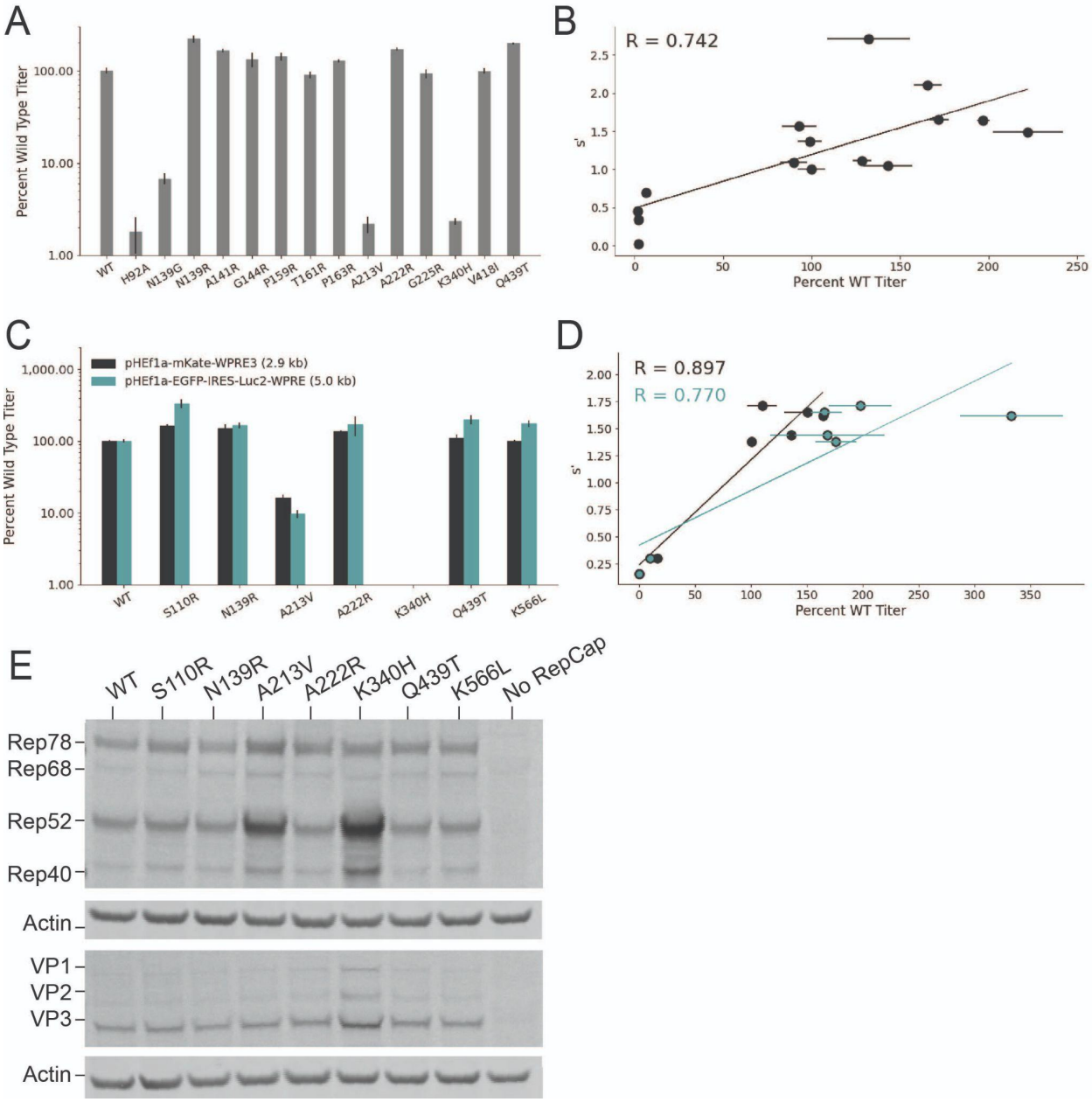

Figure 5. Validation of multiplexed AAV2 production results. (A) DRP titers for fourteen single amino acid pCMV-Rep78/68-ITR variants produced individually. (B) Relationship between normalized library selection values and pCMV-Rep78/68-ITR DRP titers from individual transfections. (C) DRP titers for two rAAV genomes produced with the indicated Rep variants. (D) Relationship between rAAV DRP titers and normalized selection values from library experiments. (E) Expression of Rep and VP proteins from variant pRepCap plasmids by Western blot.

We used these mutant pRepCap plasmids to produce rAAV vectors containing two different ITR genomes. The DRP titers for these pRepCap variants were relatively consistent across different ITR genomes (Figure 5C). Importantly, the DRP titers determined when the *rep* variants were supplied *in trans* correlated well with the selection values determined in our production assay (Figure 5D). Western blot analysis indicated that the majority of these variants had little to no effect on Rep protein or VP expression (Figure 5E). Interestingly, in the case of the deleterious

variants, A213V and K340H, Rep52/40 protein levels were increased. Codon GCG213 is located just upstream of the Rep52/40 start codon. As such, mutations to this position likely affect the strength of the p19 promoter and therefore Rep52/40 expression levels. Notably, the effects of this mutation on the p19 promoter are separate from the deleterious effects of the A213V amino acid change; the negative effects of the A213V change are observed even when Rep52/40 are expressed *in trans* (Figure 5A).

A222R and Q439T variants increase the viral genome: physical particle ratio

We assessed the viral genome: physical particle ratio for a panel of variants, including R113Q, A222R, K340H, and Q439T. We set up individual transfections with these *rep* variants and affinity purified the resulting rAAV2 vectors. Viral genome and physical particle titers were determined by qPCR and capsid ELISA, respectively (Figure S8). The previously characterized K340H variant resulted in a very low viral genome: physical particle titer ratio. rAAV vectors produced with the R113Q *rep* variant also had a reduced ratio relative to a wild-type control, although the difference was much smaller. The A222R and Q439T variants, conversely, resulted in a slightly higher ratio of viral genome: physical particle titers compared to wild-type.

Mutations in the AAV2 *rep* gene have similar effects on production of AAV2, AAV5, and AAV9 capsids

Many different AAV capsid serotypes are of clinical interest for their unique tissue targeting properties. Given the physical interaction between Rep proteins and the capsid, we hypothesized that some *rep* mutations would have unique effects on the production of different capsid serotypes. To identify *rep* variants with serotype-specific effects on production, we repeated our pCMV-Rep78/68 library production assay using AAV5 and AAV9 *cap* genes in place of AAV2 (Figures S9-12). The AAV5 capsid was chosen as it has low overall sequence identity with the AAV2 capsid (57.7% amino acid identity). The AAV9 capsid is more similar to the AAV2 capsid (81.7% amino acid identity) but is of particular clinical interest and differs from AAV2 at amino acid positions 329 and 330, which were previously identified as important for Rep-capsid interactions (AAV2: T329/T330, AAV9: V331/K332)[24]. Surprisingly, the correlation in selection values across capsid serotypes was comparable to the correlation between biological replicates, indicating that the effect of AAV2 *rep* variants on production is largely consistent across capsid serotypes (Figures 6A-B). We individually produced rAAV5 and rAAV9 vectors using a subset of our *rep* variants and confirmed that the titers with each *rep* variant are well correlated across capsid serotype (Figure S13).

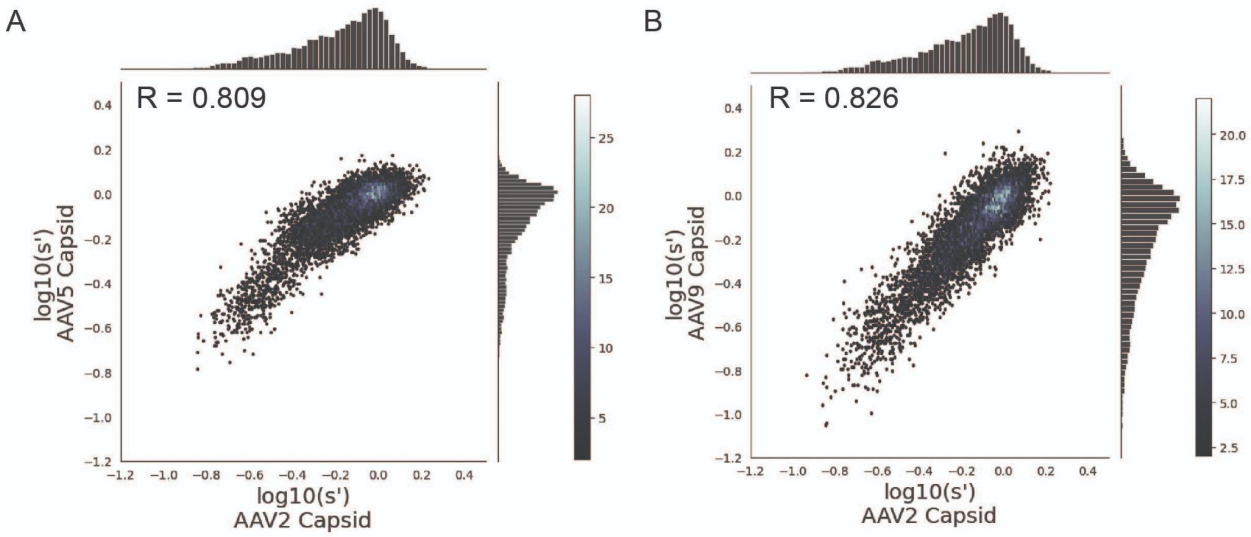

Figure 6. Mutations to AAV2 *rep* have similar effects on AAV2, AAV5, and AAV9 capsid production. Selection values from the library production assay with (A) AAV5 and AAV2 capsids and (B) AAV9 and AAV2 capsids. Pearson R correlation coefficient calculated after log transformation.

