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# An engineered helper plasmid generates differential E4orf6 and L4-22/33K gene expression increasing AAV vector production

Laura van Lieshout[1]✉, Katrina Costa-Grant[1], Dimpal Lata[1], Alejo Zacarias[1], Stacy Ota[1], Annie Adusei[1], Devin Schroeder[1], Diane Golebiowski[1] and Ifeyinwa Iwuchukwu[1]

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Helper plasmids that depend on native adenovirus gene expression have long been the standard for transient adeno-associated virus (AAV) production. Here, we demonstrate that engineering the required helper gene expression can greatly increase AAV production relative to the use of native adenovirus gene regulation. Two different engineered helper plasmid designs improved AAV vector genome (VG) titers up to 5-fold compared to a standard helper plasmid. A substantial decrease in adenovirus E4orf6 and an increase in L4 22K and 33K gene expression were associated with the improved engineered helper plasmids. VG titer improvement across capsid serotypes, plasmid transfection platforms and genome designs suggest that engineering helper gene expression is widely suited to improving AAV manufacturing yields while also maintaining consistent vector quality attributes.

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## INTRODUCTION

AAV was first isolated in 1965 as a contaminant in simian adenovirus cultures [1]. It was determined that AAV was replication defective unless in the presence of adenovirus and thus began decades of AAV production with the use of replication-competent adenovirus [2]. In 1997, the Samulski lab established the adenoviral genes that had a function in AAV replication and subcloned those genes into a helper plasmid, pXX6, effectively replacing the need for adenovirus coinfection, making the AAV production process safer and simpler by mitigating the need for removing contaminating adenovirus particles during purification [3, 4].

There are various helper plasmids available for use of different sizes, backbones and adenovirus serotypes, but minimally they all contain E2a, E4 and VA RNA as functional helper genes [5]. E1a/E1b are also required helper genes, however the standard use of HEK293 cells for AAV production, which already contain integrated E1, alleviates the need for inclusion on the helper plasmid [6]. Recently, Adsero et al. demonstrated that L4 22K and 33K, encoded within the E2a gene on the opposite strand as DNA binding protein (DBP), is also essential for AAV production [7].

We previously demonstrated that removal of some E4 open reading frames (orf) was beneficial for AAV productivity [8]. Others also supported this finding [9, 10]. We hypothesized that the increase in VG titer was due to a reduction in E4orf6 expression compared to the intact E4 gene. Adenovirus has presumably evolved to express its genes in a manner that is optimal for the adenovirus lifecycle, therefore it stands to reason that native adenovirus gene expression may not be best suited for supporting optimal AAV vector production. This led to the concept that engineering expression of the same helper genes, rather than relying on native adenovirus regulation, could provide a more optimal environment for AAV replication and packaging. In this manuscript, we demonstrate that currently used native adenovirus gene-based helper plasmids can be effectively replaced by an engineered helper plasmid resulting in over a 5-fold increase in AAV production.

## MATERIALS & METHODS

### Plasmids

All experiments utilized an OXB dual plasmid containing the AAV2 replicase (Rep; NCBI accession NC_001401.2), AAV9 capsid sequence (Cap; NCBI accession AY530579.1) and the single-stranded GFP-Luciferase (ssGFP-Luc) genome sequence, unless otherwise specified [11]. Additional AAV genome descriptions can be found in Supplementary Table 1. The Helper plasmid (pHelper) used has been previously described [8]. Engineered helper expression cassettes utilized the cytomegalovirus (CMV) promoter and bovine growth hormone (bGH) polyadenylation signal. Cloning and plasmid scale up (tier 2) was completed at GenScript (Piscataway, NJ). All plasmid sizes are available in Supplementary Table 2 and expression cassette sequences are included in the Supplemental Information.

### AAV production

VPC 2.0 HEK293 cells (ThermoFisher, Waltham, MA) were cultured in Expi239 media (ThermoFisher) at 37 °C using a New Brunswick S41i shaking incubator (Eppendorf, Hamburg, Germany) at 135 rpm (2.5 cm orbital diameter). All experiments were completed in 125 mL shake flasks (Corning 431143, Corning, NY) using a 50 mL working volume unless otherwise noted in the figure legend. Cells were transfected at a density of 2E6 cells/ mL unless otherwise noted. Transfection mixes were prepared with 0.75 µg

[1]Oxford Biomedica (US) LLC, Bedford, MA, USA. ✉email: l.vanlieshout@oxb.com

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of total plasmid/ 1E6 cells and a 2:1 PEIpro (Polyplus, Illkirch) to DNA ratio in Optipro (ThermoFisher) and incubated for 10 min before addition to cells. Equal molar plasmid ratios were utilized regardless of the number of plasmids transfected. While not necessarily optimal, between 2 and 6 plasmids were transfected in various conditions throughout these experiments and we elected to use equimolar ratios throughout to streamline the ratio variable. 48 h post transfection, cells were chemically lysed and crude lysate was clarified by centrifugation at 3000 × *g* for 10 min. The volume of crude lysate post lysis is 53.4 mL.

### VG & capsid titer quantification

Droplet digital PCR (ddPCR) was used to determine the VG titer as previously described [11]. Primer sequences can be found in Supplemental Information. Commercially available AAV2, AAV5 and AAV9 capsid ELISA kits (Progen Biotech, Wayne, PA) were used to determine capsid titer. Crude lysate titers reported reflect the post lysis volume. Calculated full vector percentages were generated by dividing the VG titer by the capsid titer.

### Flow cytometry

Prior to cell lysis, 250 µL of cell culture was collected in a 96 well plate and processed using the CytoFLEX flow cytometer (Beckman Coulter, Brea, CA). >10,000 events were collected per sample on slow speed (10 µL/min). Data was analyzed with FlowJo software using a standard gating strategy to isolate single cells and calculate the percentage of GFP-positive cells and the associated mean fluorescence intensity (MFI) of that population.

### Genome replication assay

48 h post transfection, 2E6 cells were pelleted through centrifugation at 500 × *g* for 3 min. Replicated AAV DNA was extracted from the cell pellets using QIAprep® Spin Miniprep Kit (Qiagen, Venlo, Netherlands), substituting bacterial cells for HEK293 production cells. 10 µL of the elution was mixed with 6X DNA loading dye, separated on a 1% agarose gel at 120 V for 45 min and stained with SYBR Safe (ThermoFisher). GeneRuler 1 kb plus DNA ladder (ThermoFisher) was used.

### Western immunoblotting

Rep, capsid and DBP western blots were all performed as previously described [8]. Flag westerns were completed using the DYKDDDDK Tag (9A3) primary antibody (Cell Signaling, Danvers, MA) at a 1:1000 dilution and otherwise followed the same procedure. REVERT total protein stain (LI-COR, Lincoln, NE) was used to demonstrate equal loading. For quantification, the primary antibody signal was normalized with the total protein stain signal in each lane and each condition was analyzed in duplicate unless otherwise noted. 5 µl of affinity-purified vector was loaded per sample, separated by SDS-PAGE and stained with REVERT total protein stain to calculate vector VP ratios. Analysis was performed with Image Studio software.

### RNA sequencing

Prior to harvest, 2E6 cells per replicate were centrifuged for 5 min at 500 × *g* to pellet the cells. Supernatant was removed and pellets stored at −80 °C. RNA extraction, library preparation and sequencing was completed by Azenta (Burlington, MA) using Illumina 2 × 150 bp, strand-specific poly-A. Raw FASTQ files were analyzed using condition-specific plasmid transcriptomes. Composite FASTA/ annotation files were converted to transcript FASTA with gffread and indexed with kallisto for pseudoalignment-based quantification. Transcript IDs were standardized and summarized with tximport into counts-like matrices, and differential expression across conditions was performed with DESeq2, generating normalized counts, $\log_2$ fold changes, adjusted p-values, and summary plots.

