

A Robust System for Production of Superabundant VP1 Recombinant AAV Vectors

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Recombinant adeno-associated viral (rAAV) vectors have been widely used in human gene therapy. One major impediment to its broad application is the inability to produce high-quality vectors in mass quantity. Here, an efficient and scalable suspension cell culture system for the production of rAAV vectors is described. In this system, the AAV *trans* factors, Rep78, Rep52, VP1, VP2, and VP3, were stably integrated into a single vaccinia virus carrier by maximizing the use of alternative codons between genes with identical amino acids, and the *cis* rAAV genome was carried by an E1/E3 gene-deleted adenovirus. Infection of improved, E1 integrated, suspension-cultured cells with these two viral vectors resulted in the robust production of rAAV vectors. The newly enhanced system can consistently produce $\sim 1 \times 10^{15}$ genome containing rAAV vectors per liter of suspension cells. Moreover, the capsid composition of rAAV vectors produced by this system is markedly different from those produced using the traditional system in that the VP1 protein is more abundant than the VP2 protein (19:1 versus 1:1). The unique VP1 superabundant rAAV vectors produced in this new system exhibited improved transduction *in vivo* after intravitreal injection.

INTRODUCTION

Recombination adeno-associated viral (rAAV) vectors have been widely used as gene-delivery vehicles for both basic scientific research and human gene therapy.^{1–3} They have been successfully used in clinical trials and are approved as a drug for human use.^{4–6} Although a variety of systems have been proposed to produce rAAV vectors,^{7–13} large-scale manufacturing of high-quality rAAV vectors that meet the needs of basic research in large animal models and clinical uses remains a major challenge.¹⁴

The common method used for producing rAAV requires co-transfection of adherent HEK293 cells with two or three plasmids carrying the necessary *cis* and *trans* components.^{8,14–16} The two inverted terminal repeats (ITRs) are the only *cis* elements required for rAAV replication and packaging. Although four non-structural proteins are encoded in the AAV genome, Rep78, Rep68, Rep52, and Rep40, only Rep78 and

Rep52 are required as *trans* elements for rAAV production.¹⁷ The capsid proteins VP1, VP2, and VP3, are essential for AAV capsid assembly and are therefore required *trans* elements. Besides coding VP1, VP2, and VP3, the *cap* gene also encodes an assembly-activating protein (AAP), which may further support AAV assembly.¹⁸ The AAV helper viruses, such as adenovirus (Ad), and herpes simplex virus (HSV), provide the additional *trans*-acting factors required for rAAV production.⁷ The rAAV vectors produced by the traditional transient transfection have been validated in clinical trial for their safety, convenience, and effectiveness. However, the method itself has limited scalability potential.⁹

As an alternative to the transfection method, both baculovirus and HSV-based carriers have been developed to produce rAAV vectors using a suspension culture system.^{10–13} These viral-based systems have been reported to generate $0.5 \sim 1 \times 10^5$ vector genomes (vg)/cell of rAAV vector, and when scaled-up to a 100–200 L volume, can produce 1×10^{16} vg of rAAV vector.^{14,19} However, there remains concern regarding the potency of the vectors produced, the possibility of generating replication competent AAV (rcAAV) particles, and the difficulties associated with engineering the carrier viruses, considering their limited use. Recently, a novel rAAV production method using a vaccinia carrier from our group has been described, which can eliminate the generation of rcAAV particles while retaining similar scalability.²⁰ However, this method requires the use of two vaccinia viral (VV) vectors, an Ad-AAV hybrid vector (Ad/AAV), and a wild-type Ad (wtAd), making this system rather complicated.

Traditionally, the AAV capsid is comprised of 60 subunits of VP1, VP2, and VP3 capsid proteins in a ratio of $\sim 1:1:8$. To control the protein ratio, wild-type AAV relies on the use of overlapping, and alternative mRNA splicing combined with the use of a non-canonical start

Received 30 October 2017; accepted 1 November 2017;
<https://doi.org/10.1016/j.omtm.2017.11.002>.

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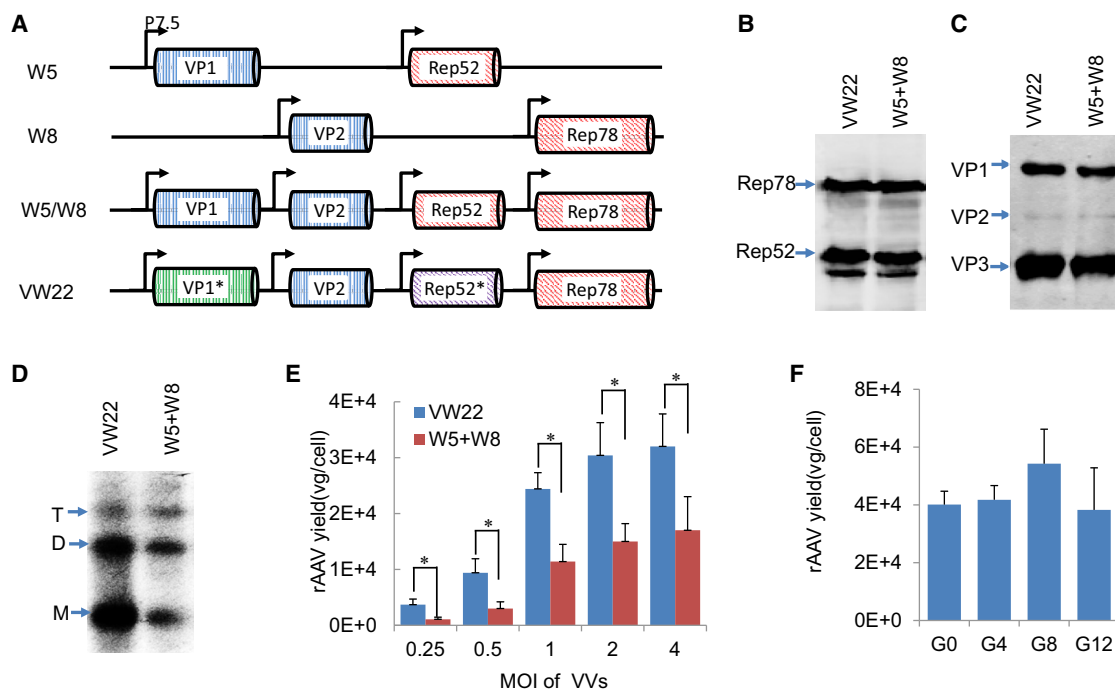


Figure 1. Constructing a Stable Single Vaccinia Carrier for rAAV Production

(A) Schematic illustration of vaccinia carriers. W5 and W8 carry AAV *trans* factors. The hypothetical W5/W8 carrier shows the long direct repeat sequences of *Rep52-Rep78* and *VP2-VP1* sequences that would have destabilized the vaccinia carrier. VW22 contains *Rep52**, the codon-optimized variant of *Rep52*, and *VP1**, the codon-optimized variant of *VP1*. The arrow stands for p7.5 promoter. (B and C) Immunoblot analysis of Rep (B) and Cap (C) proteins from lysates that were harvested at 24 hr post-VV infection. Mouse anti-Rep and mouse anti-VP1, VP2, and VP3 were used as detecting antibodies. (D) Southern blot analysis of rAAV vector genome replication in vaccinia-Ad-mediated rAAV production system. The Hirt DNA was extracted at 24 hr post-VW22-infection, and the rAAV vector genomes were detected with EGFP-specific probe. The bands corresponding to the linear monomer (M), dimer (D), and tetramer (T) of rAAV genomes are indicated. (E) rAAV vector yield comparison between VW22 and W5 + W8. rAAV vectors were harvested 36 hr post vaccinia carrier infection. The rAAV vector yields were measured by qPCR. (F) Test of the stability of VW22. VW22 were consecutively passed 12 times in BSC-1 cells and tested for the ability to complement rAAV packaging. The x axis indicated the passages of VW22 used for rAAV package. The y axis showed the vector yield. For the experiments in (B)–(F), HeLa-S3 cells were infected with wtAd (MOI = 1) and Ad/AAV-CMV-EGFP (MOI = 50), and VVs were introduced 16 hr later. **p* < 0.01. Bars represent the means of three independent experiments.

codon (ACG) for VP2 translation. This control mechanism is preserved in the transfection method for rAAV production.^{8,15} However, rAAV vectors with an untraditional ratio of capsid proteins have been reported.¹⁰ In fact, the presence of VP1 or VP2 is not required for the assembly of intact capsids.²¹ For example, rAAV5 produced in Sf9 cells only has one copy of VP1 per capsid,¹³ and rAAV capsids lacking VP2 can be generated by mutating the VP2 start codon.^{21,22} However, the absence of VP1 has a high impact on rAAV transduction, while deletion of VP2 does not change the rAAV transduction profile. The VP1-specific protein sequence motifs in the N-terminal region are essential for virus trafficking and cell-specific transduction.^{23,24} It is well documented that insufficient VP1 levels will result in reduced rAAV transduction rates,^{10,13,23–26} and recovery of VP1 expression boosts rAAV5 per-particle infectivity rates.^{13,26}

In the present study, a novel VV-Ad system was developed that efficiently produces VP1-superabundant rAAV vectors. This system uses a single VV vector to provide the necessary Rep and Cap proteins and a single Ad/AAV carrying the rAAV genome. Results demonstrated that approximately 2×10^5 vg/cell or 1×10^{15} vg/L

of rAAV vector can be produced by simply infecting the *E1a/E1b*-expressing QW158-7 suspension cells with these two viral vectors. The resulting rAAV vectors carried more VP1 and less VP2 compared with traditional rAAV vectors and displayed higher transduction efficiency after intravitreal injection.

RESULTS

Generation of a Single VV Carrier That Stably Expresses All AAV Packaging Genes

In the previously described VV-Ad system for rAAV production,²⁰ *Rep78* and *Rep52*, and *VP1* and *VP2*, were allocated into two different constructs (Figure 1A; W5 and W8), because the identical nucleotides at their 3' ends destabilized the VV carrier. Therefore, one VV carrier expressing all four genes was not generated (Figure 1A; W5/W8). Here, a novel strategy was employed that allowed the formation of a single, stable VV carrier, capable of expressing all four genes simultaneously. Through maximization of alternative coding, novel *Rep52* (*Rep52**) and *VP1* (*VP1**) genes were designed to express the *Rep52* and *VP1* proteins (Figures S1 and S2). Although *Rep52* and *Rep52** express identical polypeptides, the homology of *Rep52* and

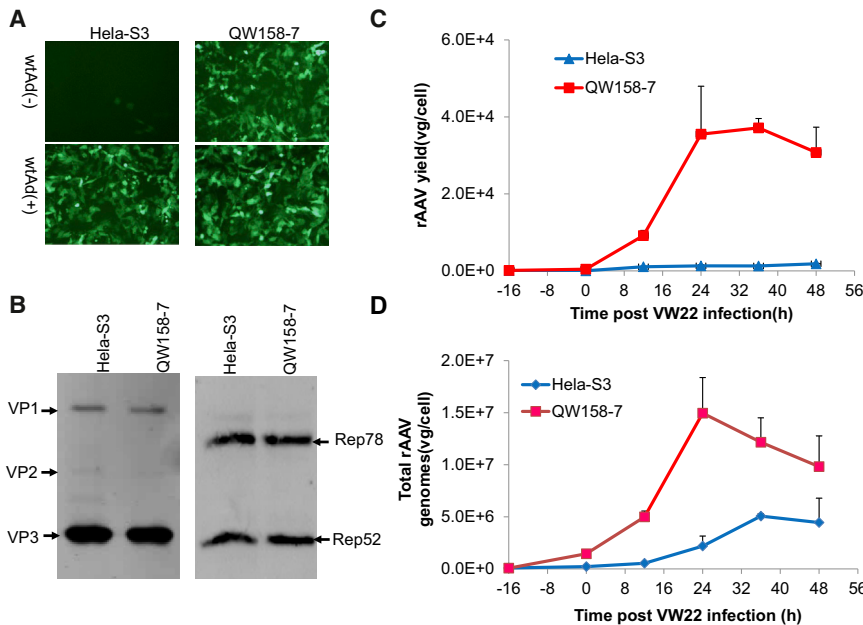


Figure 2. Establishing an Optimized Suspension Cell Line for rAAV Vector Production

HeLa-S3 cells were co-transfected with pCMV-E1a/E1b and pCI-neo (100:1), and the best cell line supporting rAAV production (QW158-7) was selected, identified, and used for rAAV packaging. (A) Adherent cultured QW158-7 cells complemented VW22 for rAAV vector production in the absence of wtAd. Both HeLa-S3 and QW158-7 cells were tested for rAAV vector packaging with or without wtAd infection. The rAAV vectors with EGFP as reporter gene were collected and assayed at 36 hr post-VW22-infection. After three rounds of freezing/thaw and inactivation treatment by heating at 56°C for 1 hr, an aliquot of final vectors were used for comparison of rAAV yield using GM16095 cells. The EGFP⁺ cells were observed and photographed at 48 hr post-infection. (B) Western blot analysis of Rep and Cap proteins expressed in QW158-7 and HeLa-S3 cells. Cell lysates were harvested and analyzed 24 hr post-VW22-infection. (C) A time course of rAAV vector packaging in 100 mL suspension cultured QW158-7 cells. rAAV vectors were harvested and assayed at various time points post-VW22-infection. HeLa-S3 cells were used as control in the absence of wtAd infection. (D) Comparison of Ad/AAV replication property in QW158-7 cells and HeLa-S3 in the absence of

wtAd. In all experiments, Ad/AAV-CMV-EGFP hybrid vectors (MOI = 50) were infected into cells 16 hr before adding VW22 (MOI = 4). The titer of rAAV vector genomes and total rAAV genomes were measured by qPCR as mentioned in the [Materials and Methods](#). Bars represent the means of three independent experiments.