Discussion

We have generated a comprehensive mutagenesis library of the AAV2 *rep* gene, containing all possible single codon substitutions and deletion variants. Multiplexed assay of this library allowed us to generate a sequence-to-function map, linking all variants to their effect on AAV production.

Many of the previous AAV mutagenesis studies have focused on the AAV *cap* gene. Previous work in our lab generated a library containing all possible single codon substitutions, deletions, and insertions of the AAV2 *cap* gene and assayed their effect on AAV2 production[32]. Notably, we observed a smaller range of selection values in our production assay with the *rep* library than with the previously reported *cap* library. This observation aligns with our knowledge of Rep and capsid biology. The Rep proteins' primary function is to facilitate AAV production, while the capsid has evolved to not only enable assembly and genome packaging, but also to facilitate cell targeting, entry, and nuclear trafficking. Additionally, the Rep proteins possess multiple enzymatic activities that require the proteins to adopt specific and dynamic conformations. The capsid, in contrast, is not known to possess enzymatic activity outside of the phospholipase domain located at the N-terminus of VP1[33]. It follows that there are greater mutational constraints on *rep* than on *cap* in the context of AAV2 production.

We observed that substitutions in the OBD and ZFD were better tolerated than substitutions in the LD and HD. A sharp drop in mutability was observed between residues D212 and A213. Previous work has identified residues V215 through Y224 as the minimal linker sequence required to facilitate Rep78/68 oligomerization[15]. In a separate study, it was reported that mutation of P214 reduced the ability of Rep to oligomerize[30]. Our data indicate that residue A213 is intolerant of mutation. A213 likely plays an important role in AAV production and, given its position, may be involved in Rep78/68 oligomerization. Additional work is needed to confirm

that mutations to A213 affect AAV production by interfering with Rep78/68 oligomerization. In both library formats, the ZFD was relatively tolerant of mutation. At some positions, even introduction of a premature stop codon was not deleterious. It was recently reported that premature stop codons introduced at positions 522 and 553 of Rep did not affect AAV2 production when supplied in a pRepCap plasmid[25]. Our results provide further evidence that the ZFD is dispensable for AAV production and identify A213 as a potential LD residue.

The majority of beneficial substitutions clustered in the OBD. In a recent investigation of *rep* hybrids, Mietzsch et al. reported that replacement of the entire AAV2 OBD with that of AAV1 or AAV8 improved the proportion of full capsids[25]. Our data supports the importance of the OBD for AAV production and identifies specific OBD regions where beneficial mutations cluster. Most substitutions of ITR-hairpin interacting residues and substitutions of residues in the RBS-interacting loop to positively charged amino acids were beneficial. Previous studies have demonstrated that Rep binding to the ITR-hairpin improves the efficiency of *trs* nicking but is not required for the nicking reaction to occur[20]. The RBS-interacting loop, on the other hand, is important for recognition of double-stranded DNA during genome replication, promoter binding, and genome integration. Taken together, our results indicate that enhancement of Rep-DNA interactions may be a fruitful avenue for further improvement of AAV production.

Inclusion of all single codon variants in our libraries allowed us to investigate the effect of synonymous mutations on AAV production. Interestingly, we observed that introduction of a c.849C>G mutation resulted in lower selection values compared to synonymous variants. Interestingly, this position is located downstream of the p5 and p19 promoters. Additionally, this mutation had a negative effect on production even when the *cap* gene was supplied *in trans,* making the activity of the p40 promoter irrelevant for AAV production. The c.849C>G mutation also does not fall near any known AAV2 splice sites[34]. While the mechanism by which this mutation affects production remains unclear, our results emphasize that the *rep* gene is optimized for AAV production at both the amino acid and nucleotide levels.

In an attempt to identify Rep substitutions with a beneficial effect on the production of specific capsid serotypes, we repeated the pCMV-Rep78/68 library assay using AAV5 and AAV9 *cap* genes in place of AAV2 *cap*. Surprisingly, the correlation in selection values across experiments was similar to the correlation between biological replicates, suggesting that the majority of AAV2 *rep* mutations have a similar effect on the production of AAV2, AAV5, and AAV9 capsids. These results suggest that either: *rep* mutations have a similar effect on Rep-capsid interaction across serotypes, that the effect of perturbing Rep-capsid interactions is obscured by the deleterious effects of the same mutations on other Rep activities, or that Rep-capsid interactions are not a limiting factor in AAV production.

While the multiplexed nature of our assay enabled us to investigate the phenotypes of all single codon *rep* substitutions and deletions, it also imposed some limitations on the types of assays we could perform. Notably, we were not able to measure the proportion of empty capsids generated by each *rep* variant in multiplex. We did, however, assess the viral genome: physical particle titer ratios for a panel of variants, including R113Q, A222R, K340H, and Q439T. Two

variants that had a beneficial effect in our library production assay, A222R and Q439T, also appeared to increase this ratio relative to a wild-type control. Further characterization will help us to understand how variants identified in our library production assay affect the production of genome-containing particles. An additional limitation of our study relates to the effects of *rep* mutations on AAV5 and AAV9 VP expression. As we supplied the *cap* genes *in trans* to our pCMV-Rep78/68 library and under the control of a CMV promoter we were not able to investigate the effects of mutating AAV2 *rep* on expression of AAV5 or AAV9 VPs. Cloning and assay of additional WT format libraries with the AAV5 and AAV9 *cap* genes could provide insight into the effect of AAV2 *rep* mutations on AAV5 and AAV9 VP expression *in cis*.

We have generated a comprehensive sequence-to-function map of the effect of all single codon AAV2 *rep* mutations on AAV2, AAV5, and AAV9 production. We have also identified mutable regions of *rep* within the OBD, laying the groundwork for further engineering of these proteins and enhancement of large scale gene therapy production.

Materials and Methods

Library cloning

We generated *rep* variant libraries through pooled oligonucleotide (oligo) synthesis (Twist Biosciences) and subsequent Golden Gate Assembly (GGA) using methods previously developed in our lab[32]. To begin, we generated two wild-type backbone plasmids, containing either the Rep78/68 ORF or the *rep* and *cap* ORFs, and removed all BsaI, BbsI, EcoRV, SphI, and XbaI sites. Within the *rep* ORF we introduced a synonymous mutation at G339 (c.1017G>C). Within the VP ORF we introduced a synonymous mutation at V118 (c.354C>G) and a coding mutation F370Y (c.1109T>A). The *rep* gene was divided into eleven tiles and cloning for each tile was carried out separately. We designed 300-mer oligos to include 207 nucleotides of *rep*-coding sequence immediately followed by a BbsI site, an EcoRV site, another BbsI site, and a unique 20 nucleotide barcode sequence. All of these elements were flanked by BsaI and primer binding sites on either end of the synthesized sequence. Unique primer binding sites were used for each tile. To enable cloning of both the Rep78/68 and WT AAV2 format libraries, oligos containing the Rep52/40 start codon (M225) were synthesized with and without the M225G mutation. All possible single codon substitutions and deletions, including synonymous variants, were included in the library. All positions from the Rep78/68 start codon to the Rep78/52 stop codon (622 codons) were mutated. We did not include the eight codons at the end of the Rep68/40 ORF in our mutagenesis as these positions overlap with the VP1 ORF. Each codon variant was represented by at least two unique barcodes. For each tile, a minimum of ten uniquely barcoded wild-type controls were included. The Rep78/68 and WT AAV2 libraries each had a total of 81,116 uniquely barcoded variants.

Following synthesis, we amplified the oligos for each tile (Q5 Hot Start High-Fidelity 2X Master Mix, NEB). In parallel, we amplified the backbone vector using primers that introduced BsaI sites. Vector PCR products were digested with BsaI-HF v2, DpnI, and recombinant shrimp alkaline phosphatase (rSAP, NEB) overnight and PCR purified (Qiagen QIAQuick PCR Purification Kit) the following day. We then performed GGA with the amplified oligos and vector PCR digest products (NEBridge Golden Gate Assembly Kit, BsaI-HF v2). GGA reactions were

cycled 100X (16°C for 5 minutes, 37°C for minutes) and then heat inactivated. GGA products were PCR purified and eluted into 25 uL of Buffer EB (Qiagen). Eluates were drop-dialyzed against 30 mL of water for 1 hour and transformed (Lucigen E. cloni 10G ELITE electrocompetent cells). Transformed cells were recovered in 1 mL Lucigen recovery media at 37°C for 1 hour and the entire volume of outgrowth was used to inoculate 50 mL LB + Kanamycin cultures, which we grew at 30°C overnight. The following day, we midi-prepped these cultures *via* alkaline lysis (Qiagen Plasmid Plus Midi-Prep Kit). This first cloning step enabled generation of intermediate products, which contained any wild-type *rep* sequence upstream of the mutated oligo followed by the mutant oligo. In this step, all wild-type sequences downstream of the mutant oligo were removed.