### Affinity purification & product quality analysis

Clarified crude lysate was 0.22 µm filtered and purified by POROS CaptureSelect AAV9 affinity resin (ThermoFisher) using an AKTA Avant (Cytiva, Marlborough, MA). The resulting affinity-purified product was subsequently analyzed by capsid western immunoblot (described above), analytical ultracentrifugation (AUC) and for residual host cell DNA (hcDNA). Quantification of intact genomes, partial genomes and empty capsid fractions were determined using an Optima AUC (Beckman Coulter). Sample analysis using the SEDFIT c(S) model yields a distribution of sedimentation coefficients (S) with each peak in the distribution representing an intact or partial genome or empty capsid. Integration of the individual peaks yield the S value and the relative concentration of each species in the distribution [12]. The linear relationship between S and the nucleotide length of the packaged genome can be used to predict the S value of an intact packaged AAV vector. The resDNASEQ human residual DNA quantification qPCR kit (ThermoFisher) was used to determine the residual host cell DNA (hcDNA) in affinity-purified vector following extraction with the QIAamp mini kit (Qiagen).

### Statistical analysis

All conditions were evaluated with two biological replicates unless otherwise noted in the figure legend. All error bars represent the mean ± standard deviation (SD). Statistical analysis was completed using GraphPad Prism software.

## RESULTS

### Native adenovirus gene expression can be effectively replaced with engineered expression

To facilitate optimization of individual gene expression, E2a, E4 and VA RNA were cloned onto separate plasmids, together termed the native helper genes (NHG; Fig. 1A). Compared to pHelper, NHG increases VG titer 2-fold but disproportionally increased capsid titer almost 3-fold, resulting in a net decrease in the calculated full capsids, a preliminary measure of AAV packaging quality (Fig. 1B–D). Despite the identical gene sequences used in pHelper and NHG, the differences in plasmid stoichiometry utilizing three helper plasmids rather than one may provide a more advantageous environment for AAV production (Fig. 1E). Cells transfected as measured by GFP expression from the AAV genome plasmid yielded similar GFP+ cells across conditions; however, the MFI was significantly higher for NHG compared to pHelper (Fig. 1F). Although not an accurate quantification of "transfection efficiency", measuring GFP expression indicates cells that successfully received the AAV genome plasmid and produced GFP. It is possible more cells were transfected but did not undergo GFP expression. More notably is the disparity in the MFI of GFP expression despite similar input of GFP-bearing plasmid, indicating differential helper gene expression could impact this output.

Further improving on NHG, each protein-coding gene was substituted for an engineered counterpart, replacing the native promoter with the strong heterologous CMV promoter. The only protein product of the E2a gene is DBP. When the native E2a plasmid was replaced with CMV-DBP, AAV VG and capsid titers were abolished (Fig. 1G, H). It is now understood that there are additional essential proteins produced from the L4 gene, encoded on the opposite strand of the E2a gene [7, 13]. Replacing E2a with CMV-DBP and CMV-L4 22K (henceforth CMV-22K) recovered AAV productivity, with a ~50% improvement in VG titer and a positive gain in calculated full vectors compared to the NHG control (Fig. 1G, I).

It has been long established that the E4 gene in its entirety is not required for AAV production and E4orf6 specifically is known to play the critical role [14–16]. We previously demonstrated E4orf1-4 were dispensable and their removal led to an increase in AAV VG titer, however there was a considerable decrease when

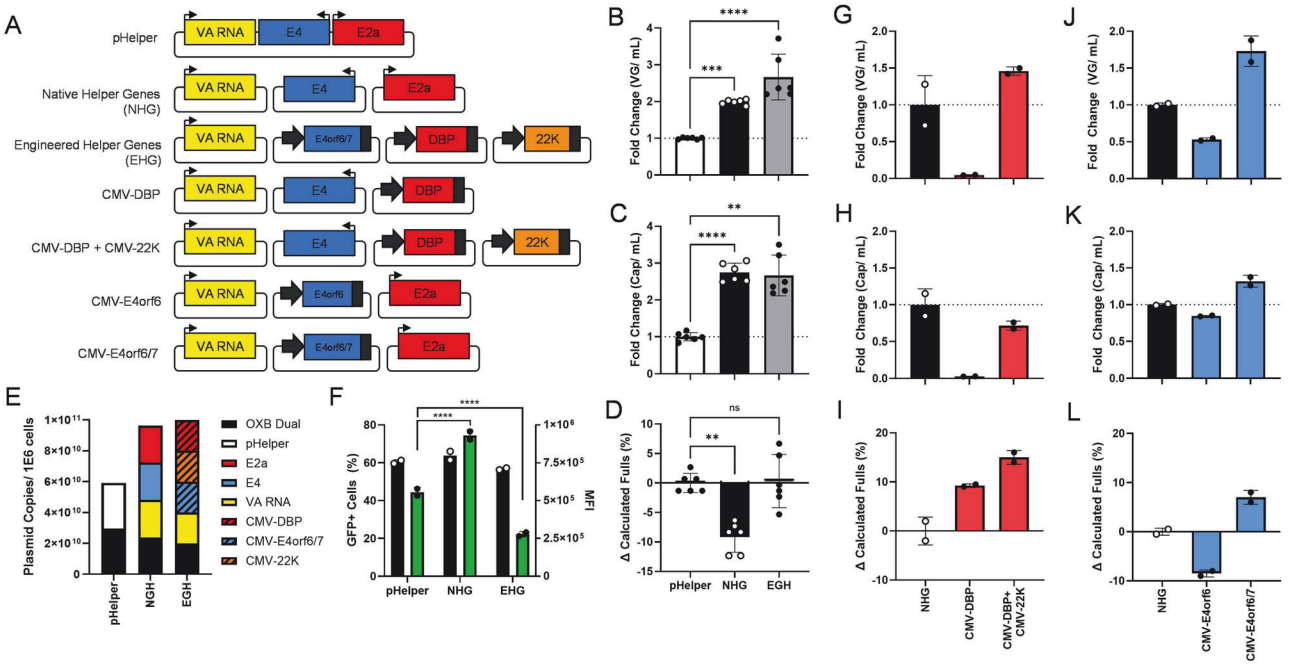


**Fig. 1 Developing a four-plasmid engineered helper gene system that increases VG productivity. A** Schematic diagram of the helper plasmids utilized in this experiment. Small black arrows represent native promoters and large black arrows represent CMV promoters. The arrow orientation indicates the direction of transcription. Black boxes represent bGH poly A signals. pHelper ($n = 6$), native helper genes (NGH; $n = 6$) and engineered helper genes (EHG; $n = 6$) were transfected with an OXB dual. Crude lysate (**B**) VG titer, (**C**) capsid titer and (**D**) calculated full vector percentage were normalized to the average pHelper values. Statistical analysis was performed using one-way analysis of variance (ANOVA). **E** Graphic representation of the plasmid input for each condition. **F** Cells transfected was assessed by flow cytometry; GFP+ cells (left axis) and mean fluorescence intensity (MFI; right axis). Statistical analysis was performed using two-way ANOVA. In another experiment testing engineered plasmids to replace native E2a and E4, crude lysate (**G**, **J**) VG titers, (**H**, **K**) capsid titers and (**I**, **L**) calculated full vector percentage were normalized to the average NGH values. Statistical significance: ns not significant, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Data is shown as mean ± SD of biological replicates ($n = 2$ unless noted otherwise above).