*Rep52** at the nucleotide level is only 61.2%. Similarly, *VP1* and *VP1** share a homology of 59.2% at the nucleotide level, despite coding for identical amino acids. *Rep52** and *VP1** exhibited similar expression profiles compared with unmodified *Rep52* and *VP1*, controlled either by cytomegalovirus (CMV) promoter or vaccinia P7.5 promoter ([Figure S3](#)). As expected, these four genes could be successfully built into a single VV construct (VW22) in which each gene was driven by a P7.5 promoter ([Figure 1A](#)).

Infection of HeLa-S3 cells with VW22 resulted in the production of Rep and Cap proteins to similar levels as HeLa-S3 cells co-infected with W5 and W8 ([Figures 1B and 1C](#)). Since VP2 is initiated by an ACG start codon, the expression of VP2 is less robust compared to VP3 in this system. When VW22 (MOI = 1) was introduced to cells 16 hr after co-infection with wtAd (MOI = 1) and Ad/AAV (MOI = 50), efficient replication of rAAV genomes from the Ad/AAV hybrid was observed ([Figure 1D](#)). The rAAV vector yield is proportional to the MOI of VW22 ([Figure 1E](#)). About 3.2×10^4 vg/cell of rAAV vector was generated when MOI was 4, approximately two-fold higher than that of W5 and W8 co-infection. VW22 is stable for at least 12 continuous replication and expansion cycles without losing its ability to complement rAAV production ([Figure 1F](#)). Taken together, all the AAV packaging genes are stably and functionally expressed from a single VV carrier.

Eliminating wtAd from the New rAAV Production System

wtAd has been demonstrated to increase rAAV packaging in HeLa-S3 cells when using the previously described VV-Ad system for rAAV production.²⁰ We compared the helper functions of wtAd and an

E1 deleted Ad, Ad375, in this system ([Figure S4A](#)). Ad375 failed to increase rAAV genome replication and was unable to improve rAAV production, indicating that the *E1a/E1b* genes, but not the E3 genes of wtAd, are primarily responsible for enhanced replication of rAAV genomes. Therefore, the *E1a/E1b* genes were introduced into HeLa-S3 cells to eliminate the using of wtAd. The resulting E1a- and E1b-expressing cell line was named QW158-7, which can be easily propagated in suspension and have similar cell morphology as the original HeLa-S3 cells. Not surprisingly, they complemented rAAV vector production quite efficiently in adherent culture conditions ([Figure 2A](#)). Results demonstrated that even in the absence of wtAd infection, over 3×10^4 vg/cell of rAAV vector can be produced using suspension-cultured QW158-7 cells when the VW22 helper virus was added at an MOI of 4 ([Figure 2C](#)). The expression profile of Cap and Rep proteins from VW22 was similar between QW158-7 and HeLa-S3 cells ([Figure 2B](#)). QW158-7 cells provide earlier and more abundant replicated rAAV genomes for AAV packaging ([Figure 2D](#)), which are generally positively correlated to rAAV yield.²⁷

Increasing MOI of the Ad/AAV hybrid from 1 to 50 significantly promoted total rAAV genome accumulation and improved rAAV yield ([Figures S5E and S5F](#)). However, a further increase of the Ad/AAV vector failed to substantially enhance rAAV production, presumably due to the increased toxicity to the host cells and interactions between the two types of viruses ([Figure S5H](#)). We have systematically evaluated whether individual Ad genes, *E1a-13s*, *E1a-12s*, *E1b-55k*, *E1b-19k*, *E2a*, *VA I RNA*, and *E4orf6*, would increase rAAV packaging or not. Each gene was expressed by a VV vector that was controlled by a P7.5 promoter. As demonstrated in [Figure S6A](#), adding *E1a-13s*,

E2a, *E4orf6* (facilitating AAV DNA replication); *E2a* and *VA I RNA* (enhancing the viral mRNA stability and efficiency of translation); *E4orf6* and *E1b-55k* (facilitating the timely transportation of viral mRNAs); and *E1b-19K* (providing additional anti-apoptotic activity) had no effect, or only a slight increase, on rAAV vector yield in the new system. These results suggested that the Ad/AAV hybrid at MOI of 50 provided sufficient replicated rAAV genomes in QW158-7 cells for vector production.

Modulating VP2/VP3 Expression Can Improve rAAV Production Yield

An increase in MOI of VW22 significantly improved rAAV vector production (Figure S5A). However, a high VW22 MOI was toxic to the host cells. It inhibited the replication of the Ad/AAV hybrid and reduced rAAV genome accumulation (Figures S5B and S5C). In an effort to further improve this system without increasing the MOI of VW22, the effect of individual AAV genes (*Rep78*, *Rep68*, *Rep52*, *Rep40*, *VP1*, *VP2*, *VP3*, *AAP*, and *X-gene*)^{28,29} on rAAV packaging was evaluated. The expression levels of VP2 and VP3 were found to be proportional to rAAV vector yield (Figures S6B and S6C). For example, enhancing VP2 expression improved vector yield by 5-fold, and the optimized expression of VP3 lead to a 3-fold increase in vector yield. In contrast, increasing VP1 expression decreased rAAV yield in the VV-Ad system. This result suggested that insufficient VP2 and VP3 might have been the primary cause of inefficient rAAV packaging in the new system.

To address the issue of inefficient VP2/VP3 expression, it was hypothesized that the universal adoption of the P7.5 promoter might lead to the unbalanced expression of AAV genes. Since there are a variety of VV promoters available with different expression profiles and potency, eight VV promoters (Leo160,³⁰ Pr4LS5E,³¹ pHyb,³² PrS5E,³¹ PrMVA13.5-long,³¹ mH5,³³ pSyn,³⁴ and ATI,³⁵ Table S1) were systematically assessed for their effect on rAAV production. To accomplish this, either individual promoter or various combinations of these promoters were used to drive the *VP2* gene (Figure 3A). As presented in Figure 3A, the VW22 carriers containing a Pr4LS5E or an mH5 promoter consistently resulted in higher rAAV yield. In fact, using these promoters resulted in the production of 3~6-fold more rAAV vectors compared with that of original VW22 carrier in adherent cultured QW158-7 cells. Moreover, the VW22 carrier with a Pr4LS5E-mH5 dual promoter (VW22-PM) led to the highest rAAV yield, 3.5×10^5 vg/cells, which is approximately an 8-fold increase over the VW22 (Figure 3A). The rAAV yield with the VW22-PM carrier in suspension QW158-7 cells produced approximately 3×10^5 vg/cell, which is about seven times higher than that of VW22 (Figures 3B and S7A). Earlier expression and accumulation of VP2/VP3 was considered as the likely reason for more efficient replication and packaging of rAAV vectors (Figures 3C and S7B). In contrast, the replication of Ad/AAV and VV in the system was not affected when the Pr4LS5E-mH5 dual promoter was used to control VP2/VP3 expression (Figures S7C and S7D). Based on this data, it was concluded that the use of a strong and early VV promoter

improved expression of VP2/VP3 and enhanced rAAV packaging in the new vector production system.

An Optimized System for Robust rAAV Production

The combination of all the discussed enhancements and optimization resulted in a new VV-Ad system for rAAV vector production using suspension cells without transfection procedures (Figure 4). This new system includes three major components: a single, cytoplasmic VV carrier (VW22-PM) providing AAV helper functions; one Ad/AAV hybrid carrier containing rAAV genomes and providing Ad helper functions; and suspension host QW158-7 cells containing the E1a/E1b functions (Figure 4, right). This is in contrast to the original VV-Ad system that required five components: two VVs (W5 and W8), two Ads (Ad/AAV hybrid and wtAd), and used HeLa-S3 as host cells (Figure 4, left).²⁰ The new system eliminates excessive procedures and cost for additional VV carrier preparation, provides a more direct method, and importantly, removes wtAd from the production system.

To further confirm that this new production system is competent for general rAAV vector production, it was used to produce the following rAAV vector-based AAV serotype 2: ssAAV-CMV-LacZ, ssAAV-CB-AAT, dsAAV-CB-EGFP, and dsAAV-CB-Gaussia luciferase (Gluc). Ad/AAV hybrids carrying the corresponding transgene cassettes and AAV ITRs were generated. The AAV genome size for these vectors ranged from 2.6 to 4.7 kb. As presented in Table 1, both single-strand rAAV vectors (ssAAV-CMV-EGFP, ssAAV-CMV-LacZ, and ssAAV-CB-AAT) and self-complementary rAAV vectors (scAAV-CB-EGFP and scAAV-CB-Gluc) were produced successfully. The yield ranged from 0.59×10^5 to 2.76×10^5 vg/cell (Table 1). Moreover, increasing the cell density as high as 5×10^6 cells/mL still allowed for a high yield of rAAV vector per cell. Under this condition, over 1×10^{15} vg rAAV vectors could be produced from 1 L of cell culture.

Since the new system uses a VV carrier and an Ad/AAV hybrid to produce rAAV vectors, the chance of residual carrier viruses in the final rAAV preparations is a safety concern. However, no residual VV or wtAd contamination could be detected using a plaque assay (the sensitive of our assay was 1 VV or wtAd out of 10^{10} AAV particles). The Ad/AAV hybrid has a peak density of 1.344 g/cm^3 , which is close to the density of empty AAV particles and is easy to inactivate by heating treatment at 56°C for 1 hr (Figures S8F and S8G). Moreover, there were less rAAV genomes detected in rAAV vectors produced by the VV-Ad system compared with that of the triple-plasmid transfection system (Figures 5B and S8E), which confirms previous results.²⁰

VP1 Superabundant rAAV Vectors Efficiently Express Transgene In Vivo

It is notable that rAAV vectors produced by the new system displayed a high abundance of VP1 (VP1 superabundant AAV). For these rAAV vectors, the ratio of VP1:VP2:VP3 is close to 1.9:0.1:8, which differs from the traditional AAV capsid with a ratio of 1:1:8

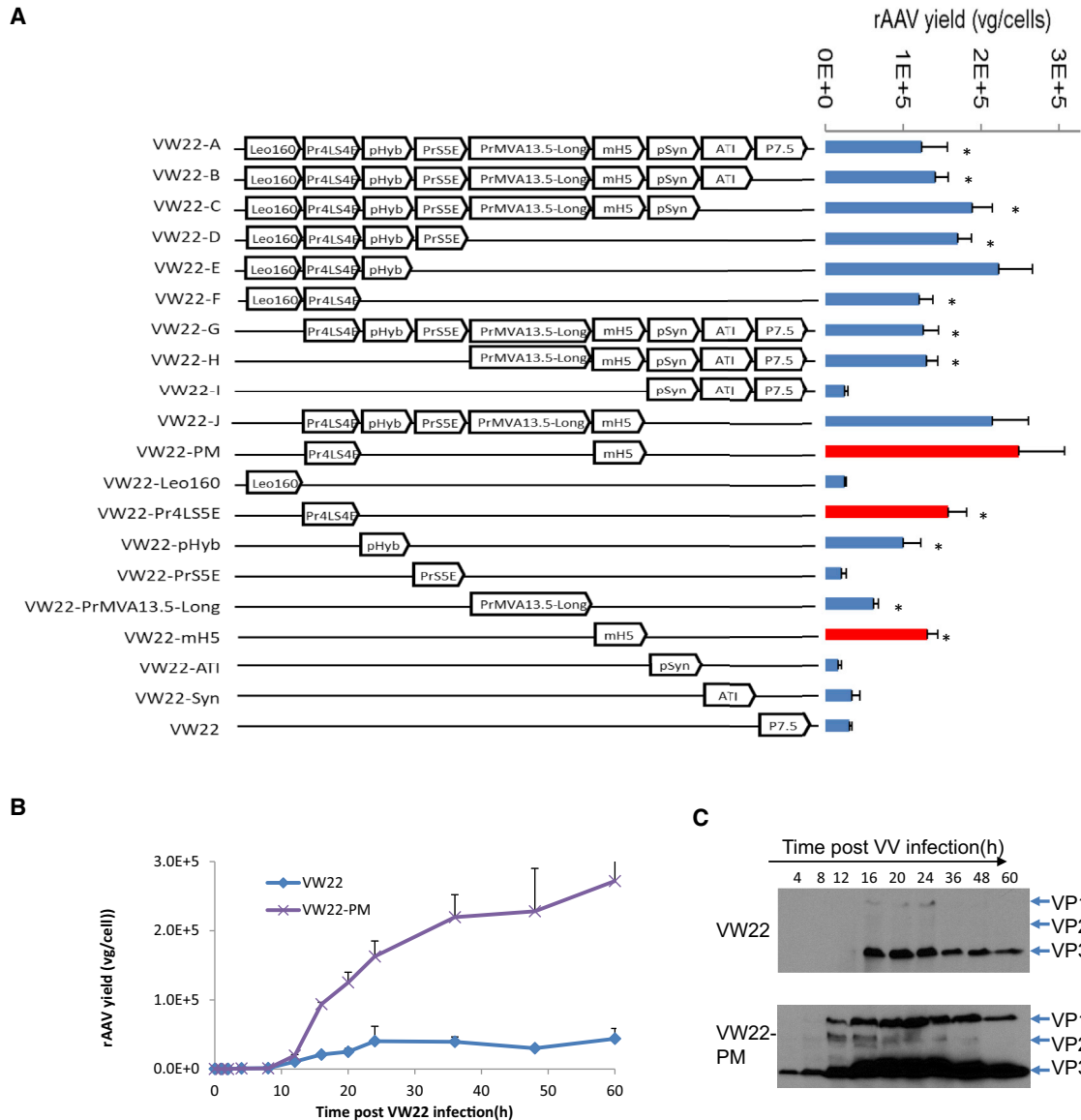


Figure 3. Regulating VP2/VP3 Expression of VV by Strong and Early Promoters Enhance rAAV Package of VV-Ad System

(A) Schematic representation of the VVs with single or combinatorial promoters (left) and their ability to produce rAAV vectors (right). The used promoters include Leo160, Pr4LS5E, pHyb, PrMVA13.5-long, PrS5E, mH5, pSyn, and ATI (Table S1). The combinatorial promoters made from different combinations of those promoters were also used to drive the expression of VP2 in VW22. The figure on the right showed the relative yield of rAAV vectors 48 hr after the specified vaccinia carrier infection (MOI = 2). (B) Time courses of rAAV yield of VV-Ad system using VW22-PM as Rep and Cap provider (with Pr4LS5E-mH5 driven VP2) in suspension QW158-7 cells. (C) Western blot analysis of Cap protein expression in VV-Ad system during VW22-PM-mediated rAAV production. Mouse anti-VP1, VP2, and VP3 were used as detecting antibodies. In all experiments, Ad/AAV-CMV-EGFP hybrid vectors (MOI = 50) were infected 16 hr before adding VW22s. The titer of rAAV vectors was measured by qPCR. $n = 4$, * $p < 0.01$.