To re-introduce these missing wild-type sequences, we used a second round of GGA and the internal BbsI sites present in each oligo. Before performing GGA, we ran rolling circle amplification (RCA) on our intermediate cloning products. 10 ng of the intermediate plasmid products were incubated with 10 uM random hexamer primers and 1X phi29 DNA polymerase buffer (NEB) at 95°C for 3 minutes and then cooled to room temperature. We then mixed these samples with RCA amplification solution (10 uM random hexamers, 1X phi29 DNA polymerase buffer, 5 U phi29 DNA polymerase, 1 mM dNTPs, 2 mg/mL recombinant albumin, 0.02 U inorganic pyrophosphatase) at a 1:1 ratio and incubated them at 30°C overnight. The resulting RCA products were directly digested with BbsI-HF, DpnI, and recombinant shrimp alkaline phosphatase (all NEB) at 37°C overnight. The following day, we ran the digest products on 1% Tris-acetate EDTA gels and extracted and purified the correctly sized products (Qiagen QIAQuick Gel Extraction Kit). In parallel, we PCR amplified the missing downstream wild-type sequences for each tile from the backbone vectors; the primers used here also added BbsI sites. We ran GGA with the RCA digest products and vector PCR products, BbsI-HF (NEB), and T4 DNA Ligase (NEB), cycling as described above. GGA products were transformed and midi-prepped as above. This second cloning step resulted in plasmids containing a full-length *rep* gene with a single codon mutation followed by a twenty nucleotide barcode. WT AAV2 library plasmids also contained a wild-type copy of the AAV2 *cap* gene between the variant *rep* genes and barcodes.

To enable packaging of variant *rep* sequences into AAV capsids, the step two cloning products were moved into ITR-containing vectors. Step two cloning products were subject to RCA as above and digested with XbaI, SphI-HF, and EcoRV-HF. The *rep* and/or *cap* ORFs in the backbone vectors were flanked by XbaI and SphI sites. EcoRV sites were part of the synthesized oligos and should only be present in step one cloning products. Digest products were gel extracted and ligated into ITR-containing vectors. In the case of the WT AAV2 format library, the ITR vector contained the p5 promoter and endogenous AAV2 polyA sequence. In the case of the Rep78/68 and Rep52/40 libraries, the ITR vector contained a CMV promoter, WPRE, and bGH polyA sequence. The integrity of the ITR destination plasmids was confirmed by SmaI digest and complete plasmid sequencing (MGH DNA Core). The integrity of the final ITR plasmid libraries was confirmed by SmaI digest.

Viral library production assay
HEK293T cells were cultured in DMEM supplemented with 10% FBS and seeded in five-layer cell culture multi-flasks (Corning 353144) at 8 x 10^7 cells/flask two days prior to transfection. We transfected HEK293T cells using polyethylenimine. For each library, replicate transfections were performed in separate cell stacks. For the pCMV-Rep78/68 library, transfections were performed with 1 ug of pCMV-Rep78/68 library plasmids, 50 ug of pHelper, 25 ug of pCMV-AAV2*cap*, and 1.5 ug of p15-Rep52/40 per cell stack. For the WT AAV2 library, transfections were performed with 1 ug of WT AAV2 library plasmids and 50 ug of pHelper. Additional plasmid ratios were tested for each library; conditions that resulted in the strongest correlation in selection values between replicate transfections were used; these are the conditions listed above. AAV5 and AAV9 capsid production assays were performed using the pCMV-Rep78/68 library as described above.

Three days post-transfection NaCl was added to a final concentration of 0.5 M and samples were incubated at 37°C for 3 hours to detach and lyse cells. Samples were transferred to fresh 500 mL bottles and incubated at 4°C overnight. The following day any precipitate was removed by pipetting and the remaining volume was passed through a sterile 0.22 um PES filter. PEG-8000 was added to a final concentration of 8% and samples were again incubated at 4°C overnight. The following day, we centrifuged samples at 3000 x g for 20 minutes to pellet the PEG-precipitated virus. Supernatants were discarded and pellets resuspended in 8 mL of DPBS. We then digested samples with a 1:10,000 dilution benzonase (Millipore Sigma 1.101695.0001) at 37°C for 45 minutes and subjected them to iodixanol gradient ultracentrifugation as previously described[32,35]. Briefly, benzonase-treated samples were underlaid with a iodixanol gradient (Millipore Sigma D1556) in polypropylene tubes (Beckman Coulter, 362183) and centrifuged at 242,000 x *g* for 1 hour at 16°C in a VTi50 rotor. Following ultracentrifugation, 500 uL fractions were collected from the 40% iodixanol layer and buffer-exchanged into DPBS using 100 kDa molecular weight cutoff centrifugal filter units (Millipore Sigma UFC910024).

Rep sequences in the purified pool represent variants capable of producing genome-containing viral particles. Barcodes from this viral pool, along with barcodes from the plasmid libraries, were amplified using flanking primer sequences. Illumina sequencing adapters and indices were added in a subsequent PCR. The resulting PCR amplicons were pooled and sequenced using the Illumina NextSeq platform (Biopolymers Facility, HMS). Barcode sequences were extracted from the resulting sequencing reads and barcode counts (c_v) from replicate transfections were summed. We calculated the frequency of each variant (f_v) in the viral library as $f_v = c/\Sigma c_v$ and then determined the selection value for each variant as $s = f_{viral}/f_{plasmid}$. Finally, selection values were normalized to that of the wild-type controls: $s = s/s_{WT}$.

Determination of DNase-resistant particle titers by qPCR
To validate the results of our multiplexed library production assay, fourteen single codon *rep* variants were selected and individually transfected into HEK293T cells. Transfections were performed in triplicate. We seeded cells at 4 x 10^5 cells/well in 6-well plates 24 hours prior to

transfection. Small amounts of pCMV-Rep78/68 format variants, 50 pg per transfection, were used to recapitulate the low plasmid levels used in the library production assay. pCMV-Rep78/68 variants were transfected *via* PEI together with pHelper (2 ug), pCMV-AAV2*cap* (1 ug), and p15-Rep52/40 (2 ng) plasmids as in the library assay. Three days post-transfection, we lysed cells by 3X freeze-thaw. Samples were then clarified by centrifugation at 15,000 x *g* for 5 minutes. 5 uL of supernatant were subjected to DNase digest (ThermoFisher, EN0521) at 37°C for 30 minutes followed by heat inactivation at 65°C for 10 minutes. We then incubated samples at 98°C for 10 minutes to denature the capsids and measured DRP titer with qPCR (IDT PrimeTime Gene Expression Master Mix). Primers and a probe binding to the 5' region of the *rep* gene were used. The sequence of the forward primer was: 5'-GAGCATCTGCCCGGCATTTC-3', the sequence of the reverse primer was: 5'-ATCTGGCGGCAACTCCCATT-3', and the sequence of the probe was 5'-HEX-ACAGCTTTG-ZEN-TGAACTGGGTGGCCGA-3IABkFQ-3' (IDT).

Transfections with *rep* variants cloned into pRepCap plasmids were performed as described above, using the following amounts of plasmid for each transfection: 1 ug of pRepCap, 1 ug of ITR plasmid, and 2 ug of pHelper.

Western blot analysis of VP and Rep protein levels

Following transfection, media was aspirated from cell culture plates and cells were washed with DPBS. We then lysed cells with 50 uL of lysis buffer per well (6-well plates). The lysis buffer contained:10 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Triton X-100, and cOmplete mini protease inhibitor (1 tablet/10 mL lysis buffer). Lysates were transferred to fresh 1.5 mL tubes and centrifuged at 15,000 x *g* for 5 minutes to clarify. Total protein concentrations were determined by Bradford assay (Thermo Scientific Pierce Coomassie Bradford Protein Assay Kit). We loaded 75 ug of total protein from each sample on 4-12% Bis-Tris gels (ThermoFisher) and ran the gels for 45 minutes at 140V. We then transferred the proteins to PVDF membranes (ThermoFisher). Membranes were blocked with 5% milk in PBST for 1 hour at 4°C. Blots were then incubated with a 1:250 dilution of B1 anti-VP antibody or a 1:100 dilution of anti-Rep 303.9 antibody (both American Research Products) in blocking buffer overnight. Both primary antibody mixtures also included a 1:20,000 dilution of anti- -actin antibody (Abcam, ab8227). The following day, blots were washed 3X with PBST and incubated with secondary antibodies for 1 hour in blocking buffer with 0.01% SDS. Goat anti-mouse IRDye 800CW (LiCor 925-32210) and donkey anti-rabbit IRDye 680RD (LiCor 925-68073) secondary antibodies were used. Blots were washed 3X with PBST and 1X with PBS, and the near-infrared fluorescence of the secondary antibodies was visualized using the Sapphire imager.

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Longevity-Related Research Fund at HMS, and the Synthetic Biology Platform at the Wyss Institute. Schematics were generated with BioRender.

Authors contributions

NKJ, PJO, and GMC conceived the study and designed experiments. NKJ performed experiments. NKJ performed data analysis with input from PJO. NKJ wrote the paper with input from all authors. GMC supervised the project and secured funding.

Competing interests

A full list of GMC's tech transfer, advisory roles, and funding sources can be found on the lab's website: http://arep.med.harvard.edu/gmc/tech.html. Harvard University has filed a patent application for inventions related to this work.

Supplemental Materials

Supplemental Files

Supplemental File 1 contains the barcode level selection values for all library production experiments.
Supplemental File 2 contains the codon level selection values for all library production experiments.
Supplemental File 3 contains the amino acid level selection values for all library production experiments.