E4orf6 alone was present rather than E4orf6/7, which expressed both orf6 and orf6/7 [8]. Here, both CMV-E4orf6 and CMV-E4orf6/7 replaced the native E4 plasmid with relative success, however CMV-E4orf6/7 was optimal for VG and capsid titer as well as vector packaging (Fig. 1J–L, Supplemental Fig. 1). Johari et al. report similar outcomes regarding E4orf6/7 and E4orf6 only expression [10].

The native VA RNA gene produces two distinct RNA species; VA RNAI and VA RNAII. Each VA RNA was placed on an individual plasmid and tested in replacement of the entire native VA RNA gene. Much of the helper function comes from VA RNAI, with VA RNAII playing a secondary role (Supplementary Fig. 2A–C), supporting the findings of Doshi et al. [17]. However, this data demonstrates an incomplete VG titer recovery from VA RNAI alone and due to the minimal size of the native VA RNA gene, we proceeded to include the entirety of the VA RNA sequence.

Combined, CMV-DBP, CMV-22K and CMV-E4orf6/7 along with the native VA RNA plasmid constitute the engineered helper genes (EHG). The EHG outperformed pHelper over 2-fold for VG titer and demonstrated comparable calculated full values (Fig. 1B, D). The MFI from the EHG condition was significantly lower than pHelper and NHG, despite similar plasmid input to NHG, indicating engineered helper expression decreases GFP output produced from the transfected AAV genome (Fig. 1E, F). Reduced transgene expression during AAV production has been shown by other avenues to increase VG production [18, 19].

### Bicistronic expression of DBP & 22K is highly variable depending on cassette design

Although the EHG increased VG titer compared to pHelper, a four-plasmid helper platform is not practical for GMP use and our aim was to rebuild a single engineered helper plasmid. Since all protein sequences in EHG utilize the same CMV promoter, there was concern regarding promoter competition and sequence fidelity during *E. coli* production of plasmid due to repetitive sequences [20–22]. As such, bicistronic gene expression of DBP and 22K to reconstitute the native E2a/L4 gene was evaluated using an internal ribosome entry site (IRES) and a 2A peptide [23]. It is known that IRES use results in attenuated expression for the orf in second position, therefore we evaluated both modalities with each orf in both arrangements (Fig. 2A) [24]. Only one of the four bicistronic cassettes demonstrated comparable or improved VG titers relative to the EHG control, CMV-DBP-IRES-22K (Fig. 2B). Capsid titers increased for all four constructs, which decreased the calculated full vectors, but CMV-DBP-IRES-22K was least reduced (Fig. 2C, D).

DBP western blot analysis demonstrated ~50% reduction in expression for both constructs with DBP in second position (Fig. 2H, Supplementary Fig. 3). While this was expected using the IRES, it was somewhat surprising for the 2A peptide as equimolar expression is thought to be an advantage of utilizing the latter [25]. A N-terminal flag tag was included on the CMV-22K plasmid to allow for protein detection since commercial antibodies are unavailable. CMV-22K plasmid versions with and without the flag tag resulted in comparable VG and capsid titers (Supplementary Fig. 2D–F). 22K expression varied drastically by each plasmid design. The best performer, CMV-DBP-IRES-22K, showed only slightly elevated 22K expression, however CMV-22K-IRES-DBP and CMV-DBP-2A-22K produced 16-fold and 45-fold more 22K than CMV-22K in the EHG control (Fig. 2I, Supplementary Fig. 3). While it is known that particular sequences or arrangement of sequences in bicistronic cassettes can have unexpected inhibitory outcomes, the magnitude of increased expression observed here is puzzling

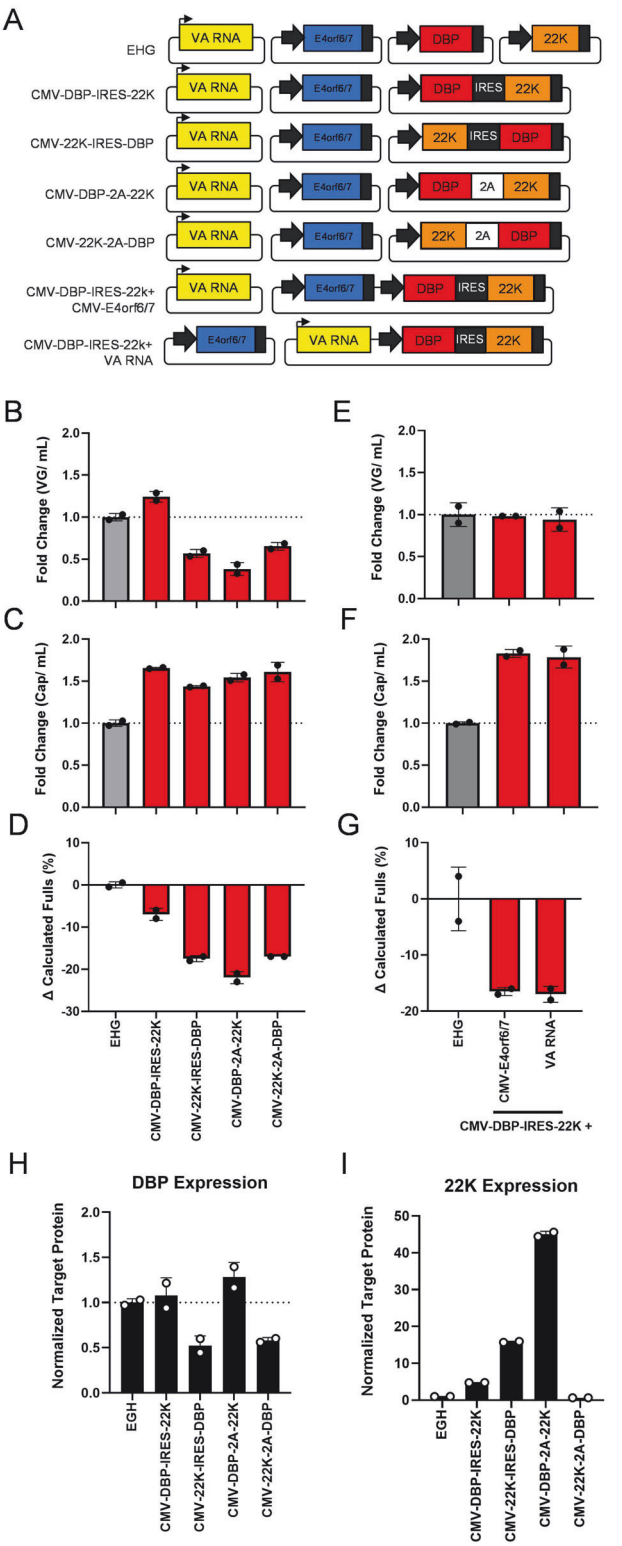


**Fig. 2 Replacing native E2a with bicistronic DBP and 22K expression. A** Schematic diagram of the helper plasmids utilized in this experiment. Small black arrows represent native promoters and large black arrows represent CMV promoters. The arrow orientation indicates the direction of transcription. Black boxes represent bGH poly A signals. IRES- internal ribosome entry site, 2A- T2A peptide. Four bicistronic DBP/22K constructs were evaluated for crude lysate (**B**) VG titer, **C** capsid titer and **D** calculated full vector percentage, normalized to the engineered helper genes (EHG). Two additional plasmids, CMV-DBP-IRES-22K + CMV-E4orf6/7 and CMV-DBP-IRES-22K + VA RNA were tested and the crude lysate **E** VG titer, **F** capsid titer and **G** calculated full vector percentage were normalized to the average EHG values. Quantification of (**H**) DNA-binding protein (DBP) and (**I**) flag-22K protein expression by western blot. Data is shown as mean ± SD of biological replicates ($n = 2$).