(Figure 5A). Those VP1 superabundant rAAV vectors demonstrated similar genome status, morphology, thermal stability, and CsCl gradient buoyant density as the canonical rAAV vectors (Figures S8A–S8D). As the abundance of VP1 is critical for infectivity of rAAV,^{23,24} the transduction efficacy of VP1 superabundant rAAV vectors were compared to canonical vectors through injection into the vitreous chamber of mice to target the retina cells. At 2 weeks post-injection, it was observed that the VP1-enhanced vectors pro-

duce higher expression in retina ganglion cells and optic nerve cells than canonical rAAV vectors (Figure 5C).

DISCUSSION

In the current study, a robust and scalable manufacturing system was described that contained significant improvements over the previous VV-Ad system for rAAV production (Figure 4). In fact, all components of the system have been substantially improved and optimized.

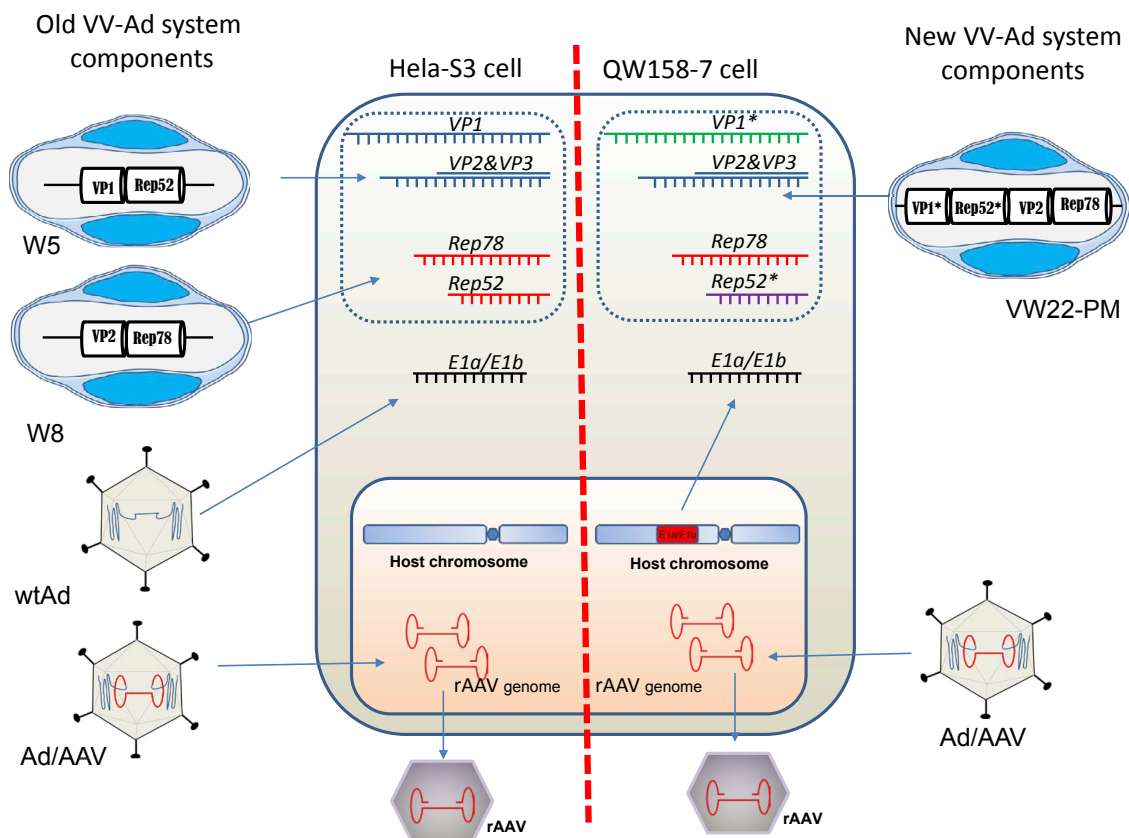


Figure 4. Schematic Comparison of the Original (Left) and New (Right) VV-Ad Systems for rAAV Vector Production

The previous system used two vaccinia vectors (W5 and W8) to provide the required Rep and Cap functions, one Ad/AAV hybrid vector to provide the rAAV genome, and one wtAd to provide E1a/E1b function in HeLa-S3 cells. In the new system, one vaccinia vector (VW22-PM) provides all Rep and Cap functions. It contains two *rep* genes (*Rep78* and *Rep52**) and two *cap* genes (*VP1** and *VP2*). *Rep52** and *VP1** genes are codon optimized from original *Rep52* and *VP1* (Figures S1 and S2). The *E1a/E1b* gene was integrated into the genome of QW158-7 cells.

A single VV carrier, VW22-PM, was engineered to consolidate all the necessary AAV *trans* factors. The wtAd was completely eliminated, and its essential functions for rAAV production have been incorporated into a HeLa-S3 derivative cell line, QW158-7 cells, which can be grown in suspension. This new rAAV vector production system performed significantly better than other rAAV production systems that have been previously described.^{9–12,17,36,37}

The main advantages of the new VV-Ad system include the following: (1) rAAV vectors can be generated in a yield greater than 2×10^5 vg/cell or greater than 1×10^{15} vg/L; (2) the scalable system can be readily implemented, since production cells can be grown in suspension and the helper carrier virus can be propagated easily; (3) it completely avoids rcAAV generation by sequestering *rep* and *cap* genes and the rAAV genomes into two separate compartments of the host cell; (4) the presence of different VV promoters (early, intermediate, late) and sequential infection of two helper viral vectors enables temporal regulation of Rep and Cap expression and genome replication, which can closely mimic wtAAV production and increase rAAV production; (5) it avoids the use of wtAd; (6)

it generated a VP1 superabundant rAAV vector, which has high *in vivo* transduction; (7) it is especially flexible for producing chimeric AAV vectors that contain a VP1 from one serotype and VP2 or VP3 from other sources.

One main obstacle of using a single VV carrier to express Rep78 and Rep52, VP1, VP2, and VP3 simultaneously is that these proteins have identical nucleotide sequences in the AAV virus. It was initially determined that viruses harboring large homologous repeats were unstable and limited their expansion for large-scale rAAV production.^{25,38} This problem was solved by maximizing the use of alternative codons for *Rep52* and *VP1* that allowed *VP1*, *Rep52*, *VP2*, and *Rep78* proteins to be expressed in a single VV without changing either the amino acid sequences or the expression profile of these proteins (Figure 1). The elimination of the extra VV carrier not only reduces the labor and cost for VV carrier preparation, but also improves the rAAV yield (Figure 1E), presumably by reducing cell cytotoxicity.²⁰ The VV carrier was able to support rAAV production without losing its effectiveness even after 12 consecutive passages. This is significantly better than systems using

Table 1. rAAV Yield of the Optimized VV-Ad System

Vector Name	Vector Size (kb)	Corresponding Ad/AAV Name	Adherent Culture			Suspension Culture		
			(vg/Cell)	vg/L	Low Cell Density (1×10^6 Cells/mL)	Modest Cell Density (2.5×10^6 Cells/mL)	High Cell Density (5×10^6 Cells/mL)	
ssAAV2-CMV-EGFP	4.0	Ad/AAV-CMV-EGFP	$2.37 \pm 0.7 \times 10^5$	$2.01 \pm 0.42 \times 10^5$	$2.01 \pm 0.42 \times 10^{14}$	$1.79 \pm 0.43 \times 10^5$	$4.48 \pm 1.08 \times 10^{14}$	$1.95 \pm 0.22 \times 10^5$
ssAAV2-CMV-lacZ	4.7	Ad/AAV-CMV-lacZ	$0.96 \pm 0.14 \times 10^5$	$0.59 \pm 0.04 \times 10^5$	$0.59 \pm 0.04 \times 10^{14}$	-	-	-
dsAAV2-CB-E GFP	2.1	Ad/dsAAV-CB-EGFP	$1.78 \pm 0.04 \times 10^5$	$1.60 \pm 0.01 \times 10^5$	$1.60 \pm 0.01 \times 10^{14}$	-	-	-
dsAAV2-CB-G luc	1.8	Ad/dsAAV-CB-Gluc	$1.54 \pm 0.27 \times 10^5$	$1.17 \pm 0.04 \times 10^5$	$1.17 \pm 0.04 \times 10^{14}$	$1.09 \pm 0.13 \times 10^5$	$2.72 \pm 0.32 \times 10^{14}$	-
ssAAV2-CB-h AAT	2.6	Ad/AAV-CB-A AAT	$4.04 \pm 0.90 \times 10^5$	$2.76 \pm 0.11 \times 10^5$	$2.76 \pm 0.11 \times 10^{14}$	-	-	$2.03 \pm 0.43 \times 10^5$

Suspension QW158-7 cells were infected with the Ad/AAV hybrid (MOI = 50) and VV22-PM (MOI = 2). The titer of rAAV vectors was measured by qPCR at 48 hr post-VV22-PM infection.

baculoviruses, which are prone for accumulating mutations and forming revertants.^{25,38} The stability of the new VV can be readily adapted for large-scale rAAV production.

For AAV Cap expression using a VV carrier, two expression cassettes are required, as the VP1 failed to produce enough VP2/VP3 (Figure S3). The weak ACG start codon was used to initiate VP2/VP3 translation, as the use of strong AUG start codon significantly decreased VP3 expression and reduced rAAV yield (data not shown). This strategy resulted in the production of much more VP1, but less VP2, compared to that of canonical rAAV vectors (Figure 5A). The varied ratio of VP1:VP2:VP3 was also reported with the use of baculoviruses to express Cap proteins.^{13,25,39} Results indicated that the high expression level of VP1 may inhibit rAAV packaging, since overexpression of VP1 significantly decreased rAAV yield (Figure S6). The VP1 capsid may have a pH-sensitive protease that can catalyze autolytic cleavage of the capsid⁴⁰ and lower the production yield. The smaller amount of VP2 may not be a critical limitation for rAAV production in this system, as additional supplementation of VP3 only also dramatically improved rAAV package efficiency (Figure S6). Consistently, a complete lack of VP2 did not affect packaging efficiency.^{21,22} As the ratio of VP1:VP2:VP3 affects rAAV production, it is important to balance the expression of three capsid proteins. In the current study, it was demonstrated that VP2/VP3 expression could be improved by using a vaccinia promoter with either stronger overall activity or with higher early activity (Figure 3).^{31,33} Further mimic wtAAV generation using different VV promoters (early, intermediate, and late; strong and weak) may continuously improve rAAV yields in this system.

The AAP protein was reported to function in assembly of the AAV capsid for some AAV serotypes.⁴¹⁻⁴⁵ AAP uses a non-conventional CTG start codon, located between the ACG start codon of VP2 and ATG start codon of VP3. In the current study, the start codon CTG of AAP at 2729 was changed to CCG in the codon-optimized VP1. Thus, there should be no AAP expressed from codon-optimized VP1. Although the open reading frame (ORF) of AAP is still present in VP2 gene, there should be no AAP transcripts when VP2 is expressed in the cytoplasm since there is no cellular promoter for AAP. However, the additional expression of AAP using a vaccinia vector did not lead to a significant increase in rAAV production yield (Figure S6). Nevertheless, the role of AAP in our VV-Ad system remains to be investigated further.

In the current system, the new suspension base cell line, QW158-7, expressed the essential *E1a/E1b* genes and eliminated the use of wtAd. The complete removal of wtAd from the system greatly simplifies the downstream processing and purification procedures. Unlike the HeLa-S3 cells, QW158-7 cells greatly supported Ad/AAV hybrid replication and provided a sufficient amount of replicated rAAV genomes. VVs were replicated efficiently in QW158-7 cells and expressed abundant Rep and Cap proteins.