Materials and Methods

Analysis of stop codons in alternate reading frames

At each codon position, the average selection value for all variants that introduced a premature stop codon into the +1 reading frame was determined. The average selection value for all variants that did not introduce a stop codon into the +1 frame but were synonymous in the Rep frame to those that did was also calculated. Ten amino acid rolling averages for the +1 stops and +1 non-stops were calculated. These average selection values were plotted and compared. The same procedure was used to determine the effect of premature stop codons in the +2 reading frame.

Affinity purification of AAV2 capsids

We purified rAAV2 vectors produced with variant pRepCap plasmids with AVB Sepharose (Cytiva) using a previously reported protocol, which we modified for use with gravity columns [36]. A 5.0 kb ITR genome (pHef1a-EGFP-IRES-Luc) was used. HEK293T cells were harvested three days post-transfection and lysed by 3X freeze-thaw. We then subject samples to benzonase digest as described above and centrifuged samples at 6,000 x *g* for 30 minutes to pellet cell debris. Samples were then passed through 0.2 um PES filters, diluted 1:1 with TD Buffer (DPBS, 1 mM $MgCl_2$, 2.5 mL KCl), and incubated with 4 mL of AVB Sepharose slurry at 4°C overnight with shaking. The following day, we loaded the samples onto gravity columns, washed with 40 mL of 1X TD Buffer, and eluted samples with 5 mL 0.1 M glycine-HCl (pH 2.6). 1 mL fractions were collected and immediately neutralized with 100 uL of 1 M Tris-HCl (pH 10). Elution fractions containing VPs were pooled and buffer-exchanged as above. rAAV viral genome titers were determined *via* qPCR using primers and a probe that bind to the EGFP ORF. Forward primer: 5’-GAACCGCATCGAGCTGAA-3’, reverse primer: 5’-TGCTTGTCGGCCATGATATAG-3’, and probe 5’-FAM-ATCGACTTC-ZEN-AAGGAGGACGGCAAC-3IABkFQ-3' (IDT). Total particle titers were determined *via* capsid ELISA (Progen) according to the manufacturer’s protocol. Transfections, affinity purifications, and titer determinations were performed in duplicate.

Supplemental Figures

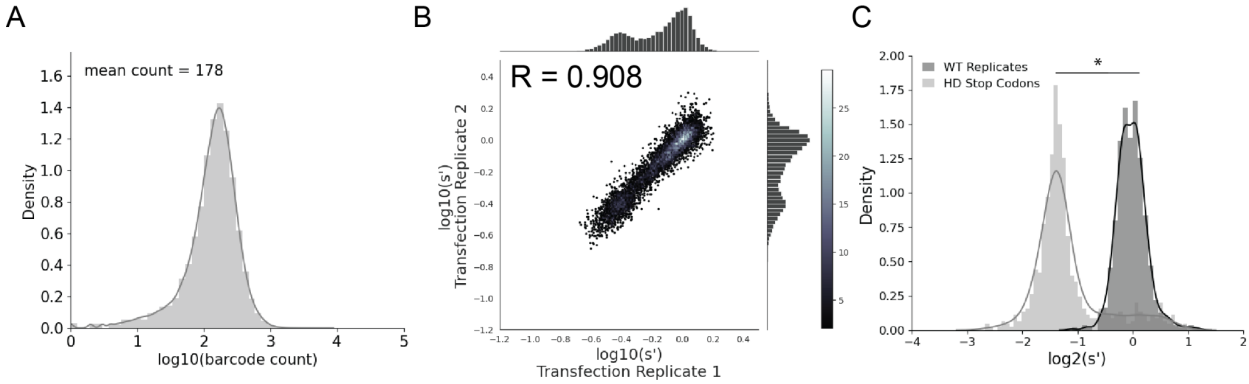

Figure S1. Comprehensive mutagenesis library design and production assay results for WT AAV2 format library. (A) Density plot of barcode counts in the WT AAV2 plasmid libraries. (B) Amino acid level selection values from replicate transfections for the WT AAV2 library. Pearson R correlation coefficient calculated after log transformation. (C) Density plot of wild-type (red) and premature stop codon (yellow) controls for the WT AAV2 library. *$p < 10^{-20}$ (Mann Whitney U test)

Table S1. Percent of expected variants sequenced in plasmid and viral particle libraries.

	pCMV-Rep78/68		**WT AAV2**	
	Plasmid	**Viral Particle**	**Plasmid**	**Viral Particle**
Barcodes	99.8%	98.0%	98.3%	97.6%
Codon Variants	100%	99.9%	99.7%	99.7%
Amino Acid Variants	100%	100%	99.9%	99.9%

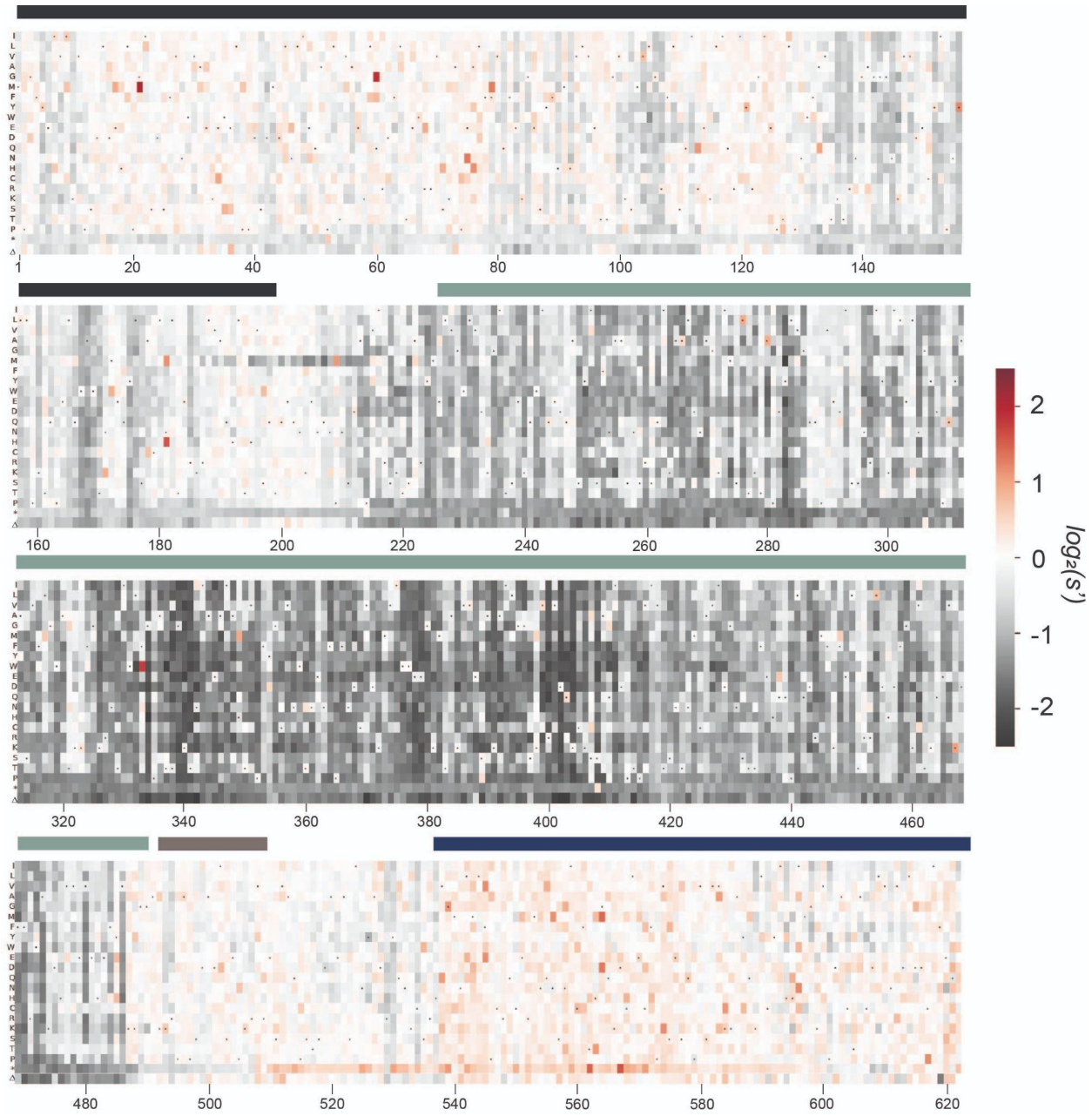

Figure S2. Effects of all single amino acid substitutions and deletions in Rep78, Rep68, Rep52, and Rep40 on AAV2 production. Amino-acid level selection values from the WT AAV2 library production assay were calculated as in Figure 1C by summing barcode counts for synonymous variants. Colored bars indicate protein domains. Black: OBD, light blue: HD, gray: NLS, and navy blue: ZFD. Black dots indicate wild type amino acid identity.