[26]. Overall, CMV-22K-IRES-DBP had the most similar DBP and 22K expression profile to the EHG and produced the most similar VG titer.

In a follow-up experiment, constructs with the addition of CMV-E4orf6/7 or VA RNA to CMV-DBP-IRES-22K were tested. Both resulted in similar VG titers as the EHG control, however elevated capsid titers resulted in decreased calculated full values (Fig. 2E–G). Notably, CMV-DBP-IRES-22K + CMV-E4orf6/7 contains two identical CMV promoters and did not negatively impact AAV productivity or plasmid production.

## Inclusion of L4 33K is necessary for optimal AAV output

The EHG was benchmarked against pHelper, NHG and OXB_Helper_3, a previously published minimized helper plasmid containing E2a and E4 deletions shown to increase AAV productivity (Supplementary Fig. 1) [8]. OXB_Helper_3 outperforms all other conditions including EHG, suggesting the engineered platform was still missing a component (Fig. 3A, Supplementary Fig. 3A, B). It was determined that the CMV-22K plasmid should be modified to also express 33 K (Fig. 3C–F Supplementary Fig. 1). This EHG + 33K condition was subsequently evaluated and produced higher VG titers than OXB_Helper_3 and EHG expressing only 22K (Fig. 3B). This demonstrates that both 22K and 33K play vital roles in AAV production and although CMV-22K alone was sufficient to restore VG titers to NHG levels (Fig. 2A), both are required for optimality.

Based on the preliminary success of CMV-DBP-IRES-22K + CMV-E4orf6/7 and CMV-DBP-IRES-22K + VA RNA (Fig. 2E), an engineered helper (eHelper) plasmid containing CMV-DBP-IRES-22K + CMV-E4orf6/7 + VA RNA was generated, termed eHelper_v1 (Fig. 3C). As discussed, optimum AAV production requires 22/33K, therefore a second version was produced with this inclusion, termed eHelper_v2 (Fig. 3C). These two plasmids are identical with the exception of the length of the L4 coding region. Both eHelper plasmids were evaluated alongside the appropriate 4-plasmid helper controls, EHG and EHG + 33 K. Again, there is a significant increase in the EHG VG titer with the inclusion of 33K (Fig. 3G). eHelper_v1 resulted in similar titers as EHG, demonstrating the four helper plasmids can be successfully combined and achieved the same output. However, eHelper_v2 underperformed the EHG + 33K control, suggesting translation to a single plasmid was less successful than the 22K only version. Both eHelper plasmids demonstrated a trend towards increased capsid production and reduction of calculated full vectors, which is not ideal (Fig. 3H, I).

The CMV-22/33K plasmid also includes an N-terminal flag tag that did not impair VG or capsid titers (Supplementary Fig. 2D–F). A flag western blot showed 22K expression for EHG and eHelper_v1, with elevated expression from the latter (Fig. 3J, Supplementary Fig. 4). However, the expression of 22/33K is dissimilar between EHG + 33K and eHelper_v2. In EHG + 33K, 22/33K are expressed directly from a CMV promoter and both proteins can be visualized but in eHelper_v2 expression is facilitated by an IRES and 22K protein is present but 33K expression is diminished (Fig. 3J, Supplementary Fig. 4E). While the IRES can facilitate the expression of 22K only, it does not appear to be capable of producing sufficient spliced 33K. This likely explains the reduction in VG titer for eHelper_v2 compared to EHG + 33K, again highlighting the importance of 33K expression for maximum AAV output. Rep protein expression was similar

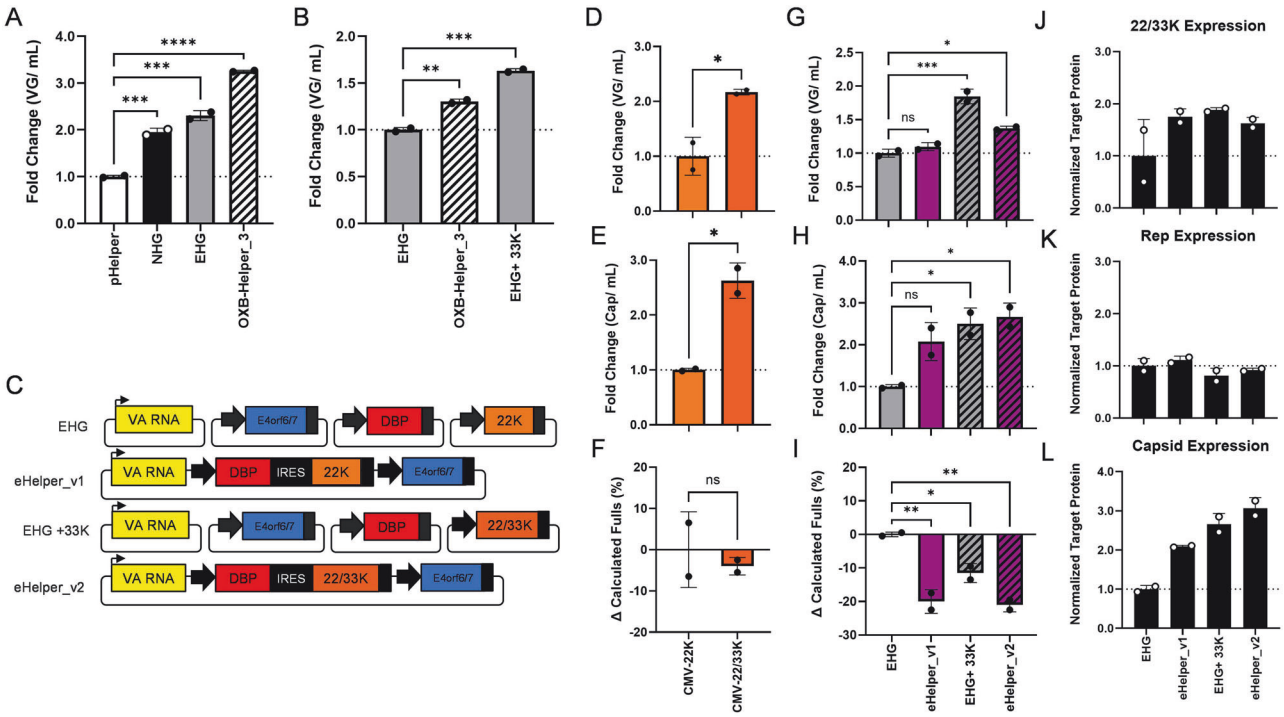


**Fig. 3 Addition of 33K expression and re-building engineered helper plasmids.** Crude lysate (**A**) VG and (**B**) capsid titer normalized to the average pHelper values. Statistical analysis was performed using one-way ANOVA. **C** Schematic diagram of the helper plasmids utilized in these experiments. Small black arrows represent native promoters and large black arrows represent CMV promoters. The arrow orientation indicates the direction of transcription. Black boxes represent bGH poly A signals. Crude lysate **D** VG titer, **E** capsid titer, and **F** calculated full vector percentage using EHG with the CMV-22K or CMV-22/33K plasmid. Statistical analysis was performed using a Student's *t* test. Crude lysate **G** VG titer, **H** capsid titer, and **I** calculated full vector percentage were normalized to the average EHG values. Statistical analysis was performed using one-way ANOVA. Normalized quantification of **J** flag-22K and flag-33K, **K** Rep, and **L** capsid protein expression by western blot relative to EHG expressing 22K only. Statistical significance: ns not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Data is shown as mean ± SD of biological replicates ($n = 2$).

across all four helper modalities (Fig. 3K, Supplementary Fig. 4E). Capsid protein expression for eHelper_v1, EHG + 33K and eHelper_v2 are all elevated relative to the EHG control (Fig. 3L, Supplementary Fig. 4E), which closely reflects the capsid titers (Fig. 3H).