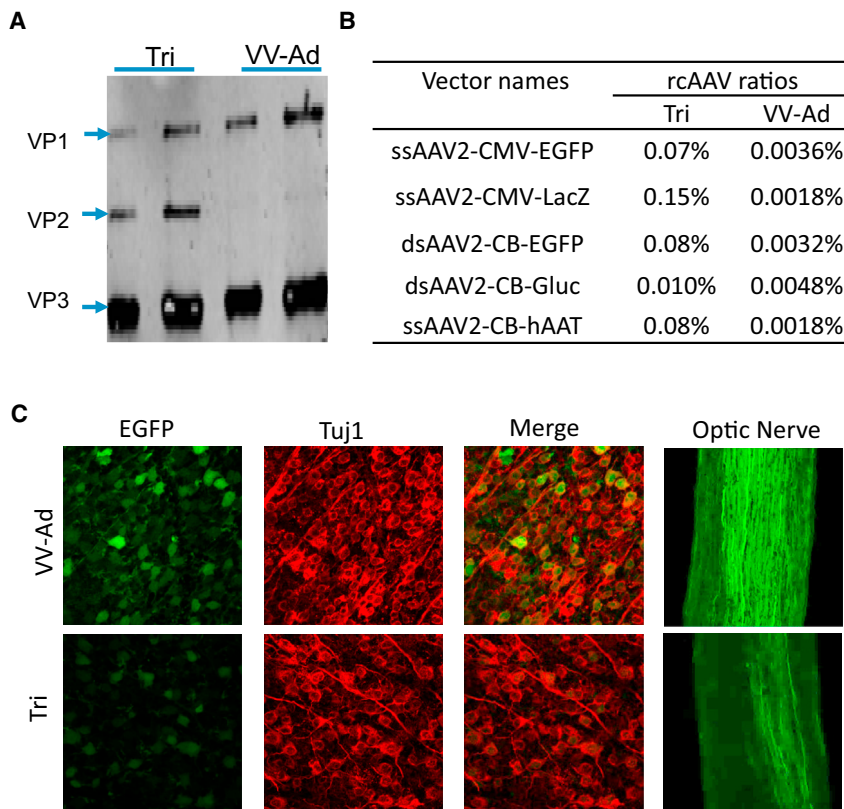


Figure 5. Comparison of rAAV Vectors Produced by the VV-Ad System and Triple Plasmid Transfection Methods

(A) Capsid composition of rAAV vectors produced by VV-Ad and triple plasmid transfection (Tri) methods. rAAV vectors ($\sim 1 \times 10^{10}$ particles) purified after two rounds of CsCl ultracentrifuge were electrophoresed and visualized by silver staining. (B) rcAAV contamination in rAAV vectors was determined by measuring the percentage of *Rep78* containing genomes in total rAAV genomes. (C) Transduction activity of dsAAV2-CB-EGFP vectors *in vivo* after intravitreal injection. Confocal images of flat-mounted retinas showing EGFP⁺ RGCs and Tuj1 immunostaining, and EGFP⁺ axons in optic nerve (n = 5, *p < 0.05).

Moreover, the additional Repts did not significantly increase rAAV yield, which indicated that the *Rep78* and *Rep52* expressed by VW22 is enough for rAAV genome rescue and replication in QW158-7 cells. The robust accumulation of replicated rAAV genomes and Cap proteins in QW158-7 cells resulted in efficient rAAV packaging (Table 1). Experiments utilizing a modestly higher cell density (5×10^6 cells/mL) conducted in a 6-L stir-tank bioreactor demonstrated a linear increase in vector production. Optimization of rAAV production for high cell density ($>1 \times 10^7$ cells/mL) should be able to further increase rAAV yield. Moreover, QW158-7 is stable up to 50 passages. All those properties should make QW158-7 an attractive cell line for large-scale rAAV production.

Taken together, a robust system for packaging high-quality rAAV vectors was presented. This novel system is transfection independent, efficient, stable, safe, and easy to scale up. The ability to produce VP1 superabundant and rcAAV-free rAAV vector makes this system optimal for producing the AAV needed for large animal studies or human clinical trials.

MATERIALS AND METHODS

Cell Lines

All cells (HEK293, HeLa-S3, GM1609, BSC-1, and QW158-7) were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS) (Sigma), and 100 U/mL of penicillin/streptomycin (Invitrogen). All cells were maintained in a humidified, 37°C incu-

bator with 5% CO₂. The suspension HeLa-S3 and QW158-7 cells were grown in DMEM media with 10% FBS and 0.1% pluronic F-68 in a 1-l Cel-stir* Spinner Flask (Wheaton Science Products, Millville, NJ).

Generation of Stable Cell Lines

To generate stable cell lines carrying the Ad *E1a/E1b* genes (QW158-7), HeLa-S3 cells were co-transfected with pCI-CMV-E1a/E1b and pCI-neo (100:1) by Lipofectamine 2000 and selected using 1,000 µg/mL G418. Single-cell-derived cell lines were then evaluated by

their abilities to produce rAAV vectors by infecting with Ad/AAV-CMV-EGFP and VW22 in 24-well plates.

Construction and Generation of Recombinant VV Vectors

The plasmid pRB21 (with P7.5 promoter) was digested with *Sma*I (NEB) and used as the backbone to construct all plasmids having individual AAV and Ad genes. *Rep68*, *Rep40*, *X-gene*, and *AAP* were generated using PCR amplified from pH22. *E1a-13S*, *E1b-55K*, *E1b-19k*, *E2a*, *E4orf6*, and *VA I RNA* were amplified using the pFΔ6 plasmid as a template. The PCR products were digested by *Eco*RV and blunt-end ligated into pRB21. pRB21-E1a-12S plasmid was generated by replacing *Bsg*I/*Bsm*I fragment in *E1a-13s* with the corresponding sequence from the *E1a-12s* gene.

*Rep52** and *VP1** (Figures S1 and S2) genes, together with p7.5 promoter and transcription stop signal, were synthesized by integrated DNA technologies (IDT) and blunt-end ligated into pRB21. To generate pRB21-W8* that contains both *VP1** and *Rep52** genes, the synthesized fragments were assembled into the pRB21. The pRB21-VW22 construct was obtained by cloning an *Xho*I-digested fragment from pRB21-W8* into the pRBW1-W5 plasmid, which was used to generate W5 vectors.²⁰ To generate pRB21-VW22 with different promoters, a DNA sequence containing all promoters was synthesized in the following order: Leo160/Pr4LS5E/pHyb/PrMVA13.5-long/PrS5E/mH5/pS/ATI (Table S1). The sequence was used to replace the p7.5 promoter, which is used to control

VP2 expression, and obtained VW22-A (Figure 3A). The VW22 constructs with individual promoters or promoter combinations were generated by deleting additional promoter sequences.

To generate VVs, BSC-1 cells were infected by vRB12 at an MOI of 0.05 for 1 hr followed by Polyjet (SignaGen) transfection of pRB21-derived plasmids. Each kind of VV was subject to three rounds of plaque purification and then propagated in BSC-1 cells. The titer of each VV was determined by counting plaques formed in BSC-1 cells after serial dilution. W5, W8, and VVs carrying *Rep78*, *Rep52*, *VP1*, *VP2*, and *VP3* have been previously.²⁰

Ads

Adenoviral vectors used in the current study are based on Ad5. Ad375 was purchased from OD260 Inc. Ad375 is an E1-deleted Ad, with the E3 region intact. All the Ad/AAV hybrids are E1/E3 gene-deleted Ads that contain transgene cassettes flanked on their 5' and 3' ends by AAV ITR vector sequences. Ad/dsAAV-CB-EGFP, Ad/dsAAV-CB-Gluc, and Ad/AAV-CB-AAT were obtained from a contract company. The Ad/AAV-CMV-EGFP hybrid vector was purchased from Penn Vector Core (Philadelphia, PA). The Ad/AAV-CMV-LacZ was previously described.²⁰ All Ad vectors used were grown in HEK293 cells and purified by CsCl ultracentrifuge.

Production of rAAV Vectors Using VV-Ad System

To produce rAAV vectors using the VV-Ad system in adherent cells, host cells were infected with Ad/AAV hybrid vectors 16 hr before VV infection. At 36 hr after VV infection, the cells were collected. To produce rAAV vectors in suspension cells, QW158-7 or HeLa-S3 cells were grown in a 1L-Flask (Wheaton Science Products, Millville, NJ). When the cell density reached 1×10^6 cells/mL, Ad/AAV hybrids were added (MOI = 50) and incubated for 16 hr. The cells were then collected and re-suspended in 100 mL sera-free DMEM media. Next, VW22 (MOI = 2) was added and incubated at 37°C for 2 hr. After that, the DMEM media was supplemented with 10% FBS and 0.1% Pluronic F-68 at a total volume of 400 mL. rAAV vectors generated by the new Ad-VV system, as well as by the triple plasmid transfection method,⁴⁶ were purified by two rounds of CsCl gradient ultracentrifugation. After extensive buffer exchange against PBS with 5% D-sorbitol, the purified vectors were incubated at 56°C for 1 hr and stored at -80°C.

Titration of rAAV Yield, rAAV, Ad/AAV, and VV Genomes Using qPCR

To titer the rAAV genomes in the culture system, 2 mL of cell culture infected with VV and Ad/AAV were removed and sonicated to lyse the cells efficiently. After sonication, 10 μ L of each sample were added into 90 μ L DNase I solution (500 μ g/mL, 5 mM CaCl₂, 5 mM MgCl₂, 50 mM Tris-HCl [pH 8.0]) and incubated at 37°C for 1 hr. DNase I was inactivated by adding 7 μ L 0.5 M EDTA (pH 8.0) followed by incubation at 70°C for 20 min. Next, 93 μ L of proteinase K solution (40 μ g/mL proteinase K, 1 M NaCl, 1% Sarkosyl) were added and incubated at 55°C for at least 2 hr. Samples were then boiled for 20 min to inactivate the proteinase K. After inactivation, samples

were serially diluted, and 10 μ L were taken to analyze using qPCR. To measure the total rAAV genomes, Ad/AAV genomes, and VV genomes, samples were treated using the same protocol but without DNase I treatment. For titration of purified rAAV, Ad, or VV vectors, 10 μ L of each vector were treated with DNase I and proteinase K. Then, the DNA samples were diluted to different ratios and used for analysis via qPCR. The qPCR was conducted using Sybr green reagents in an Eppendorf MasterCycler RealPlex.⁴⁶ Samples were denatured at 95°C for 5 min, followed by 40 cycles of amplification (95°C for 10 s, 60°C for 30 s) and terminated after generating a melting curve. A plasmid containing corresponding targeted sequence was used to make the standard curve.

Analysis of rAAV Vector Genomes Using Southern Blot

For detection of replicated rAAV genomes using a Southern blot, Hirt-extracted DNA was digested by *DpnI* overnight. For detecting rAAV genomes in the vectors, rAAV vectors ($\sim 1 \times 10^{10}$) were treated with DNase I and proteinase K, and the DNA was extracted using a GeneJET gel purification kit. All samples were electrophoresed on a 0.8% agarose gel, and a Southern blot was performed using a ³²P-labeled EGFP-specific probe.

Silver Staining Analysis of rAAV Capsid Components

The capsid composition of the rAAV vectors was determined by silver staining analysis. Briefly, rAAV vectors ($\sim 1 \times 10^{10}$ particles) were electrophoresed using a 10% SDS-PAGE gel, and the standard silver staining protocol was followed according to the manufacturer's procedures (Pierce Silver Stain Kit, Thermo Scientific, Rockford, IL).

Western Blot

Total proteins were extracted with lysis buffer, which consisted of 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 10 mM DTT, and 1 \times protein inhibitor (Roche, Indianapolis, IN). Cell lysates were resolved on 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, California). After blocking the membrane with 5% non-fat dry milk in TBST buffer, which contains 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20, the membrane was incubated with the primary antibody, anti-AAV capsid (B1, American Research Products, Belmont, MA), or anti-AAV Rep (303.9, American Research Products) at a dilution of 1:500 at 4°C overnight. The membrane was washed and incubated with a horseradish peroxidase (HRP)-conjugated sheep, anti-mouse immunoglobulin G (IgG) antibody (Sigma, St Louis, MI) at a dilution of 1:10,000. The membrane was developed using an enhanced chemiluminescent substrate (Amersham-Pharmacia Biotech, Piscataway, NJ).