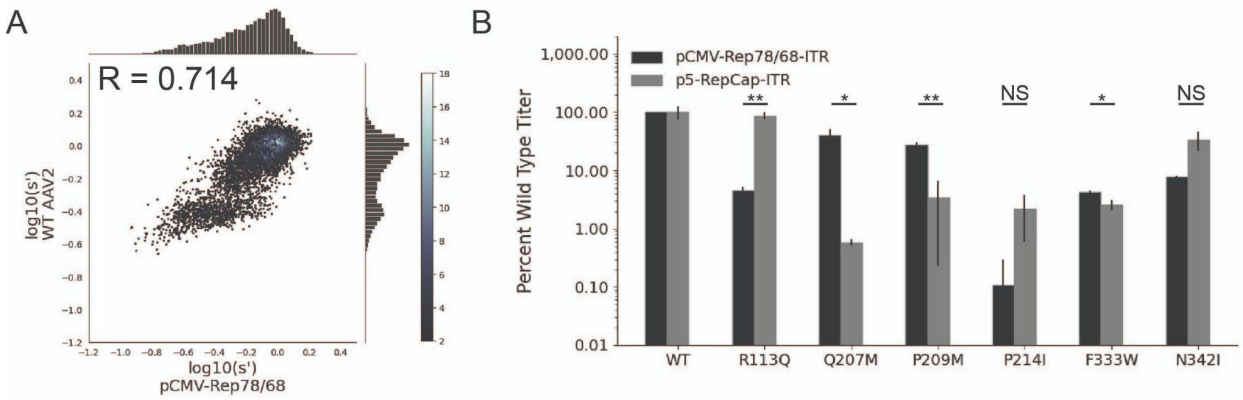

Figure S3. Mutations to AAV2 *rep* have similar effects on AAV2 production in pCMV-Rep78/68 and WT AAV2 format libraries. (A) Selection values for library production assay with pCMV-Rep78/68 and WT AAV2 format libraries, Pearson R correlation coefficient calculated after log transformation, (B) DRP titers for AAV2 capsids produced with pCMV-Rep78/68-ITR or p5-RepCap-ITR plasmids containing the indicated Rep substitutions. *p < 0.05, **p < 0.01 (Welch's t test)

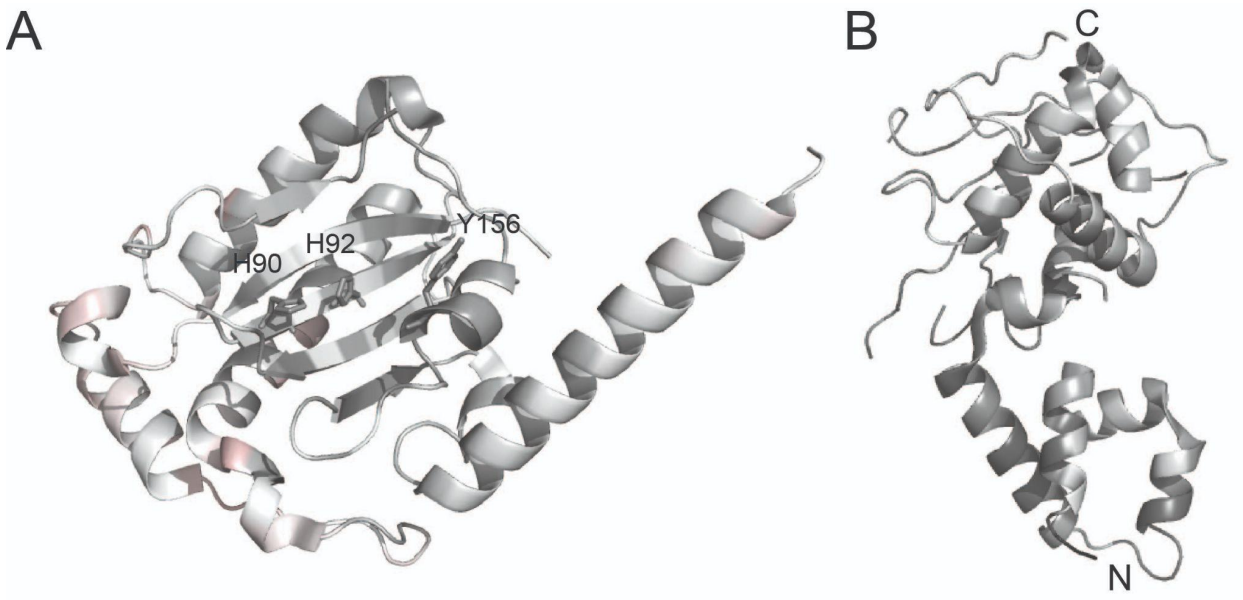

Figure S4. Average selection values from pCMV-Rep78/68 library mapped onto (A) the structure of the OBD active site (PDB 5DCX) and (B) the structure of one HD monomer (PDB 1S9H). H90, H92, and the active site nucleophile, Y156, are shown as sticks in (A).

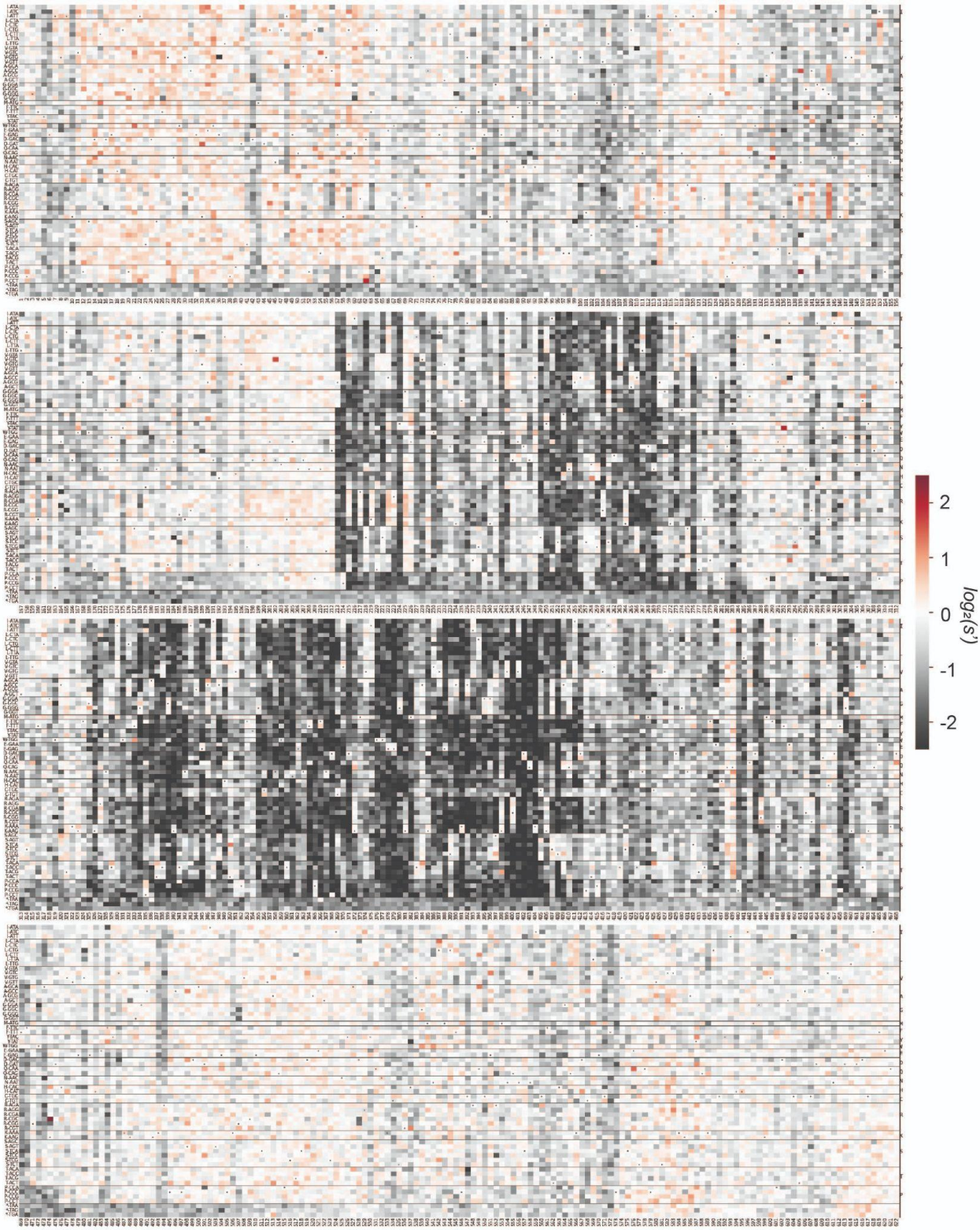

Figure S5. Codon level selection values for the pCMV-Rep78/68 format library.