## Engineered helper plasmid increases AAV productivity five-fold

Due to the obstacle posed by bicistronic expression of 22/33K in combination with successful evaluation of CMV-DBP-IRES-22K + CMV-E4orf6/7, reducing concerns regarding duplicate promoters, a third eHelper design was cloned that simply combined all EHG + 33K cassettes onto a single plasmid, eHelper_v3 (Fig. 4A). Both eHelper_v2 and eHelper_v3 resulted in over 4-fold higher VG titers than pHelper (Fig. 4B). Capsid output was higher for eHelper_v2 than eHelper_v3, resulting in a lower calculated full value for the former and a similar value to pHelper for the latter (Fig. 4C, D). The MFI of cells transfected with eHelper plasmids were significantly lower than pHelper, similar to the previous EHG results (Figs. 1F and 4E). Under high cell density conditions reflective of the OXB AAV production platform, but otherwise the same process described for low cell density, eHelper_v3 produced 1.4E12 VG/ mL or 1.4E15 VG/ L, 5.1-fold more than pHelper (Fig. 4B). These results demonstrate that there are significant gains available when helper gene expression is altered to best suit AAV replication and packaging.

Rep protein expression was comparable from all three helper plasmids (Fig. 4F, Supplementary Fig. 5). Capsid protein expression was almost 4-fold higher for eHelpers compared to pHelper (Fig. 4G, Supplementary Fig. 5), which corresponds to the capsid titers as well (Fig. 4C). DBP protein expression was similar across all three helper conditions (Fig. 4H, Supplementary Fig. 5). 22/33K protein expression was mediated through an epitope tag, which was not present on the pHelper plasmid, therefore that comparison was not feasible. However, eHelper_v3 expresses 6-fold more 22/33K than eHelper_v2 (Fig. 4I, Supplementary Fig. 5). Unfortunately, we were unable to obtain any viable western blot data for E4orf6/7 despite multiple attempts to generate a polyclonal antibody and trialing epitope tags.

To further characterize the gene expression profile of the eHelper plasmids, RNA sequencing was employed. First, the adenovirus and AAV gene expression profile using pHelper was examined, as to the best of our knowledge this has not previously been reported. AAV capsid transcripts were the most abundant, followed by DBP, Rep, E4orf6/7, E4orf6, 33K, 22K, VA RNAI and finally VA RNAII (Fig. 4J). E4orf6/7 and 33K transcripts, both products of splicing, outnumbered their unspliced counterparts E4orf6 and 22K. 22K and 33K were the least expressed viral protein transcripts.

Overall, the viral transcript profiles between the three helper plasmids were reasonably similar with two major exceptions. There was a reduction in E4orf6 transcripts and a substantial increase in 22K and 33K expression from eHelper plasmids compared to pHelper (Fig. 4K). We previously reported reduced E4orf6 transcripts in association with increased VG titers, which aligns well with this data [8]. However, here we also show increased 22K and 33K transcripts in association with increased VG productivity. The abundance of 22K and 33K transcripts relative to the total adenoviral gene transcripts in both eHelper plasmids compared to pHelper is noteworthy (Fig. 4L). The proportion of 22K and 33K transcripts produced from eHelper_v2 and eHelper_v3 correspond with the protein expression profiles