TEM of rAAV Particles

For TEM analysis, formvar was coated on a microscope slide and floated onto a water bath. Cleaned 200 mesh grids were placed on this film and collected using parafilm. Five microliters of purified AAV ($\sim 1 \times 10^{13}$ vg/mL) was placed on the grid and allowed to dry, then 8 μ L of 1% phospho-tungstic acid (Electron Microscopy Science, Hatfield, PA) was applied and drawn off. After drying, the

grids were observed using a Philips Transmission Electron Microscope CM 12 (Philips, the Netherlands) with an accelerating voltage of 100 KV and imaged with a DVC detector controlled by AMT software (Danvers, MA, USA).

rAAV Transduction *In Vitro*

For each experiment, 50,000 viable GM16095 cells were seeded into a 24-well plate 24 hr before transduction. rAAV particles carrying EGFP or Gluc genes were added directly to each well. Transgene expression was examined 48 hr post-infection. EGFP expression was observed 24 hr post-infection using a fluorescent microscope. Gaussia luciferase (Gluc) expression in the medium was determined by adding 5 μ L of coelenterazine substrate (CLZ, 200 μ g/mL) in 0.1M Tris buffer (pH 7.5) containing 0.5 M NaCl using a POLARstar omega bioluminescence plate reader to measure bioluminescence (BMG Labtech, Germany). All experiments were performed in triplicate, and the results are presented as averages of the experimental data.

rAAV Transduction *In Vivo*

In vivo transduction experiments were carried out in C57BL/6 mice. All mice were housed in a specific pathogen-free environment, with a normal diet, and treated in accordance with NIH guidelines as approved by IACUC at Temple University. Mice were anesthetized by xylazine and ketamine based on their body weight (0.01 mg xylazine/g + 0.08 mg ketamine/g). For each AAV intravitreal injection, a micropipette was inserted into the peripheral retina of 3-week-old mice just behind the ora serrata and advanced into the vitreous chamber so as to avoid damage to the lens. Approximately 2 μ L of the vitreous fluid was removed before injection of 2 μ L dsAAV2-CB-EGFP (6.5×10^{12} vg/mL) into the vitreous chamber. Eye ointment containing neomycin (Akorn, Somerset, New Jersey) was applied to protect the cornea after surgery.

Immunohistochemistry with Whole-Mount Retina

Retinas were dissected out from 4% paraformaldehyde (PFA)-fixed mouse eyeballs and washed extensively in PBS before blocking in staining buffer (10% normal goat serum [NGS] and 2% Triton X-100 in PBS) for half an hour. All antibodies were diluted in the same staining buffer. Antibodies used were as follows: mouse neuronal class β -III tubulin (clone TUJ1, 1:200 dilution; Covance). Floating retinas were incubated with primary antibodies overnight at 4°C and washed three times for 30 min each with PBS. Secondary antibodies (Cy3-conjugated) were then applied (1:200; Jackson ImmunoResearch) and incubated for 1 hr at room temperature. Retinas were again washed three times for 30 min each with PBS before a coverslip was attached with Fluoromount-G (Southernbiotech).

Statistical Analyses

Two-tailed Student's *t* tests and one-way ANOVA with Bonferroni multiple comparisons post-test were performed. The differences were considered significant when *p* was < 0.05. The analysis was performed using the SPSS 11.0.

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures and one table and can be found with this article online at <https://doi.org/10.1016/j.omtm.2017.11.002>.

AUTHOR CONTRIBUTIONS

Q.W. helped with study design, performed experiments, analyzed the data, prepared figures, and wrote the manuscript. Z.W., J.Z., H.W., and Z.Z. performed experiments. L.M. and Y.H. performed animal studies. J.F. and L.L. performed TEM experiments. J.F. and Y.D. helped with manuscript writing and editing. W.X. directed the study and wrote the manuscript.

ACKNOWLEDGMENTS

This work is supported by NIH grants (R01HL080789, R01HL114152, and HL130871), the Fujian Class A technology project (JA12012), and the Natural National Science foundation of China (81271691, 81371669, 81371672). We would like to acknowledge Joseph Uknalis for taking the TEM pictures.

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OMTM, Volume 7

Supplemental Information

A Robust System for Production of Superabundant

VP1 Recombinant AAV Vectors

Qizhao Wang, Zhongren Wu, Junping Zhang, Jenni Firrman, Hongying Wei, Zhengjing Zhuang, LinShu Liu, Linqing Miao, Yang Hu, Dong Li, Yong Diao, and Weidong Xiao

Supplementary Figure2: Codon Optimization of VP1 from AAV2

'VP1' is original VP1 of AAV2

'VP1#' is optimized VP1 of AAV2

* indicates that the base remains unchanged

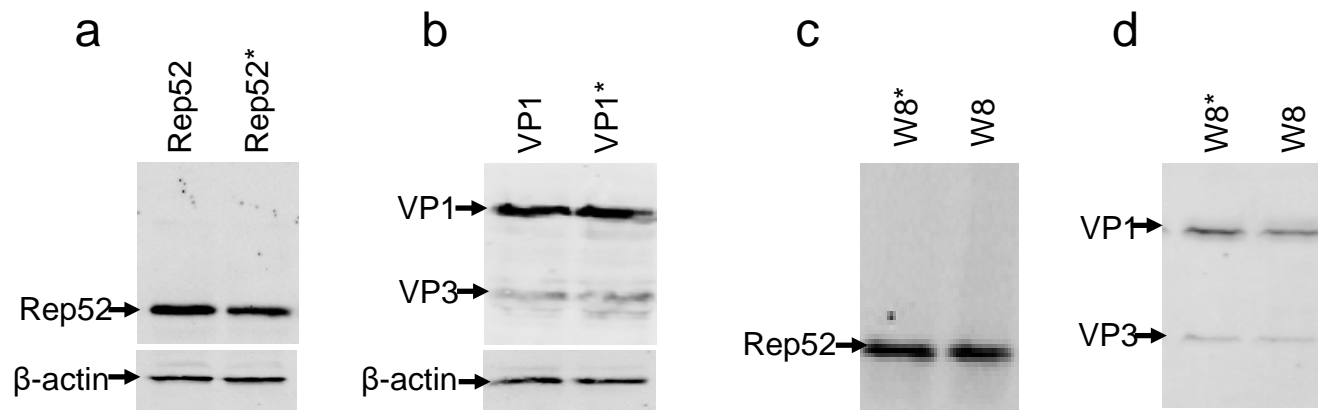
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VP1#     (1)  ATGGCCGCTGACGGCTACTTGCCCGACTGGTTGGAAGATACCTTGAGCGA
***** ** ** ** ** * ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** **
VP1      (51) AGGAATAAGACAGTGGTGGAAAGCTCAAACCTGGCCCACCACCACCAAGC
VP1#     (51) GGGCATCCGGCAATGGTGGAAATGAAAGCCGGACCTCCACCTCCCAAAC
** ** * ** ***** * ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** **
VP1      (101) CCGCAGAGCGGCATAAAGACGACAGCAGGGGTCTTGTGCTTCCTGGGTAC
VP1#     (101) CAGCCGAAGACAAAGATGATTCTCGGGCTTGGTCTTGCCGGCTAT
* ** ** * ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** **
VP1      (151) AAGTACCTCGGACCCTTCAACGGACTCGACAAGGGAGAGCCGGTCAACGA
VP1#     (151) AAATATTTGGCCCTTTTAAATGGCTTGGATAAAGCGAACCCGTGAATGA
** ** * ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** **
VP1      (201) GGCAGACGCCCGGCCCTCGAGCACGACAAAGCCTACGACCGCAGCTCG
VP1#     (201) AGCCGATGCTGCGCTTTGGAACATGATAAAGCTTATGATAGACAATTGG
** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** **
VP1      (251) ACAGCGGAGACAACCCGTACCTCAAGTACAACCACGCCGACGCGGAGTTT
VP1#     (251) ATTCTGGCGATAATCCCTATTTGAAATATAATCATGCTGATGCCGAATTC
*   ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** **
VP1      (301) CAGGAGCGCCTTAAAGAAGATACGTCTTTTGGGGCAACCTCGGACGAGC
VP1#     (301) CAAGAAAGATTGAAGGAGGACACCAGCTTCGGCGGGAAATTTGGGCAGGGC
** ** * ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** **
VP1      (351) AGTCTTCCAGGCGAAAAGAGGGTTCTTGAACCTCTGGGCCTGGTTGAGG
VP1#     (351) CGTGTTCAGCCAAAGAAACCGGTGTGAGCCCTTAGGGTTAGTGAAG
** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** **
VP1      (401) AACCTGTTAAGACGGCTCCGGGAAAAAGAGGCCGGTAGAGCACTCTCCT
VP1#     (401) AGCCCGTGAAAACCGCCCTGGCAAGAAACGCCCGTGGAACATAGCCC
* ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** **
VP1      (451) GTGGAGCCAGACTCCTCCTCGGGAACGGAAAGCGGGCAGCAGCCTGC
VP1#     (451) GTCGAACCCGATAGTAGTAGCGGCACAGGCAAGCCGGCAACAACCCGC
** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** **
VP1      (500) AAGAAAAAGATTGAATTTGGTCAAGTGGAGACGCAGACTCAGTACCTG
VP1#     (500) CCGGAAAGCGGCTCAACTTCGGCCAAACCGCGATGCCGATAGCGTGCCG
* ** * ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** **
VP1      (550) ACCCCAGCCTCTCGGACAGCCACCAGCAGCCCCCTCTGGTCTGGGAACT
VP1#     (550) ATCCTCAACCCTTGGGCCAACCACCGCCGCTCCTAGCGGCTTAGGCACC
* ** ** ** * ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** **
VP1      (600) AATACGATGGCTACAGGCAGTGGCGCACCAATGGCAGACAATAACGAGGG
VP1#     (600) AACACTATGGCCACCGGTCCGGTGCCTATGGCCGATAACAATGAAGG
** ** ***** ** ** ** ** ** ** ** ** ** **
VP1      (650) CGCCGACGGAGTGGTAATTCCTCGGAAATTGGCATTGCATTCCACAT
VP1#     (650) GGCTGATGGCGTGGCAAAGTAGCGGCAACTGGCACTGTGACAGTACCT
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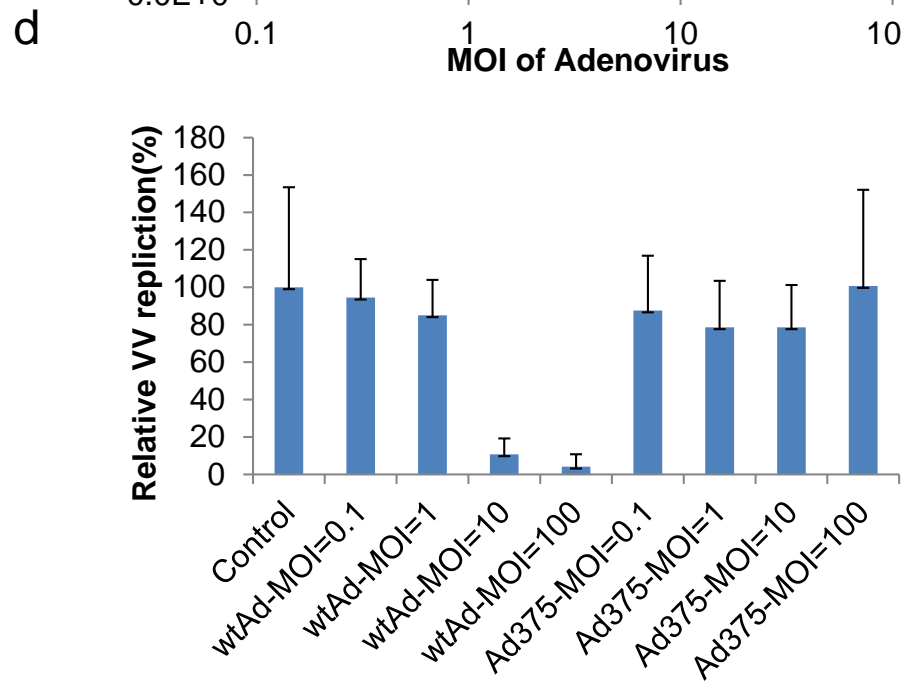
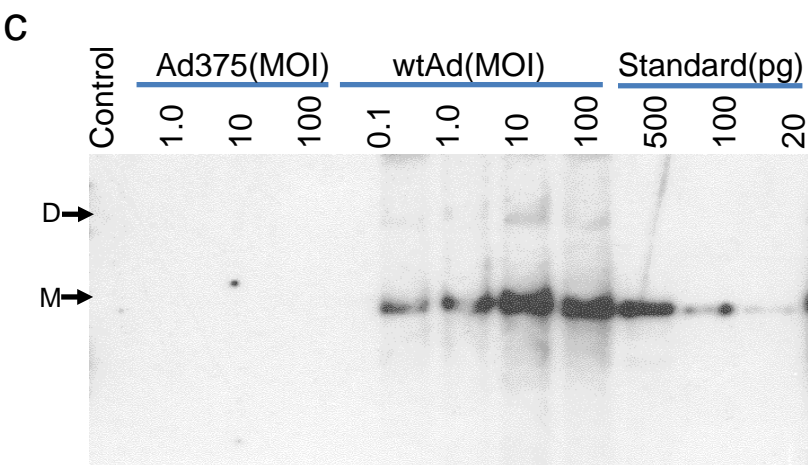
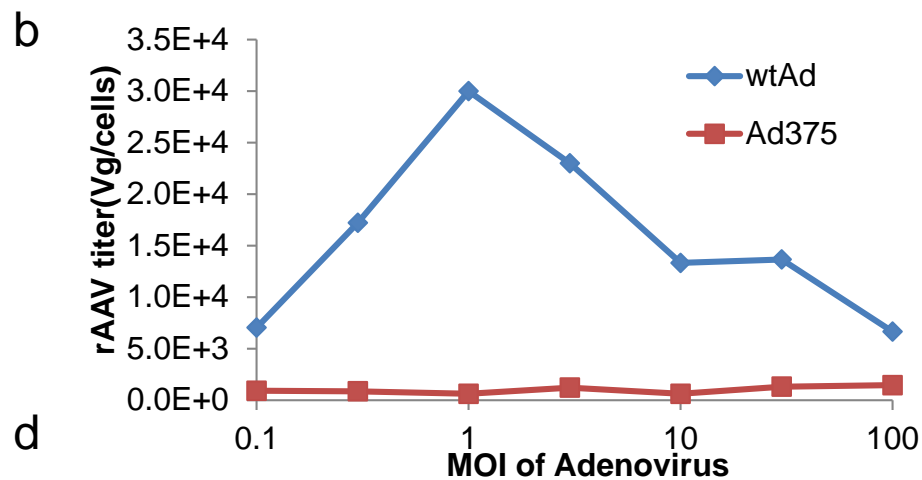
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** ** * * * * *
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VP1# (1649) CAACGTCGATATTGAGAAAGTGATGATCACCGATGAGGAAGAGATTGCGCA
** ** * * * * *
VP1 (1699) CAACCAATCCCGTGGCTACGGAGCAGTATGGTTCTGTATCTACCAACCTC
VP1# (1699) CCACAAACCTGTGCGCACCGAACAATACGGCAGCGTGAGCACAAATTTG
* ** * * * *
VP1 (1749) CAGAGAGGCAACAGACAAGCAGCTACCGCAGATGTCAACACACAAGGCGT
VP1# (1749) CAACGGGGTAAATCGGCAGGCCGCCACAGCCGACGTGAATACCCAGGGAGT
** * * * * *
VP1 (1799) TCTTCCAGGCATGGTCTGGCAGGACAGAGATGTGTACCTTCAGGGGCCCA
VP1# (1799) GTTGGCCGGGATGGTGTGGCAAGATCGGGACGTCTATTTGCAAGGCCCTA
* ** * * * *
VP1 (1849) TCTGGGCAAGATTCCACACACGGACGGACATTTTCACCCCTCTCCCCTC
VP1# (1849) TTTGGGCCAAAATCCCTCATACCGATGGCCACTTTCATCCTAGCCCTTTG
* * * * *
VP1 (1899) ATGGGTGGATTTCGACTTAAACACCCCTCCTCCACAGATTCTCATCAAGAA
VP1# (1899) ATGGGCGGCTTTGGCTTGAAGCATCCTCCGCCCAAATCTTGATTAATAA
* * * * *
VP1 (1949) CACCCCGGTACCTGCGAATCCTTCGACCACCTTCAGTGCGGCAAAAGTTTG
VP1# (1949) TACACCGTGCCCGCAAACCCAGCACAAATTTCCGCCGCCAAATTCG
** ** * * * * *
VP1 (1999) CTTCC TTCATCACACAGTACTCCACGGGACAGGT CAGCGTGGAGATCGAG
VP1# (1999) CCAGT TTCAT TACCAATA TAGTACGGCCAAGTGTCTGTGAAATTGAA
* * * * *
VP1 (2049) TGGGAGCTGCAGAAGGAAAACAGCAAACGCTGGAATCCCGAATT CAGTA
VP1# (2049) TGGGAATTACA AAAAGAGAA TTCTAAGAGATGGAA CCTGAGATCCAATA
* * * * *
VP1 (2099) CACTTCCA ACTACA AAGTCTGTTAATGTGGACTTTACTGTGGACACTA
VP1# (2099) TACAGTAA TTATAACAA AAGCGTGAACGTGCGATTTACCGTTCGATACCA
** ** * * * * *
VP1 (2149) ATGGCGTGTATTCAGAGCCTCGCCCCATTGGCACCAGATACCTGACTCGT
VP1# (2149) ACGGGGT TACAGC GAACCAGACCTATCGGGACACGGTATTTAAC CAGA
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** * **

