High Productivity HEK293 AAV Production Platform Enabled by Novel Transfection Reagents and Proprietary Plasmid Expression Systems

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Introduction: HEK293 AAV Improvement & Bottlenecks

Adeno-associated virus (AAV) remains a cornerstone of gene therapy due to its favorable safety profile, variation in tissue tropism and optionality to deliver payloads by local or systemic injections depending on serotype. However, as AAV-based gene therapy advances toward larger patient populations and broader indications, the field faces a major bottleneck: the high cost and complexity of manufacturing. Historically, these costs are largely driven by low productivity in mammalian expression systems, especially in traditional HEK293-based platforms using adherent formats and fetal bovine serum. The industry's successful transition in HEK293 AAV production from adherent to suspension, the removal of the large-T antigen and ability to produce high titer AAV products in chemically defined medium is propelling the HEK293 platform to remain a favorable choice for gene therapy clinical manufacturing. However, the costs to manufacture continues to increase with the costs of new reagents. High costs stifle patient access, discovery and development efforts and usage in larger indications including the continuation of therapeutics with promising clinical results.

To further address significant manufacturing costs, as we have mentioned in previous presentations of Tenaya's icosahedral rearrangement technology (IRT), developers are investing in dual plasmid transfection platform development (e.g. Tang et al. 2020; Lieshout et al. 2023; Velasquez et al. 2023), which possesses a clear advantage in starting material cost of plasmid manufacturing and streamlines complexity in cGMP transfection operations. Stable cell lines offer similar advantages in reduction of starting material costs per production, but offer limited flexibly needed in preclinical development, incur long development lead time and require high upfront capital investment as well as significant expertise in cell line generation to succeed.

With this background, Tenaya conducted studies in our research use HEK293 platform intended to increase productivity, while preserving safety, purity, and potency of AAV gene therapy drug product. Tenaya Process Development has successfully generated HEK293 producer cell lines, demonstrated comparable productivity to a benchmark commercial cell line and development work with proprietary transfection and expression systems. These innovations reduce raw material costs, simplify upstream operations, and enable consistent performance across batch scales and have achieved up to 6-fold increases in AAV9 titer and 4-fold increases in percent full capsid.

Figure 1.

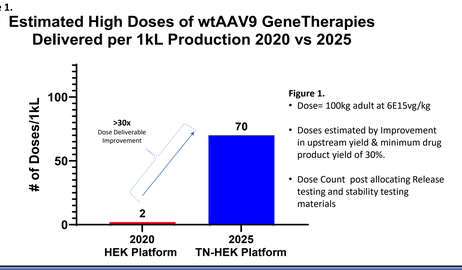
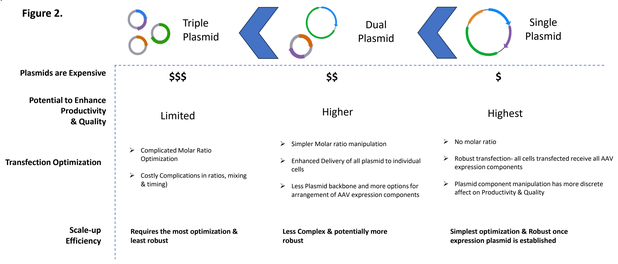


Figure 1.

- Dose= 100kg adult at 6E15vg/kg
- Doses estimated by Improvement in upstream yield & minimum drug product yield of 30%.
- Dose Count post allocating Release testing and stability testing materials

Figure 2.