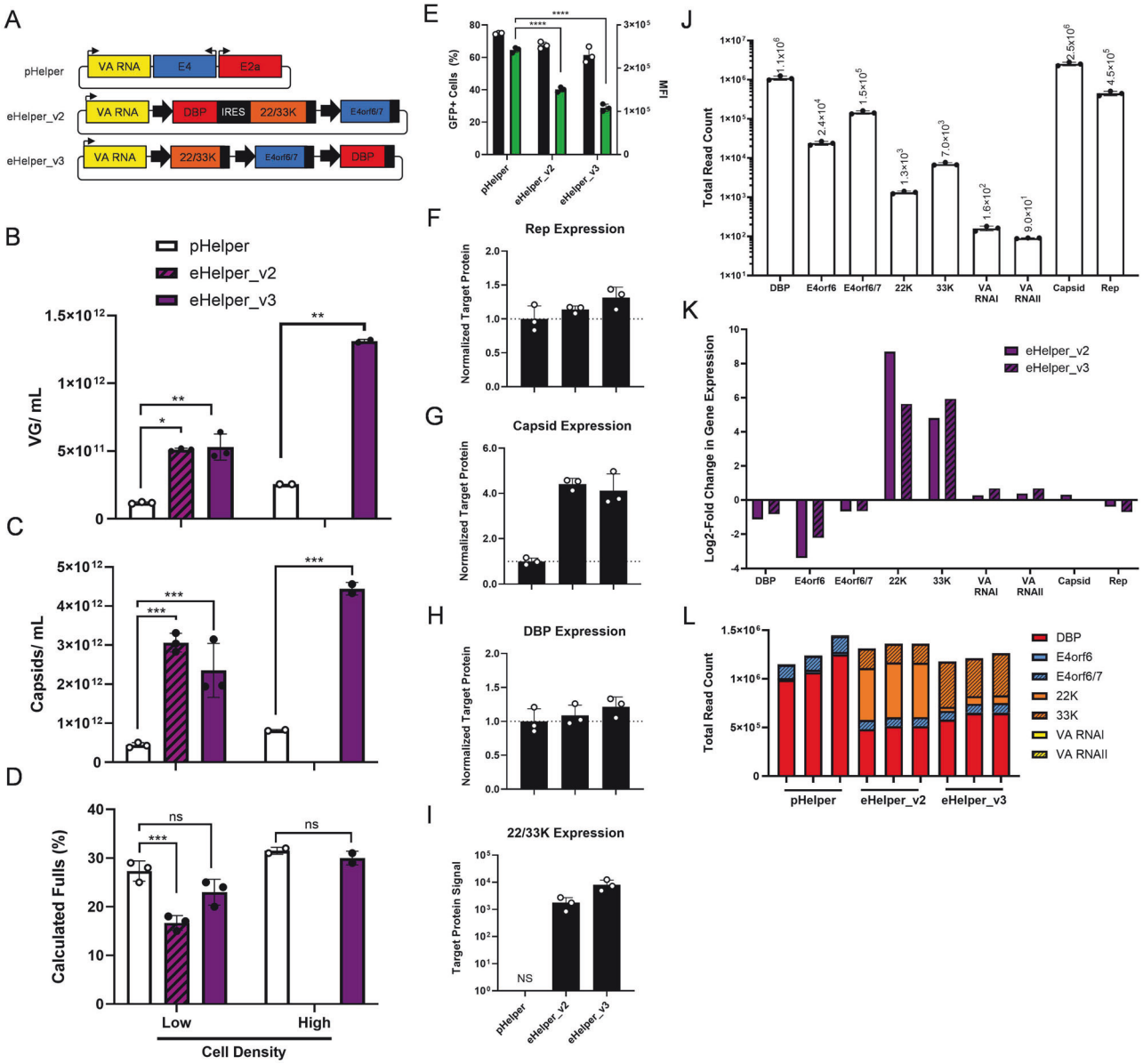


**Fig. 4 Engineered helper plasmids increase AAV production and alter helper gene transcript profile. A** Schematic diagram of the helper plasmids utilized in this experiment. Small black arrows represent native promoters and large black arrows represent CMV promoters. The arrow orientation indicates the direction of transcription. Black boxes represent bGH poly A signals. Crude lysate **B** VG titer, **C** capsid titer and **D** calculated full vector percentage produced at low cell (2E6 cells/ mL; $n = 3$) and high cell (4E6 cells/ mL; $n = 2$) transfection density. **E** Cells transfected was assessed by flow cytometry; GFP+ cells (left axis) and mean fluorescence intensity (MFI; right axis). Normalized quantification of **F** Rep, **G** capsid and **H** DNA-binding protein (DBP) protein expression by western blot relative to pHelper. **I** For flag-22K and flag-33K protein expression, the total signal was shown due to the lack of flag tagged proteins in pHelper to enable normalization. NS no signal. **J** Adenoviral helper gene, total capsid (VP1, VP2, VP3) and total Rep (Rep78, Rep68, Rep52, Rep40) transcript counts from **J** pHelper samples and **K** normalized transcript counts from eHelper_v2 and eHelper_v3 samples relative to pHelper. **L** Adenoviral helper transcripts produced from pHelper, eHelper_v2 and eHelper_v3. Statistical analysis was performed using two-way ANOVA. Statistical significance: ns not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Data is shown as mean ± SD of biological replicates ($n = 3$, unless noted otherwise above).

(Supplemental Fig. 5). The IRES utilized in eHelper_v2 skews towards higher 22K expression while expression of 22/33K directly from the CMV promoter yields greater 33K output (Fig. 4L, Supplementary Fig. 5). Interestingly, despite capsid protein expression increasing 4-fold for eHelper plasmids, an increase in capsid transcripts was not detected.

### Lack of 22/33K alters rep protein profile & abolishes capsid protein expression

Further characterizing the role of the adenovirus helper genes in AAV production, each individual helper plasmid from the EHG + 33K condition was removed and replaced with an empty pBluescript plasmid to ensure the total plasmid transfected across each condition remained consistent (Fig. 5A). The number of cells transfected was similar across all conditions with a reduction in the MFI for ΔVA RNA (Fig. 5B). Removal of each plasmid had an impact on VG and capsid titer, however ΔVA RNA was the least impactful on VG titer, while removal of all the other genes resulted in massive impairment of VG production (Fig. 5C). ΔCMV-22/33K had the greatest reduction of capsid titer, while ΔE4orf6/7 was the least impactful (Fig. 5D). Overall, removal of the CMV-22/33K plasmid had the most substantial impact on VG and capsid titers.

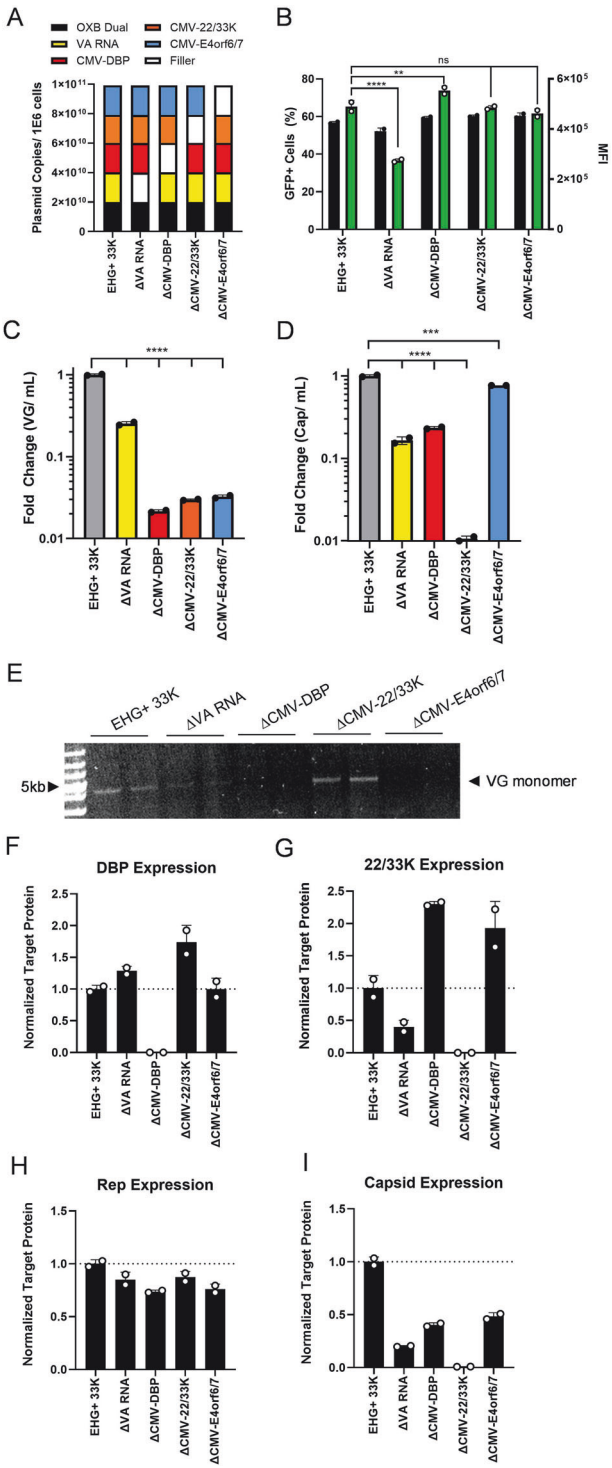


**Fig. 5 Removal of each engineered helper gene has a unique impact on AAV production. A** Graphic representation of the plasmid input for each condition. Empty pBluscript was used as the filler plasmid (white bar). **B** Cells transfected was assessed by flow cytometry; GFP+ cells (left axis) and mean fluorescence intensity (MFI; right axis). Statistical analysis was performed using two-way ANOVA. Crude lysate (**C**) VG titer and **D** capsid titer were normalized to the average EHG + 33K values. Statistical analysis was performed using one-way ANOVA. **E** AAV genome replication species separated on an agarose gel. VG monomers are expected to be 4183 nucleotides long. Normalized quantification of **F** DNA-binding protein (DBP), **G** flag-22K and flag-33K, **H** Rep and **I** capsid protein expression by western blot relative to EHG + 33K. Statistical significance: ns not significant, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Data is shown as mean ± SD of biological replicates ($n = 2$).

AAV genome replication was not detected in ΔCMV-DBP and ΔCMV-E4orf6/7 conditions and appears reduced for ΔVA RNA. While this assay is only qualitative, it is interesting to note that genome monomers are visible in ΔCMV-22/33K that are consistent with the control output. This suggests that genomes are replicated without the presence of 22/33K despite the absence of these proteins severely impairing AAV VG and capsid titers.