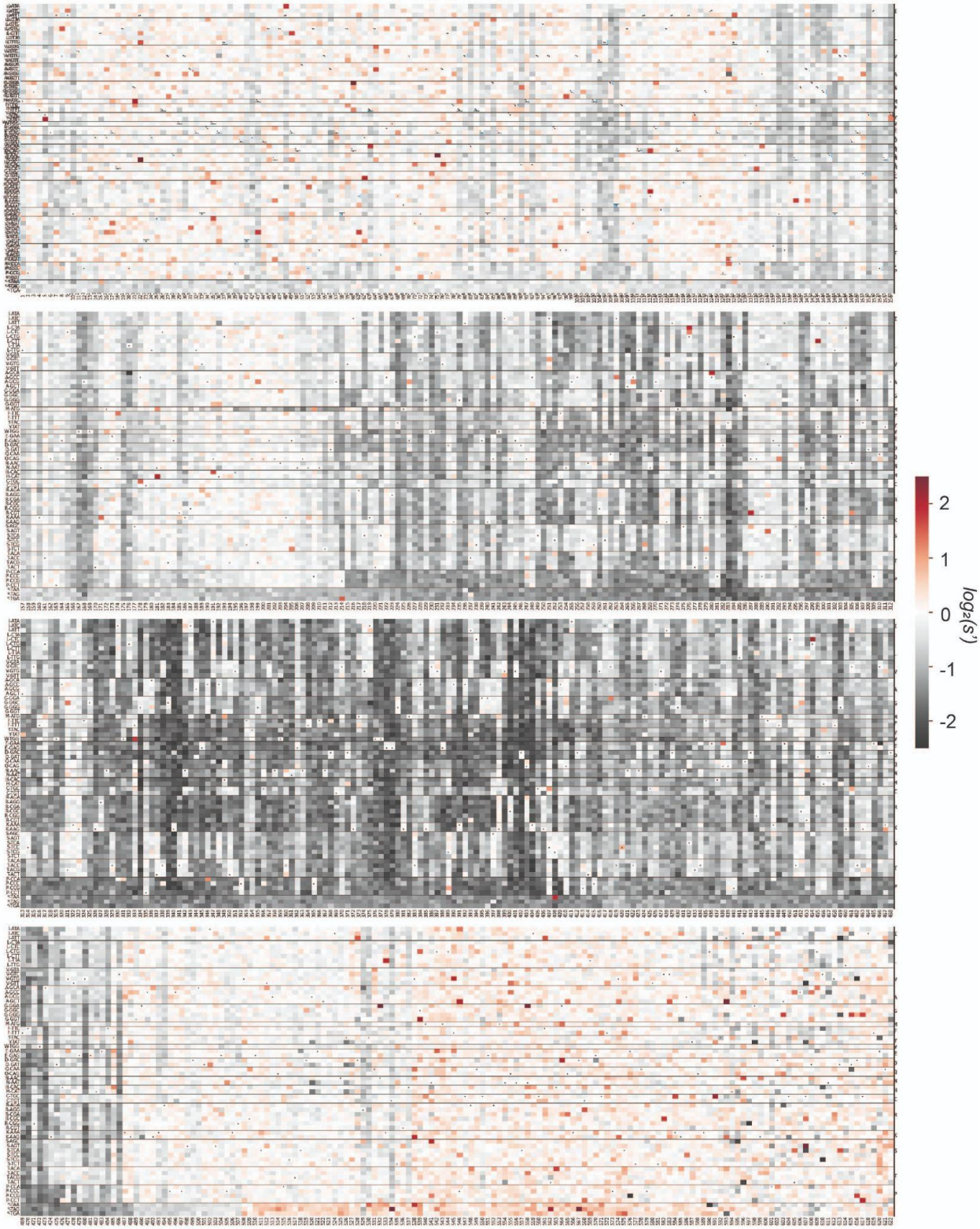

Figure S6. Codon level selection values for the WT AAV2 format library.

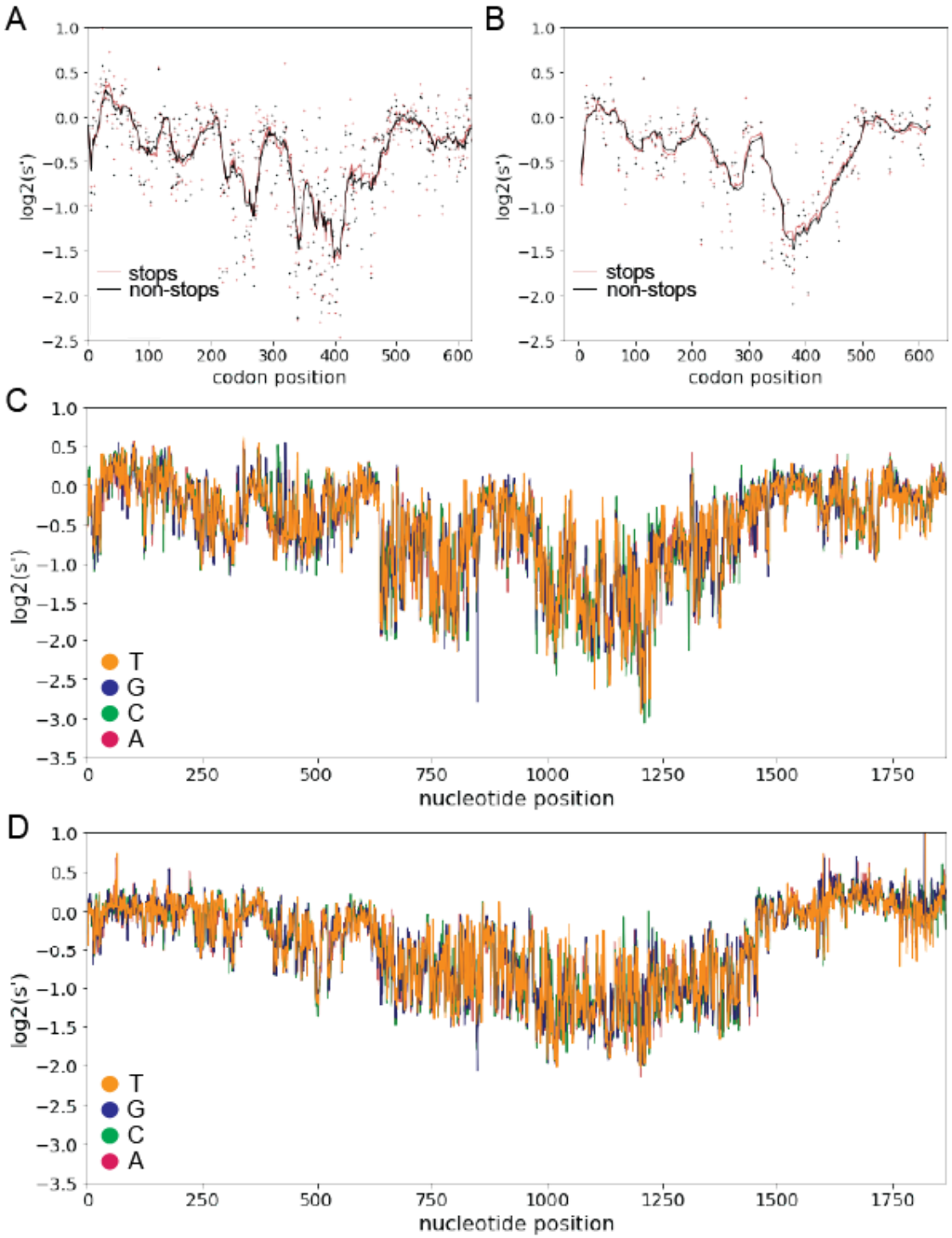

Figure S7. Inclusion of synonymous codon variants enables interrogation of nucleotide-level effects. Analysis of pCMV-Rep78/68 variants with premature stop codons in +1 (A) and +2 (B) reading frames does not identify any frameshifted ORFs. Pink dots: average selection values for mutations that introduce stop codons into +1 or +2 frame, black dots: average selection values for mutations synonymous to +1 or +2 stop codon mutations in Rep ORF, lines indicate 10 bp rolling averages. (C) Average selection values at each nucleotide position for variants that introduce the indicated nucleotide change in the pCMV-Rep78/68 library, orange: T, blue, G, green: C, red: A. (D) Average selection values for variants that introduce the indicated nucleotide change in the WT AAV2 library.

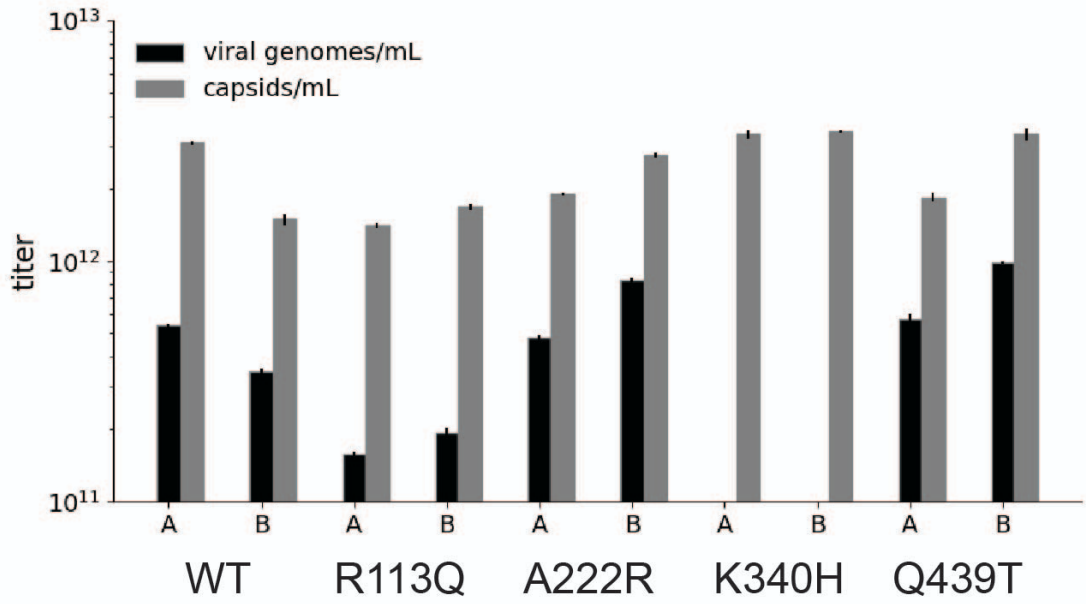

Figure S8. Effect of single amino acid Rep substitutions on the viral genome: physical particle ratio of affinity purified rAAV. Capsid (gray) and viral genome (black) titers for pHef1a-EGFP-IRES-Luc2 rAAV2 produced with the indicated Rep variant and affinity purified with AVB Sepharose. For each variant samples A and B represent replicate transfections and affinity purifications.