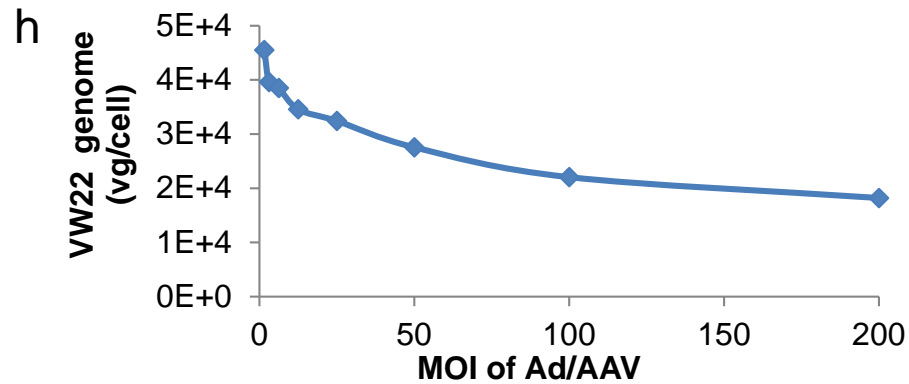
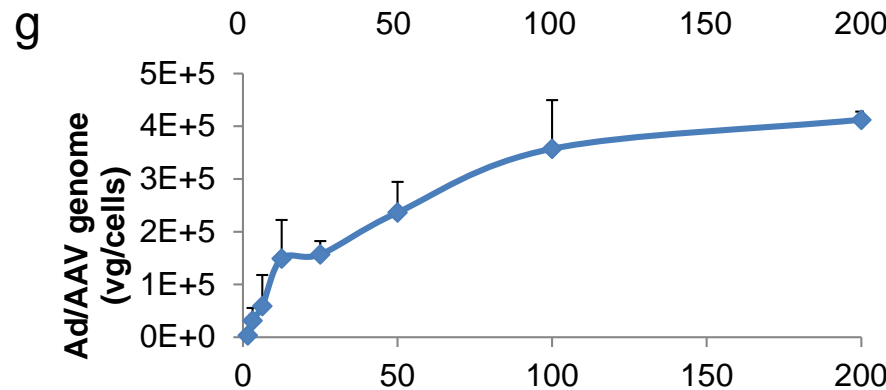
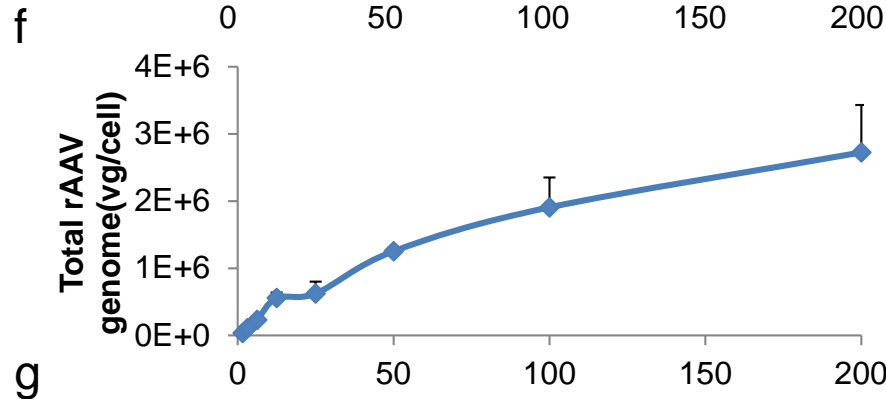
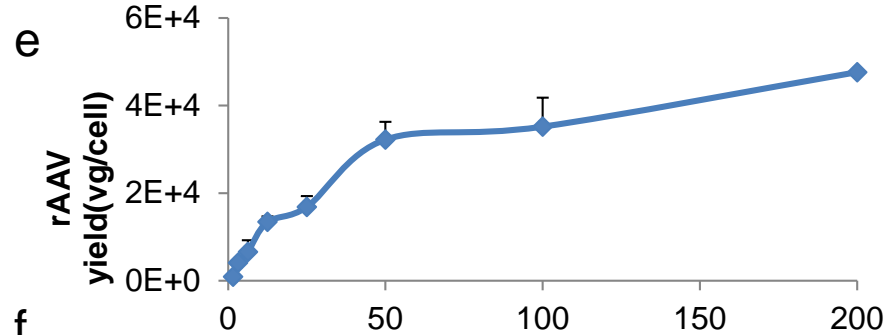
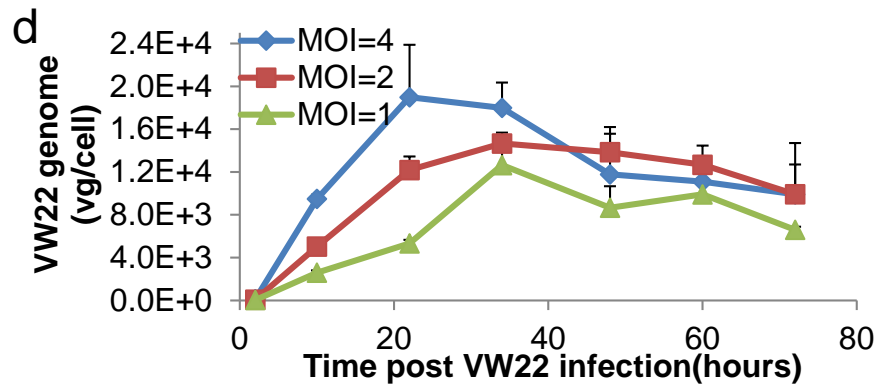
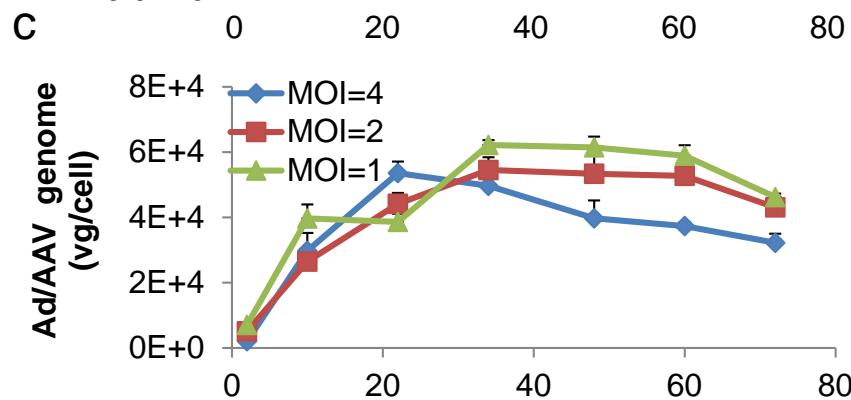
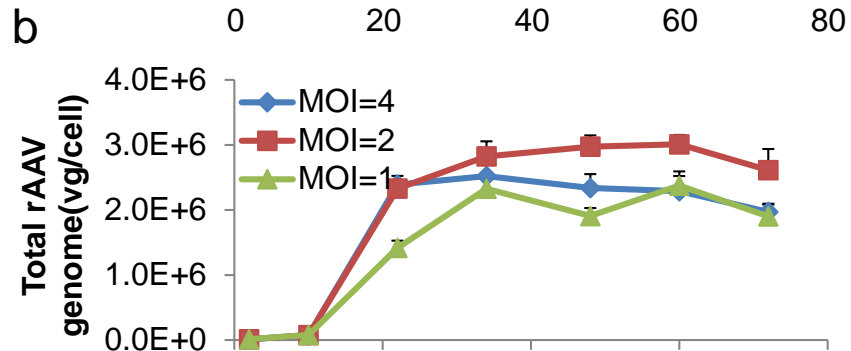
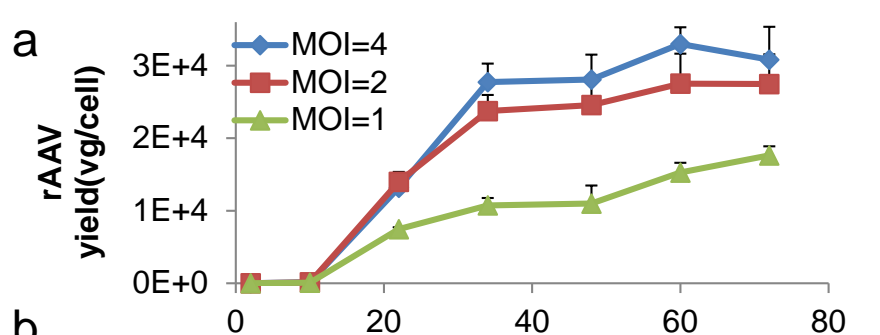
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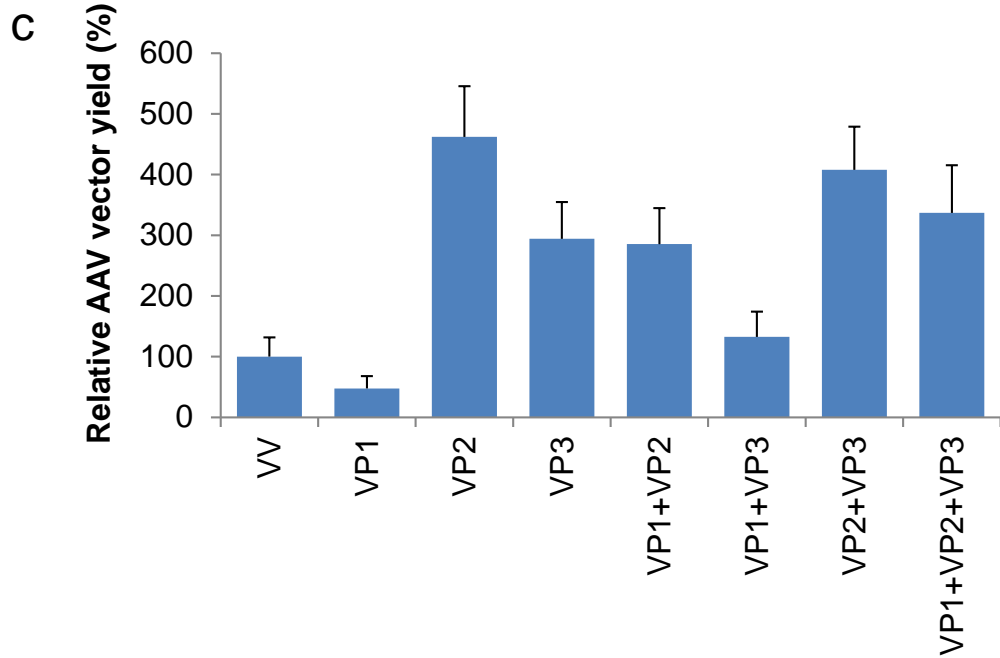
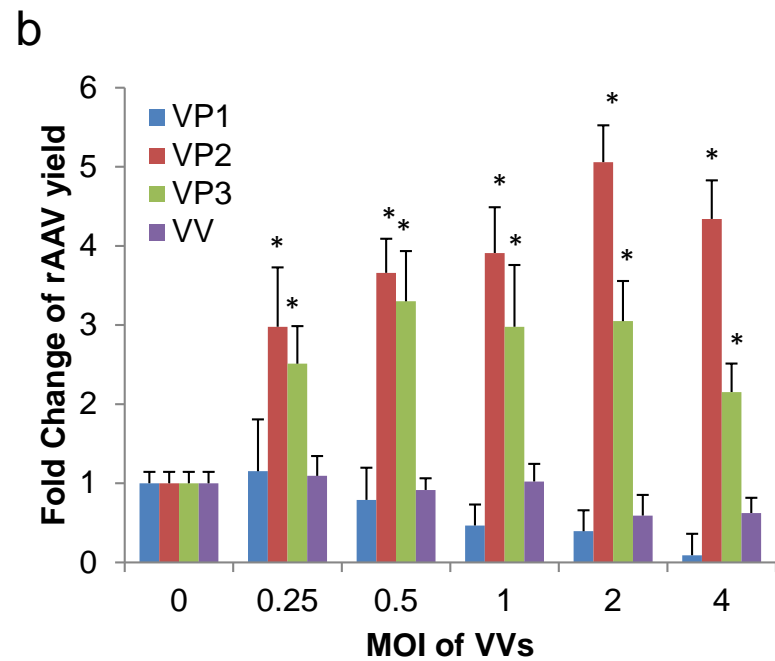
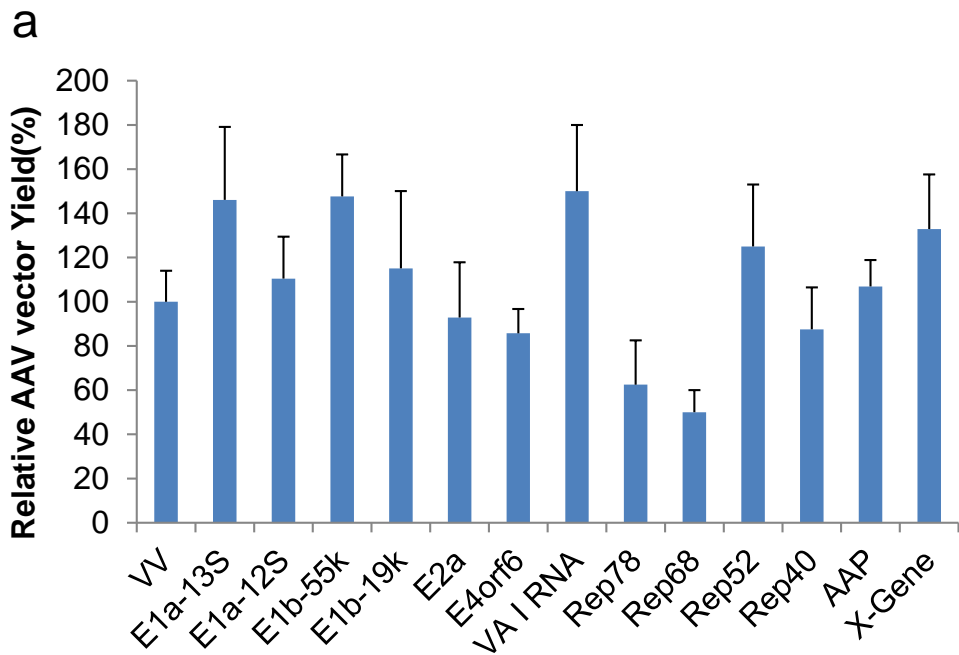
Supplementary Fig.3 Western blot analysis protein expression from codon-optimized *Rep52* and *VP1*. (a,b) HeLa-S3 cells were transfected with plasmids encoding codon-optimized *Rep52*(*Rep52**) and *VP1*(*VP1**) under control of the CMV promoter. Standard *Rep52* and *VP1* were used as controls. The protein levels of Rep52 and VP1 were detected at 48 hs post transfection. (c, d) HeLa-S3 cells were infected with W8 (Fig.1a) or W8* at MOI=1. The W8 vaccinia viral vector encodes regular *Rep52* and *VP1*, while the W8* encodes *Rep52** and *VP1**. All the genes were under control of the p7.5 promoter. The Rep and Cap proteins were analyzed 24 hs post-infection. In all experiment, Mouse anti-Rep and Mouse anti-VP1,VP2&VP3 were used as detecting antibodies.