Lack of CMV-22/33K somewhat increased DBP protein expression and lack of DBP or E4orf6/7 increased 22/33K protein expression (Fig. 5F, G). Total Rep protein expression was consistent but the isoform expression profile for ΔCMV-22/33K showed an increase in Rep78/Rep68 and a decrease in Rep52 (Fig. 5H, Supplemental Fig. 6). Rep40 expression was weak even in the control, but it was abolished in the ΔCMV-22/33K condition. Capsid protein expression was severely reduced without CMV-22/33K, only VP3 was faintly visible, aligning with the low capsid titers (Fig. 5I, Supplementary Fig. 6, Fig. 4D).

## Purified AAV produced with eHelper_v3 demonstrates similar downstream recovery and vector quality

All transfections to this point utilized an OXB dual plasmid to provide the AAV genome and RepCap functions using the AAV9 capsid sequence. Evaluation of eHelper_v3 with AAV5 and AAV2, two evolutionarily distinct serotypes from AAV9, demonstrated VG titer increased over 2-fold and 5-fold compared to pHelper, respectively (Fig. 6A). Capsid titers also increased somewhat greater than VGs, resulting in slightly reduced vector packaging, in line with observations with previous AAV9 experiments (Fig. 6B, C). Additionally, eHelper_v3 was used in traditional triple transfection using three distinct AAV vector genome constructs. Both single-stranded constructs performed similarly with eHelper_v3, yielding 4-fold VG increases and similar or minorly improved calculated full vector values compared to pHelper (Fig. 6D–F). The self-complementary (sc) genome tested also increased VG production but to a lesser extent (Fig. 6D). Despite this, capsid production from eHelper_v3 remained similar across all three genomes, resulting in an 11% decrease in the calculated full vector value compared to pHelper (Fig. 6F). Overall, these results suggest eHelper_v3 widely increases AAV productivity across transfection platforms, serotypes and AAV genome designs, however there was some variation in the magnitude depending on the input.

Larger crude lysate volumes were produced to enable affinity purification and preliminary product quality evaluation using pHelper and eHelper_v3. Similar VG titer, capsid titer and vector packaging trends were generated compared to the previous experiment using triple transfection (Fig. 6G–I). Crude lysate was purified using affinity chromatography. VG and capsid recoveries were similar for pHelper and eHelper_v3 (Fig. 6J). Purified AAV viral protein (VP) ratios were examined by SDS-PAGE. Vector produced with pHelper and eHelper_v3 showed similar VP ratios (Fig. 6K). AUC enabled quantification of the intact genome, partial genome and empty capsid fractions within vector samples. pHelper and eHelper_v3 produced similar profiles minorly favoring pHelper for lower empty and higher intact proportions (Fig. 6L). Compared to crude calculated full vector values, AUC on affinity products trended similarly but suggested an overall higher full or intact genome proportion and less variation between the helper plasmids. In both cases, the vector profile can be further refined through additional polishing steps such as anion exchange chromatography. Residual hcDNA decreased by 40% in the vector generated with eHelper_v3 (Fig. 6M). In summary, the product

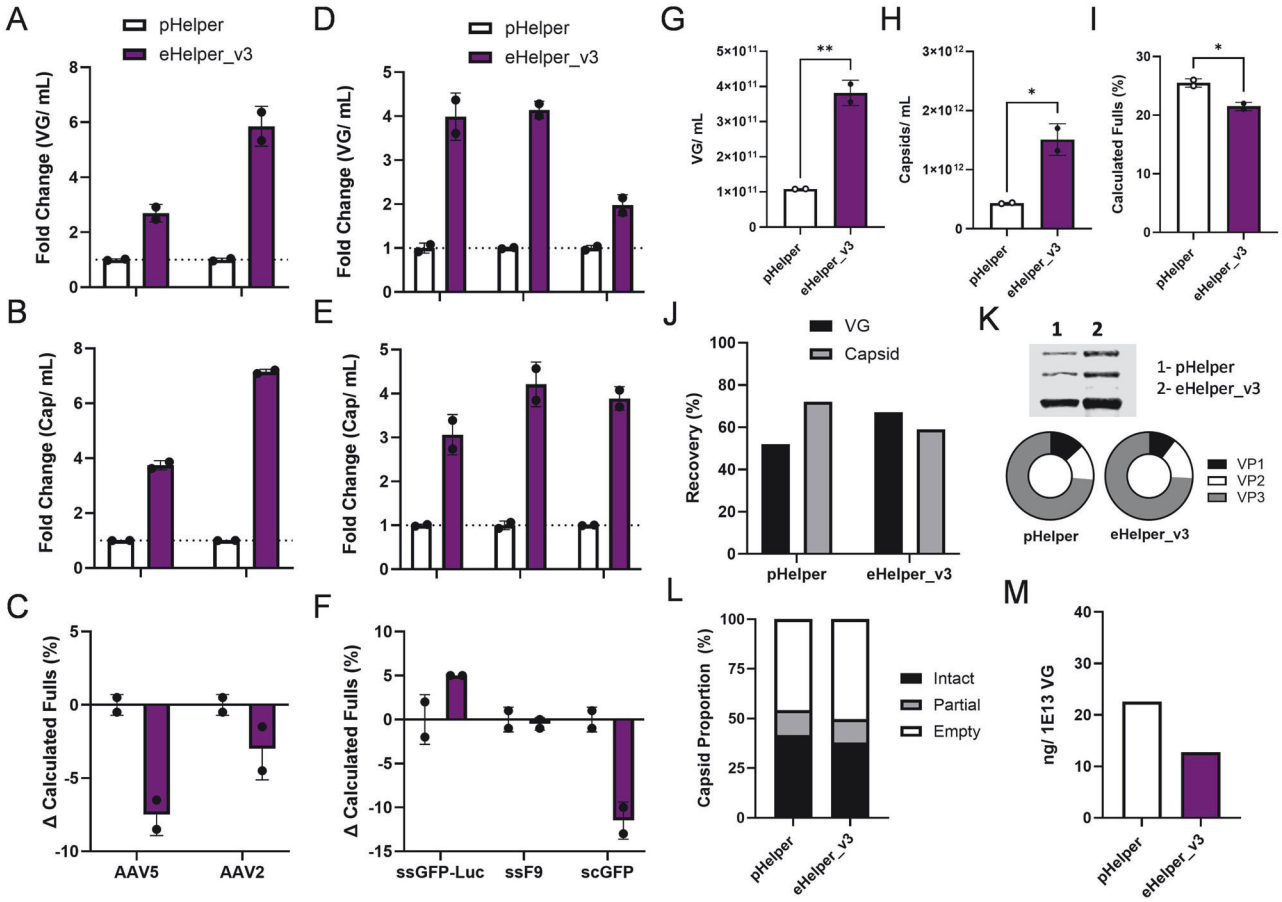


**Fig. 6 eHelper_v3 performance with additional serotypes, triple transfection and preliminary vector quality comparison.** OXB dual plasmid transfection using the ssGFP-Luc genome bearing AAV2 or AAV5 capsid sequences produced crude lysate **A**, **D** VG titer, **B**, **E** capsid titer and **C**, **F** calculated full vector percentage normalized to the average pHelper values for each condition produced at 2E6 cells/ mL transfection density. Triple transfection of the ssGFP-Luc, ssF9 and scGFP genomes and an AAV9 RepCap plasmid produced crude lysate **G** VG titer, **H** capsid titer, **I** calculated full vector percentage produced at 4E6 cells/mL transfection density in 250 mL working volume in 500 mL shake flasks. **J** Total downstream recovery of VGs and capsids of crude lysate following affinity purification. Affinity purified material was analyzed to compare **K** viral protein (VP) ratios using total protein stain, **L** capsid packaging profile using AUC and **M** residual hcDNA using qPCR in vectors produced with pHelper and eHelper_v3. Statistical analysis was performed using a *t*-test. Statistical significance: $*p < 0.05$, $**p < 0.01$. Data is shown as mean ± SD of biological replicates ($n = 2$), except where single data points are shown due to the pooling of upstream replicates for downstream affinity purification (**J**–**M**).

quality attributes evaluated were largely comparable and within expected ranges for affinity purified material, however this was a preliminary proof of concept experiment that warrants additional development prior to final conclusions regarding vector comparability using eHelper_v3.