Objectives

- Generate an economical HEK293 suspension AAV Production Platform with priority on in-house development of high cost upstream starting materials where possible.
- Benchmark Tenaya Clonal Produce cell line to commercial cell lines with off the shelf transfection reagents and enhancers
- Focus on additional high-impact starting materials that couple improvement to productivity and quality of our AAV: Alternative plasmid expression systems, transfection reagents, and enhancer molecules

Methods

- Clonal HEK293 suspension cell lines were first derived from ATCC CRL-1573.3 by mini-pool followed by limiting dilution. All clones adapted to suspension successfully were evaluated for AAV productivity and quality. Pre MCB of the top clones were then made for further use in the studies presented.
- AAV expression plasmids were designed *in silico* in a variety of formats including wildtype and proprietary arrangements in dual and single plasmid expression systems
- Plasmids were built in stages, when necessary, from PCR and synthesized DNA fragments and assembled by Gibson assembly
- Commercially available transfection reagents and enhancers were evaluated against top performing screens of reagents either from literature or discovered in-house. The protocols for transfection vary depending on the reagent type and enhancers used
- Characterization: VG titer by ddPCR, % full capsid by ddPCR and Capsid ELISA or Dip n' Check, Flowcytometry on NovoCyte or SH800S Cell Sorter,
- *In vivo* murine wildtype model C57BL/6 injected at 1E13vg/kg were sacrificed 3 weeks post injection, organs harvested and characterized by GFP ELISA (AbCam)

Benchmarking Commercial Transfection Reagents

The selected studies below represent significant improvements in commercially available transfection reagents and cell lines over the past several years. As the gene therapy field has matured, vendors have invested in GMP-ready, chemically defined reagents tailored for viral vector production - ideal for pipeline products, though often costly. To improve both productivity and quality we established benchmark processes with these reagents and developed complementary assays and screening methods. These include flow cytometry as a cost-effective alternative to other methods like ddPCR and Capsid ELISA for primary screening and reproducibility checks.

Figure 3.

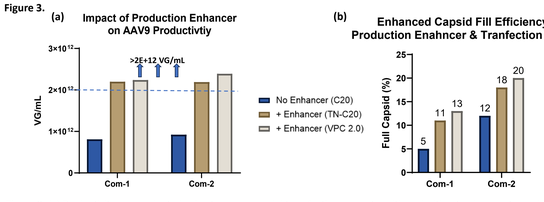


Figure 3. (a) Productivity was evaluated using two commercial transfection reagents, with and without enhancer, to package wtAAV9 with a 4.8kb genome in Tenaya's single plasmid expression system. Productions were performed in Tenaya's clonal HEK293 producer cell line (C20) and benchmarked against VPC 2.0. The addition of the enhancer resulted in significant and comparable increase in productivity across both transfection reagents and cell lines at ~1.7 and ~2x, respectively. **(b)** Further improvements in AAV quality were observed in upstream % full capsids. The combination of Com-2 with enhancer yielded the greatest improvement, reaching ~4x greater full capsids than Com-1 without enhancer. Capsid titer was determined using Dip'n'Check assay with an internal reference. Based on our correlation to AUC the predicted actual percent full capsid for the top results range from 40%-60%.

Figure 4.
Evaluation of Transfection Reagents & Enhancers by Flow Cytometry

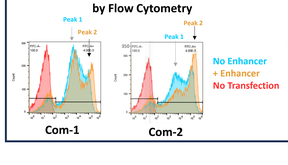
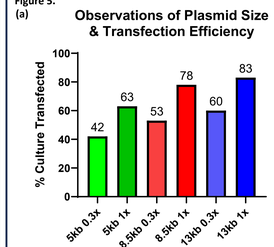


Figure 4. A GFP reporter embedded in Tenaya's proprietary single plasmid AAV expression system was used to assess transfection performance. Both commercial transfection reagents (Com-1 and Com-2) were tested with and without the addition of an enhancer (+ enhancer; orange). The enhancer significantly increased GFP magnitude, with a more pronounced effect observed with Com-2. This increase in reporter magnitude correlates with higher capsid fill rate in high-productivity AAV systems for this and other enhancers (data not shown). Reporter intensity is broadly used to benchmark the performance of Tenaya's proprietary transfection complexes and enhancers though not represented this figure or presentation.