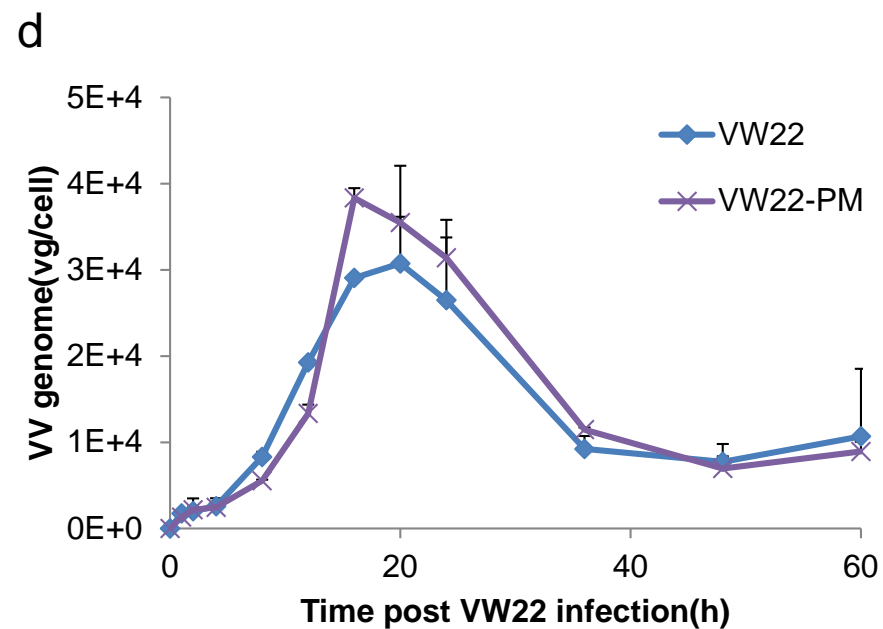
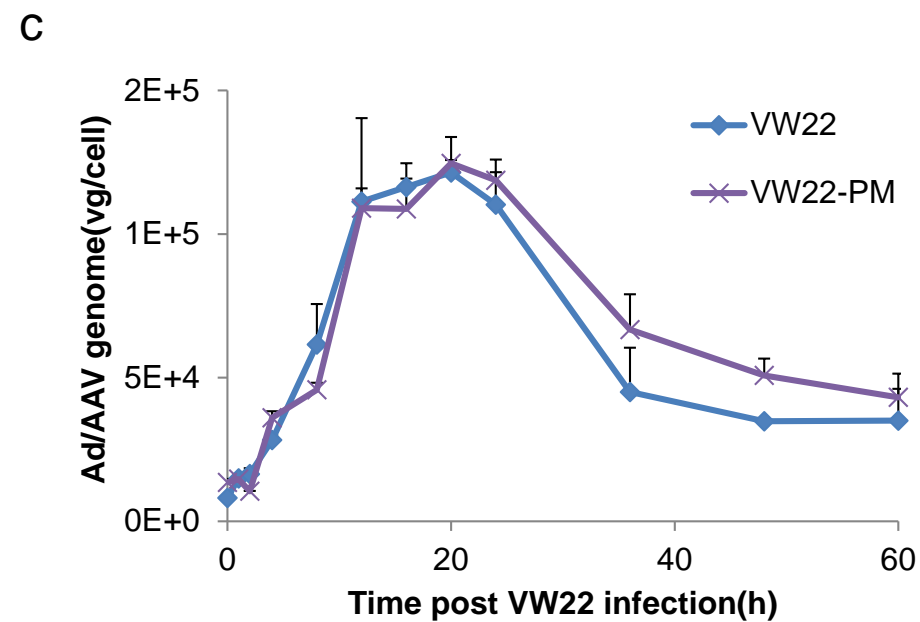
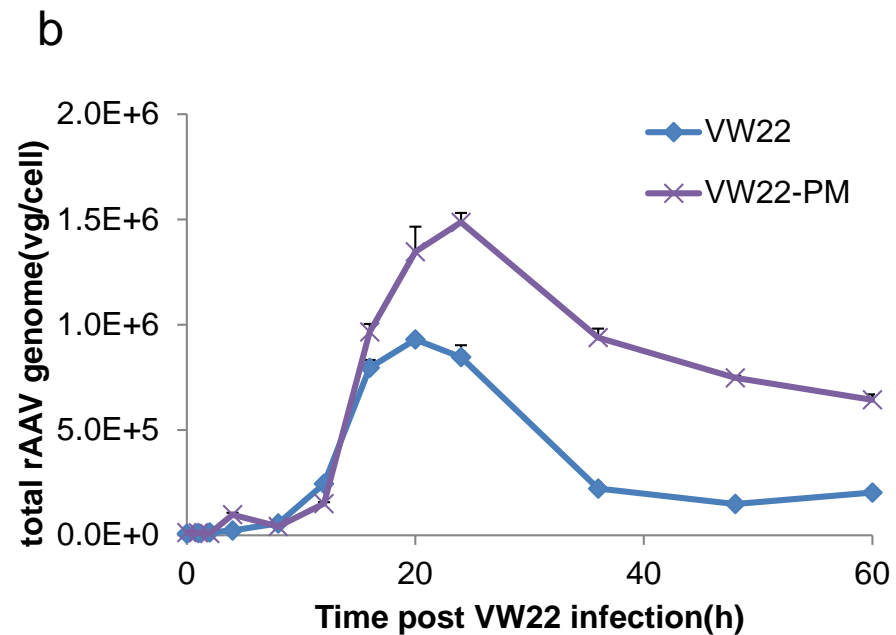
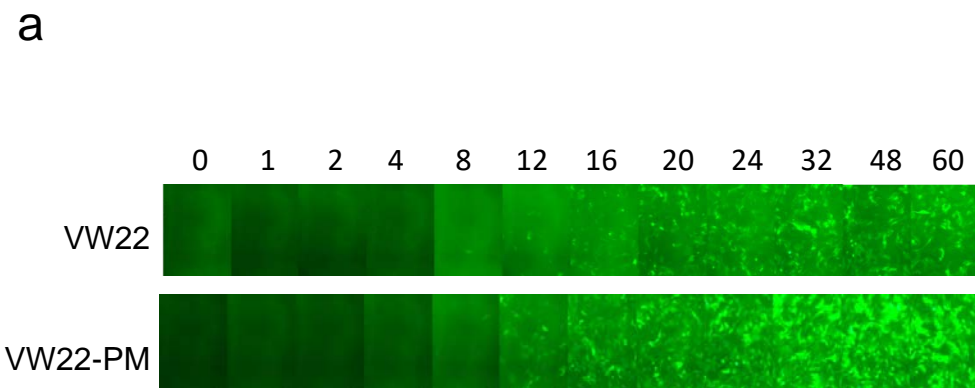
Supplementary Fig.4 Effects of wtAd and Ad375 on rAAV production, rAAV genome replication, and VV genome replication in the VV-Ad system. (a) Illustration depicting the structural differences of wtAd, the Ad/AAV hybrid, and Ad375 vectors. wtAd contains both the E1 and E3 region. For the Ad/AAV hybrid vectors, the E3 region was deleted, and the E1 region was replaced with the rAAV genome. The Ad375 adenovirus contains the E3 region, but lacks E1 region. (b) The effect of wtAd and Ad375 on rAAV packaging in Hela-S3 cells. wtAd or Ad375, at different MOIs, were co-infected with Ad/AAV-CMV-EGFP 16 hours before VW22(MOI=1) infection. The rAAV vectors in Hela-S3 cells was harvested 48 hours post VW22 infection. (c) Rescue of rAAV genomes from the Ad/AAV hybrid by VW22 in the present of varied amounts of wtAd or Ad375. Southern blot was performed using a ³²P labelled EGFP-specific probe. The bands corresponding to the linear monomer (M) and dimer (D) of rAAV genomes are indicated. (d) Replication of VW22 was effected by wtAd, but not Ad375. The control group shows VW22 replication in VV-Ad system without the addition of any wtAd or Ad375 viruses. Bars represent the means of three independent experiments.