## DISCUSSION

There has been a trend in recent years towards reducing the number of plasmids required for AAV transfection to two or even a single plasmid [9, 11, 27]. This reduces plasmid costs and has been theorized to potentially increase vector production by improving plasmid co-transfection in producer cells. There is growing consensus that the latter is unlikely to be true and data in this manuscript supports the notion that fewer plasmids does not directly translate to increased AAV production as well. The EHG condition utilized four individual helper genes plus an OXB dual plasmid, a total of five plasmids (Fig. 1E), which outperformed the two-plasmid transfection of pHelper and OXB dual plasmid by over 2-fold (Fig. 1B). However, while functional, a five-plasmid transfection is impractical for numerous reasons and our goal was ultimately to translate these EHG learnings into a single engineered helper plasmid.

The number of GFP+ cells was largely similar across all transfection conditions, regardless of the number of plasmids. However, the MFI for cells transfected with any version of engineered helper gene expression demonstrated significantly lower MFI compared to pHelper and transfection with the NHG resulted in greater MFI than pHelper (Figs. 1F and 4E). Fernandes et al. note increased MFI when helper plasmids with deletions to the native E4 gene are used and correlated that with poorer vector packaging [28]. This data also supports the link between increased MFI and lower calculated full vector produced (Fig. 1D).

Doshi et al. challenge the requirement of E4orf6 to produce AAV in adherent HEK293 cells. In our hands, there is a clear necessity for E4orf6/7 to produce practical VG titers, as exclusion results in over a 30-fold reduction (Fig. 6C) and generates no visible replication monomers (Fig. 5E), however capsid titer was only minimally impacted (Fig. 5D). Perhaps the differential requirement for E4orf6 is due to the adherent or suspension nature of the cell linages. Doshi and colleagues also evaluated constructs expressing both E4orf6 and E4orf6/7 together or E4orf6 alone and found

neither version was essential for their process [17]. Our data shows a 50% reduction in VG titer when E4orf6 is expressed alone compared to a greater than 50% increase in titer with expression of E4orf6 and orf6/7 relative to the native E4 gene expression (Fig. 1J). This is an important distinction regarding E4 gene nomenclature and the sequence present when referring to "E4orf6". For example, Yang et al. refer to inclusion of E4orf6 on the mini-pHelper-1.0 plasmid, however the sequence for the same plasmid publicly available on Addgene (plasmid # 230933) contains both E4orf6 and orf6/7 despite only annotating the former in the publication [9]. This is unlikely to be a unique circumstance and may be a common omission throughout previous literature describing E4orf6 to be aware of. On this note, Fernandes and colleagues report the intact E4 gene results in the highest vector packaging and E4orf6 alone causes a reduction [28]. They specifically state that E4orf6/7 was not included in their evaluation, and our data suggests that may explain their findings (Fig. 1L).

While a recent development, the concept that L4 22/33K are functional helper genes for AAV production has quickly become established, supported by this manuscript and a number of others [7, 8, 10, 17, 28, 29]. Despite differing conclusions on E4orf6, Doshi et al. support our findings that 22K is the major contributing factor of the L4 genes, however both 22K and 33K are required for optimal vector production [17]. The role of these proteins in the context of AAV manufacturing is still largely unknown. Nie et al. suggest that 22K plays a role in AAV transcript abundance while 33K regulates splicing of these transcripts [29]. Our data cannot separate the role of the two proteins but supports the general idea that they have some function associated with regulating AAV transcripts. Removal of 22/33K expression resulted in negligible capsid protein and an altered Rep profile with limited expression of small Rep52/ Rep40 proteins produced from the p19 promoter (Supplementary Fig. 6). However, spliced Rep68 protein was still present in the absence of 22/33K, while Rep40 was not, potentially questioning the role in all Rep transcript splicing. Furthermore, there are no clear differences in Rep or capsid protein profiles for plasmids with 22K only (EHG, eHelper_v1) or both 22/33K (EHG + 33K, eHelper_v2; Supplementary Fig. 4). Notably, genome replication occurs in the absence of 22/33K, while capsid production and overall VG yield are markedly reduced, suggesting these proteins are not required for genome replication but contribute to capsid expression regulation, perhaps via splicing mechanisms (Fig. 5C–E).

We demonstrated that the L4 promoter included in pHelper results in weaker 22/33K transcript production relative to the other adenovirus native promoter protein products (Fig. 4J). Engineering 22/33K expression resulted in a massive increase in both transcripts, associated with significant VG titer increases. eHelper_v3 outperformed eHelper_v2 in terms of packaging efficiency, with similar calculated full vectors to pHelper (Fig. 4D). The two plasmids are identical with the exception of 22/33K expression mediation. eHelper_v3 yields stronger 33K expression than 22K, but the IRES in eHelper_v2 produces more 22K (Fig. 4L, Supplementary Fig. 5). This suggests that the magnitude of 22/33K expression was a factor in improved AAV production, but the ratio of these proteins may be key to vector quality.

AAV was initially discovered alongside adenovirus, explaining why it is the most thoroughly studied helper virus for AAV, however, the question remains is it the most synergistic helper for AAV? Other large DNA viruses, such as herpesvirus and baculovirus, can serve as helpers as well [5]. Providing supplemental viral proteins in addition to the standard adenovirus genes is another strategy already employed. Polyplus produced a commercially available plasmid expressing the bocavirus NS2 and herpesvirus UL12 genes, and others have supplemented various human papillomavirus genes with success [30, 31]. While extensive evaluation of non-adenovirus helpers and supplemental helper genes are eventualities, optimization of the known adenoviral helper genes should not be overlooked. After all, it has been less than three years since 22/33K were established as essential helper genes after decades of related study. This data provides strong evidence that modulation of DBP, 22/33K, E4orf6/7 and VA RNA expression profiles at the transcript and protein level can directly lead to higher vector yields. This approach warrants further refinement and optimization to finely calibrate the quantities and proportions of each protein and RNA needed to extract maximum output, but this proof of concept showed potential to increase VG titers 5-fold. The days of relying on poorly characterized and crudely extracted sections of an adenovirus clone to provide helper functions for AAV manufacturing are quickly becoming a thing of the past, as we usher in a new era of highly tailored and innovative helper development.

## DATA AVAILABILITY

The data used to prepare this manuscript is available upon request to the corresponding author.

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## AUTHOR CONTRIBUTIONS

LvL conceptualized and designed experiments with input from KCG, DL, AZ, SO, AA, DS, DG and II. Experiments were completed by KCG, DL, AZ, SO, AA and DS. LvL drafted the manuscript with support from KCG, DL and AZ. DG and II provided supervision and manuscript review.

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## ADDITIONAL INFORMATION

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**Correspondence** and requests for materials should be addressed to Laura van Lieshout.

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