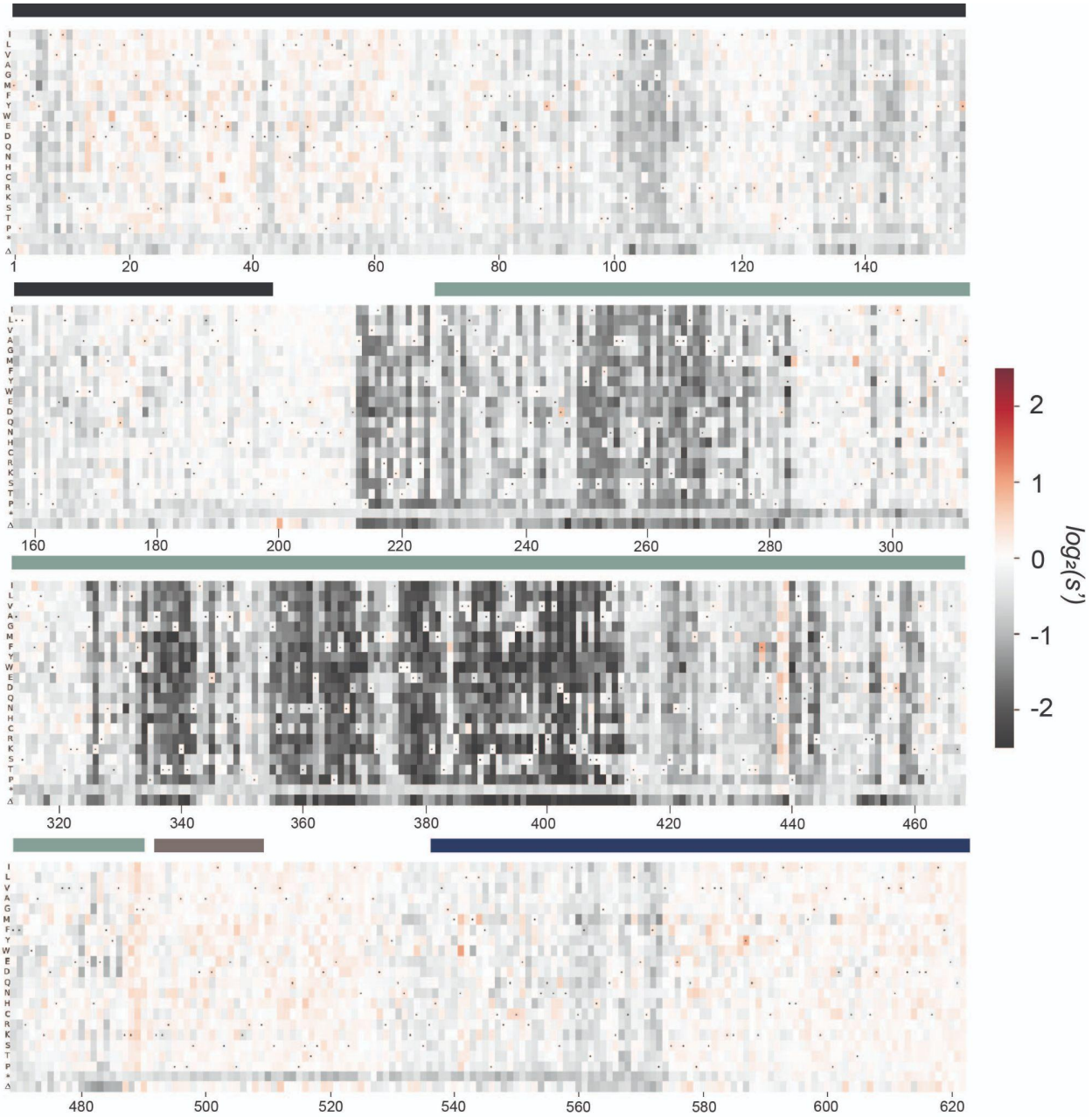

Figure S9. Effects of all single amino acid substitutions and deletions in the Rep78/68 proteins on AAV5 production. Amino acid level selection values from the pCMV-Rep78/68 production assay were calculated as in Figure 1C by summing barcode counts for synonymous mutations. Colored bars indicate protein domains. Black: OBD, light blue: HD, gray: NLS, and navy blue: ZFD. Black dots indicate wild type amino acid identity.

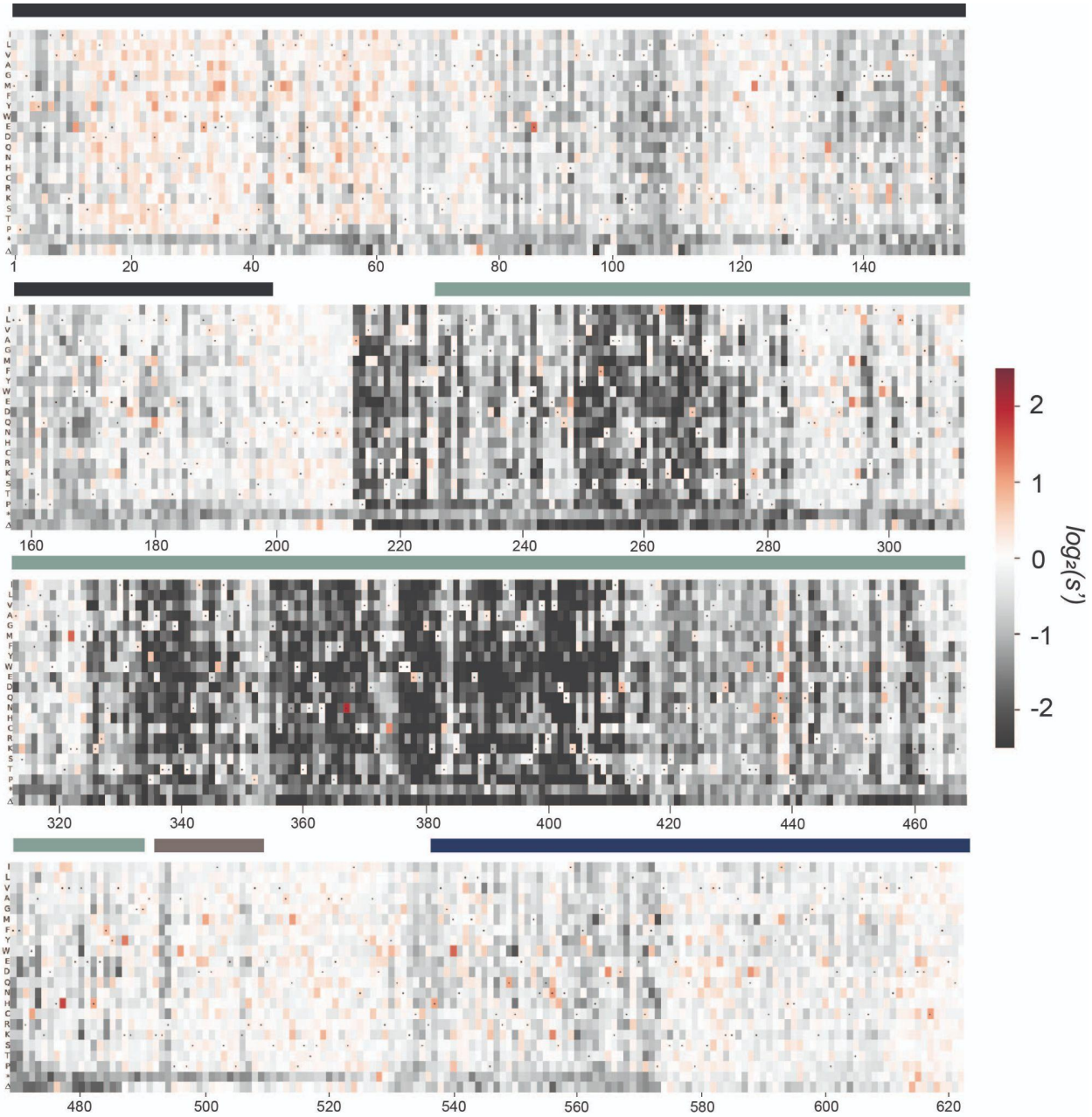

Figure S10. Effects of all single amino acid substitutions and deletions in the Rep78/68 proteins on AAV9 production. Amino acid level selection values from the pCMV-Rep78/68 production assay were calculated as in Figure 1C by summing barcode counts for synonymous mutations. Colored bars indicate protein domains. Black: OBD, light blue: HD, gray: NLS, and navy blue: ZFD. Black dots indicate wild type amino acid identity.