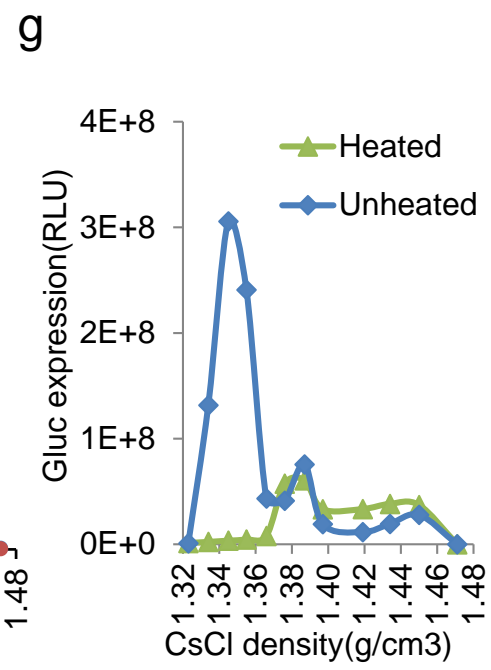
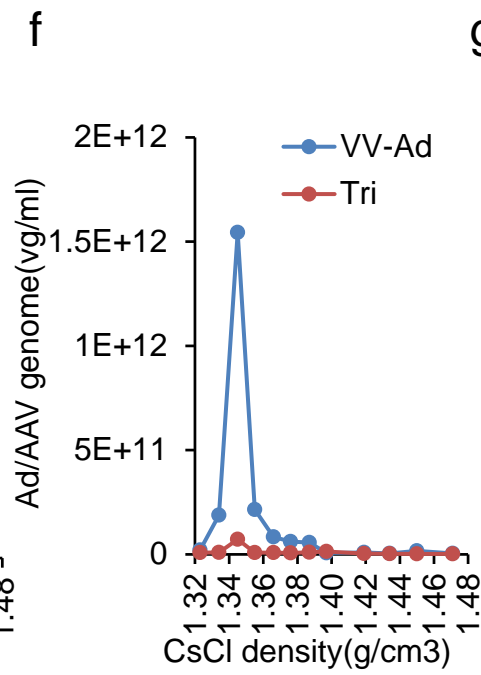
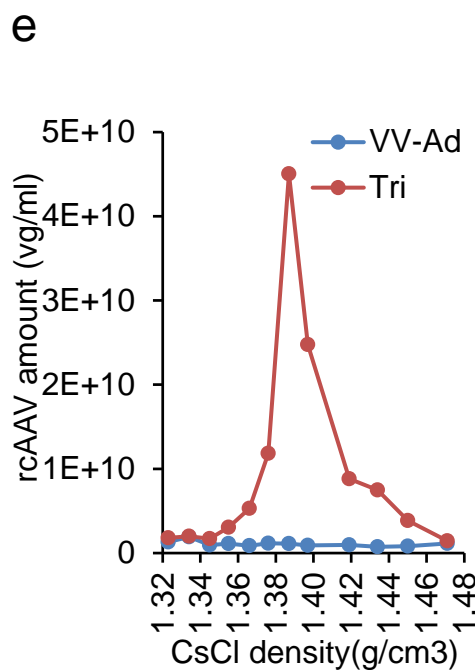
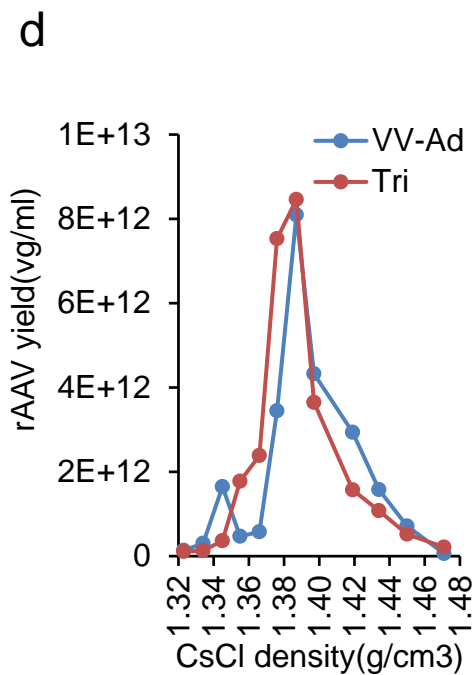
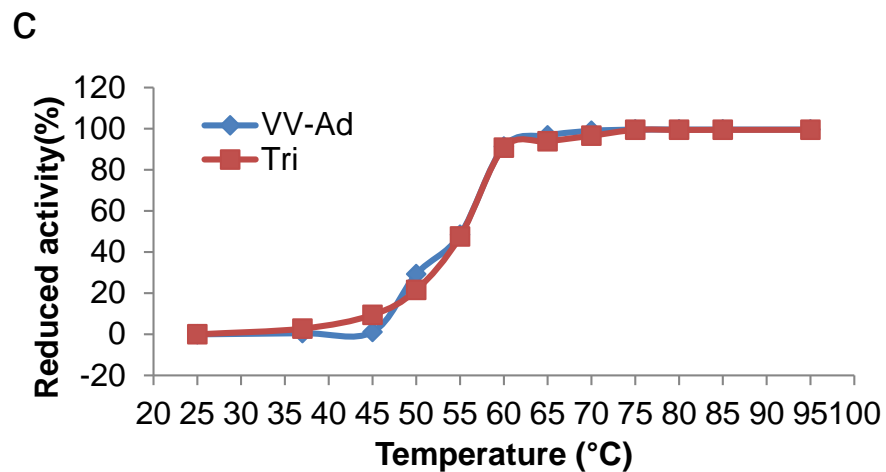
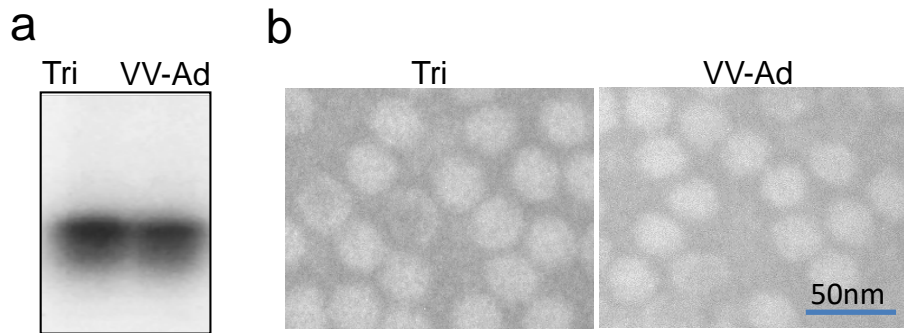
Supplementary Fig.5 Determination of the optimal multiplicity of infection (MOI) for VW22 and Ad/AAV hybrid vectors in the new VV-Ad system. The rAAV yield (a,e), total rAAV genomes(b,f), total Ad/AAV genomes(c,g), and total VW22 genomes(d,h) was determined by qPCR as mentioned in “MATERIALS AND METHODS”. (a-d) Effect of VW22 MOI in the VV-Ad system on (a) rAAV yield, (b) rAAV genome accumulation, (c) Ad/AAV replication and (d) VW22 replication. Suspension QW158-7 cells (100 ml, 2×10^6 cells/ml) were infected with Ad/AAV-CMV-EGFP (MOI=50) and VW22 (MOI=1,2,4) was introduced 16 hs later. 1 ml of cultured sample was harvested at different points post VW22 infection. The X axis present the time points. (e-h) Effect of Ad/AAV MOI in the VV-Ad system on (e) rAAV yield, (f) rAAV genome accumulation, (g) Ad/AAV replication and (h) VW22 replication. Suspension QW158-7 cells (100 ml, 2×10^6 cells/ml) were infected with Ad/AAV-CMV-EGFP at varied MOIs, and VW22 (MOI=2) was introduced 16 hs later. The X axis present the MOIs of Ad/AAV-CMV-EGFP. Samples were collected 36 hours post VW22 infection. Bars represent the means of three independent experiments.



Supplementary Fig.6 Screening of Ad and AAV genes that may contribute to rAAV vector production in the VV-Ad system. (a) Genes that failed to significantly increase rAAV production in the VV-Ad system. The Ad related genes included *E1a-13s*, *E1a-12s*, *E1b-55k*, *E1b-19k*, *E2a*, *VA I RNA*, and *E4orf6*. The AAV genes included *Rep78*, *Rep68*, *Rep 52*, *Rep40*, *AAP*, and *X gene*. All the genes were expressed from VV vectors under the control of p7.5 promoter and introduced to the system with MOI=1. The VV control is a vaccinia virus without any exogenous gene expression. (b) Dose-dependent effects of VP1, VP2 and VP3 on rAAV vector production. VVs carrying different genes (MOI=0~4) were co-introduced with VW22 (MOI=1) at 16 hs post Ad/AAV-CMV-EGFP (MOI=50) infection in QW158-7 cells. Y axis presents the fold changes compared with the VV control. (c) The combination effect of VP1, VP2 and VP3. Each VV was infected as MOI=1. In all the experiments, rAAV vectors were harvested at 36 h post VW22 infection and titrated by qPCR. Bars represent the means of three independent experiments.



Supplementary Fig.7 VW22-PM improved rAAV production in the VV-Ad system. The VW22-PM carrier a Pr4LS5E-mH5 dual promoter to drive VP2/VP3 expression, which led to earlier VP2/VP3 expression and more abundant of VP2/VP3 accumulation (Fig.3). Suspension QW158-7 cells (100 ml, 2e6 cells/ml) were infected with Ad/AAV-CMV-EGFP (MOI=50) and VW22-PM(MOI=2) or VW22 (MOI=2) was introduced 16 hs later. 1 ml of cultured sample was harvested at different points post VV infection. (a) Infectious titers of rAAV vectors produced by the VV-Ad system using either VW22 or VW22-PM. 5 μ ls of sample was exposed to freeze/thaw, and heat inactivation, then added into each well of a 24-well plate containing GM16095 cells. EGFP was observed 48 h post rAAV transduction. (b-d): Comparison of the effects of VW22-PM and VW22 on VV-Ad system. The titer of total rAAV vectors (b), Ad/AAVgenomes (c) and VV genomes (d) in Ad-VV system were determined by qPCR. Bars represent the means of four independent experiments.



- **Supplementary Fig.8: Comparison of rAAV vectors produced by the VV-Ad system (VV-Ad) and triple plasmid transfection (Tri) methods.** (a) Southern blot analysis of the genome status of purified rAAV vectors. (b) The morphology of rAAV vectors under transmission electron microscopy (TEM). (c) The thermal stability of rAAV vectors. Thermal profile (shown as reduced activity (%) versus temperature (°C)) of rAAV vectors (after 1 hour treated at different temperature) was determined by Gluc expression in GM16095 cells. (d-g) Characterization of rAAV vectors obtained from different densities of CsCl after two rounds of ultracentrifugation. (d) The distribution of rAAV vectors in CsCl gradient. rAAV genome titer in each sample was determined by qPCR. (e) rcAAV contamination in different CsCl gradient of rAAV vectors. rcAAV genomes were determined by qPCR targeting *Rep78*. (f) Determination of Ad/AAV hybrid contamination in different CsCl gradient of rAAV vectors by qPCR. (g) Inactivation of Ad/AAV hybrid vectors. “Heated” present the Gluc expression from fractions after heating at 56 °C for 1 hour. In this group, the Gluc activity is only expressed from rAAV vectors. The “Unheated” presents the Gluc expression of fractions without any treatment. In this group, Gluc activity could be either from rAAV vectors or from dsAd/AAV-CB-Gluc Ad/AAV hybrid.

Supplementary Table 1. Promoter sequences used in the paper.

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PHYB	ACGCGTGTTTAAACGTTTTGAAAATTTTTTATAATAAATATCCGGTAAAATTGAAAACTATTCT AATTTATTGCACGGTCCGGAAAAATTGAAAACTATTCTAATTTATTGCACGGTCCGGTAAAAATT GAAAACTATTCTAATTTATTGCACGGTCCGGTCCGGTAAAAATTGAAAACTATTCTAATTTATT GCACGGTCCGGTCCGGTAAAAATTGAAAACTATTCTAATTTATTGCACGGTCCGGTCCGGA
PRMVA1 3.5-LONG	TAAAAATAGAACTATAATCATATAATAGTGTAGGTTGGTAGTATTGCTCTTGTGACTAGAGACTT TAGTTAAGGTACTGTAAAAATAGAACTATAATCATATAATAGTGTAGGTTGGTAGTA
PRS5E	AAAAATTGAAATTTTATTTTTTTTTTTGGAATATAAATAAAAAATTGAAAACTATTCTAATTTATT GCACGGTCCGGTAAAAATTGAAAACTATTCTAATTTATTGCACGGTCCGGTAAAAATTGAAAA CTATTCTAATTTATTGCACGGTCCGGTAAAAATTGAAAACTATTCTAATTTATTGCACGGTCCGGT AAAAATTGAAAACTATTCTAATTTATTGCACGG
MH5	CTCGAGAAAAATTGAAAATAAATACAAAGGTTCTTGAGGGTTGTGTTAAATTGAAAGCGAGAAAT AATCATAAATAA
ATI	GTTTTGAATAAAAATTTTTTATAATAAAT
PS	AAAATTGAAATTTTATTTTTTTTTTTGGAATATAAATAGCTAGCTCGAG
P7.5	AAAAATTGAAATTTTATTTTTTTTTTTGGAATATAAAT