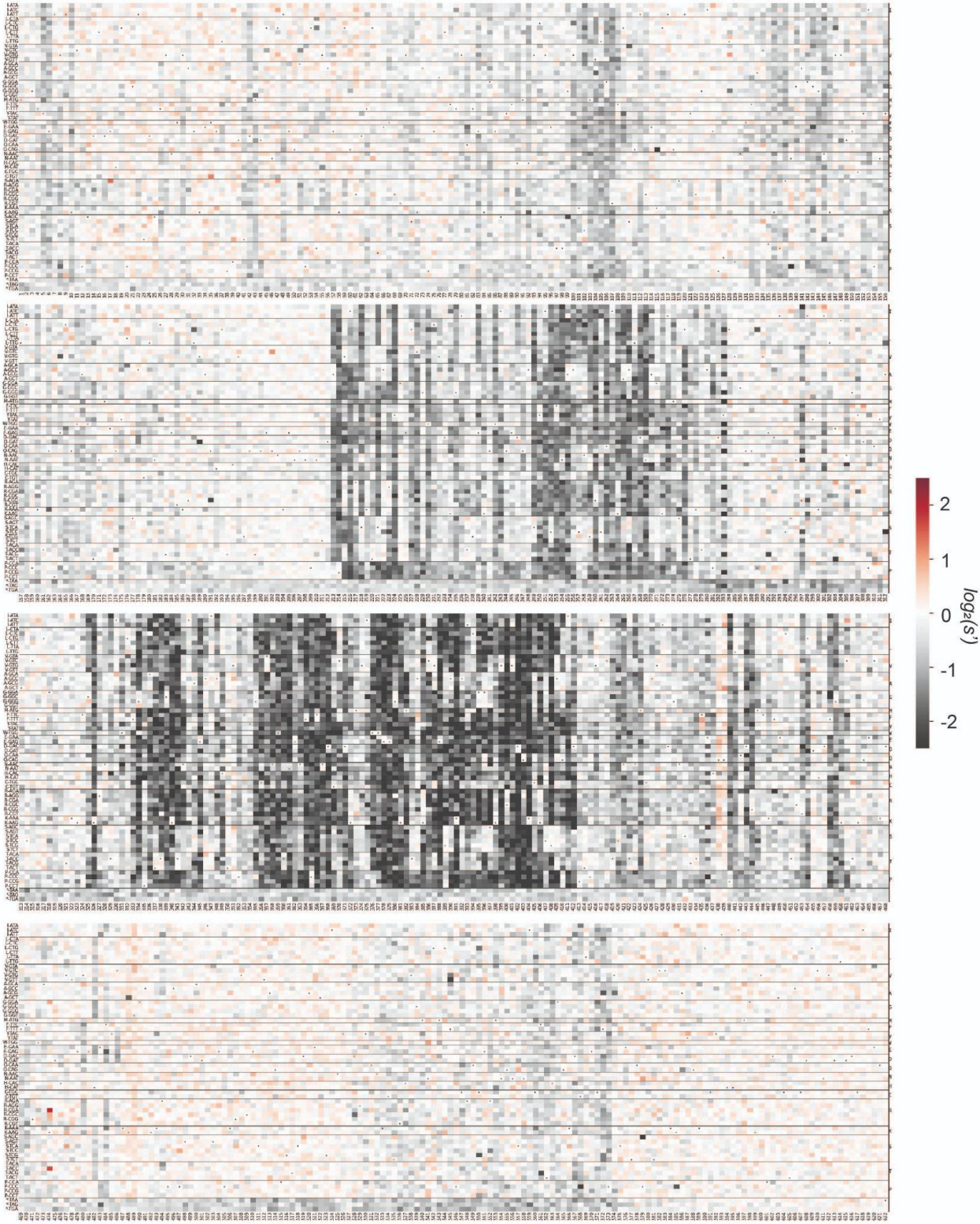

Figure S11. Codon level selection values for the AAV5 capsid production assay.

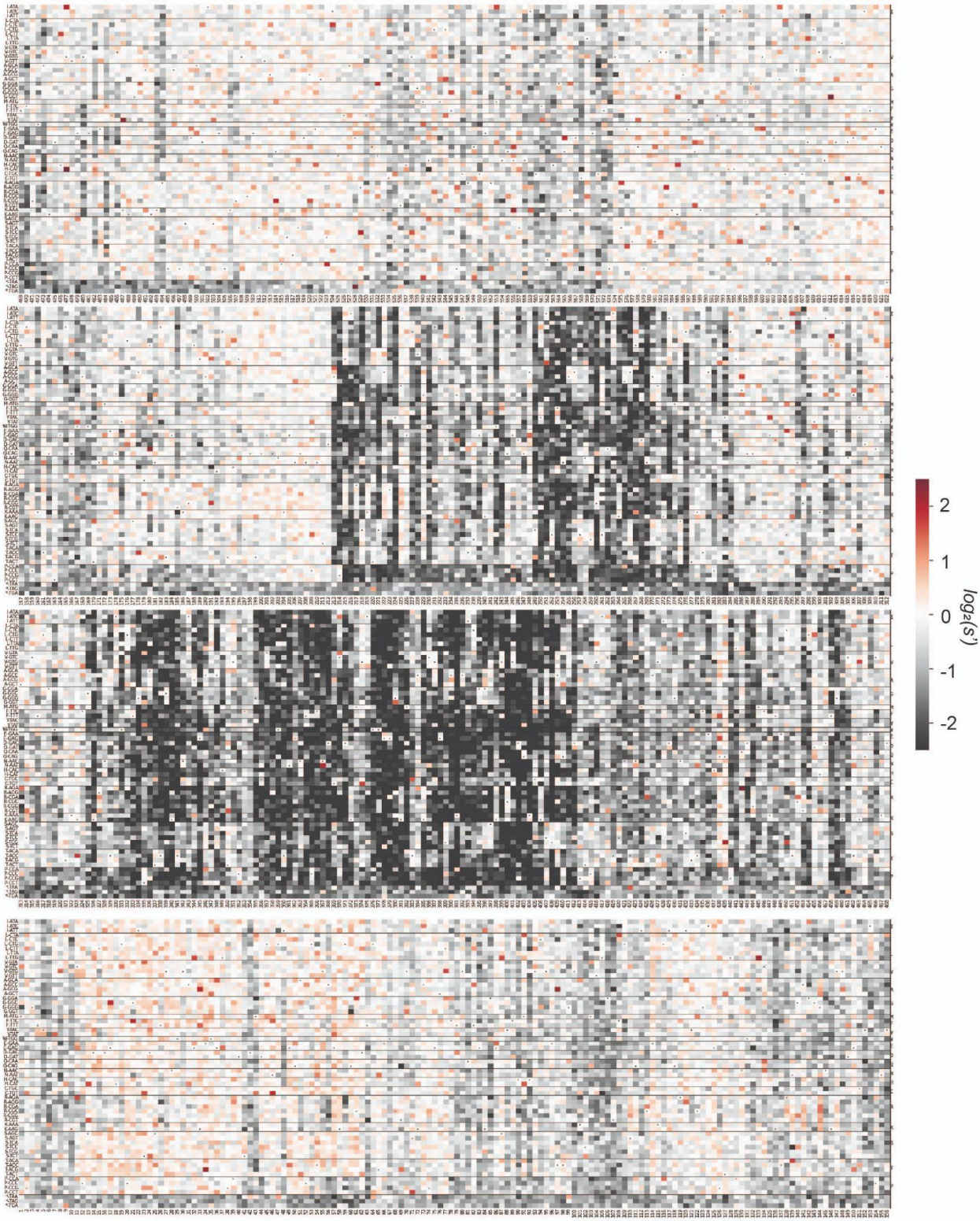

Figure S12. Codon level selection values for the AAV9 capsid production assay.

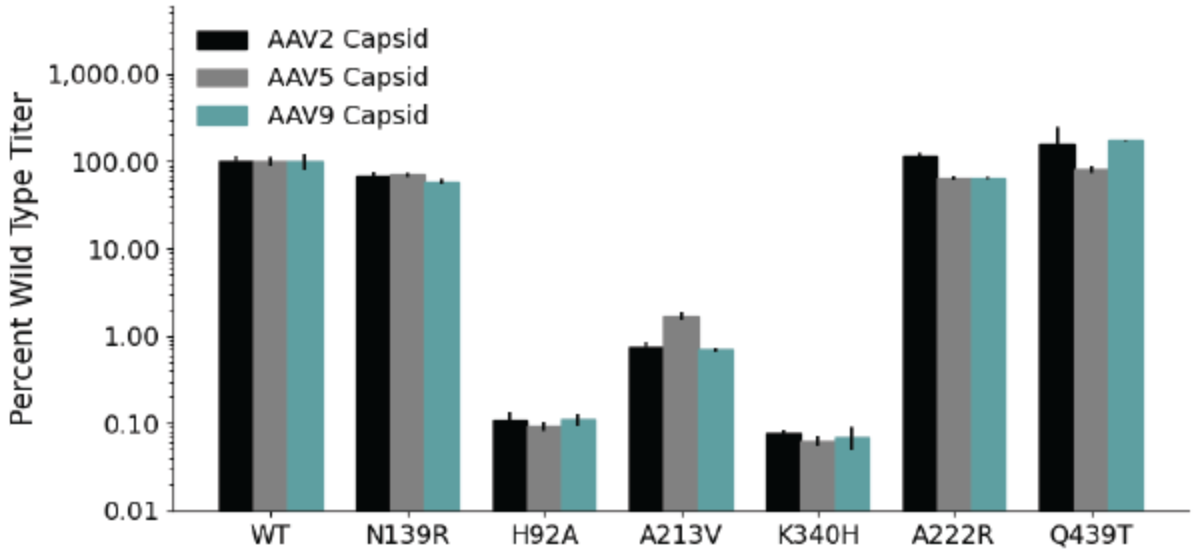

Figure S13. DRP titers for AAV2, AAV5, and AAV9 capsids produced individually with the indicated Rep variants.

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