Transfection Efficiency of Single Plasmid System

Numerous studies across academia and industry have been performed to optimize triple plasmid transfection by total DNA mass and molar ratios. In some instances, it has been reported that titers are improved, though often the best rationale for adjusting molar ratios has been demonstrated for increasing % full capsids or limiting toxicity caused by the ITR- transgene cassette to be packaged. These modified ratios are often coupled with loss in productivity. Here we present that single plasmid systems requiring no molar ratio variation will more efficiently deliver all AAV expression and packaging components without incurring greater total plasmid DNA requirements retain reproducible productivity.

Figure 5.



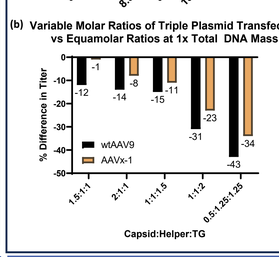
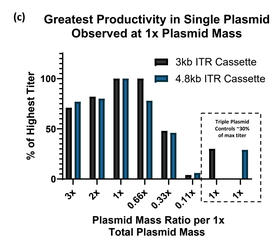


Figure 5.
(a) Individual plasmids of various sizes were transfected and assessed for % of culture transfected by flow cytometry. Single plasmids at 1x DNA mass had up to ~80% delivery while .3x mass observed ~50% delivery. Statistically we hypothesized 3 plasmid productivity was not dependent on molar ratio, but the probability of all required AAV components being delivered to a single cell to be between 12.5% to 51% (1:1:1 molar ratio).

(b) Limited study representing variable molar ratios in triple plasmid transfection negatively effects productivity.

(c) Confirmation by AAV productivity of single plasmid system efficient delivery of all AAV components at 1x DNA mass compared to 30% relative productivity of triple plasmid at 1x DNA Mass and 1:1:1 molar ratio. Additional observation of potential impact of DNA total mass toxicity at 2x and 3x mass.



In-House Transfection Cocktails

Though commercial reagents have significantly improved the cost is extremely high for routine productions in house at scale. These improved reagents have come to the market relatively quickly and are based on legacy products for general DNA transfection. Our approach to identify alterative transfection reagents, complexation/delivery enhancers, and productivity enhancers that can be developed in-house pushes us closer in our goal to have an efficient platform to deliver materials in our Vector Core PD function and continuing building data on these products to later produce a GMP-ready, economical and high-quality HEK293 AAV manufacturing platform.

Figure 6.

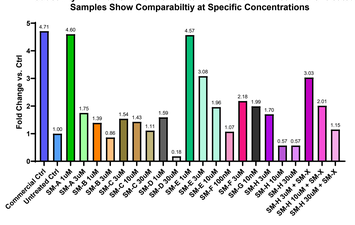
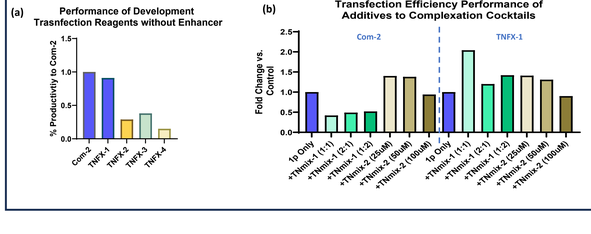


Figure 6. (left)
A representative sample of compounds, which at specific concentrations and timing are applied can have both enhancing or limiting effects on productivity. Screening methods for toxicity followed by productivity were developed in these evaluations. Products comparable to control have been pursued in subsequent rounds of testing. Note that the scale-down model used here limits productivity and exaggerates beneficial impact .

Figure 7. (Bottom)
(a) Propriety transfections reagents have been identified with TNFX-1 showing comparable productivity to Com-2. (b) Initial experiments in improving transfection complex with additives also have promising results to continue pursuing

Figure 7.



Scale-up & *In Vivo* Confirmation

Figure 8.

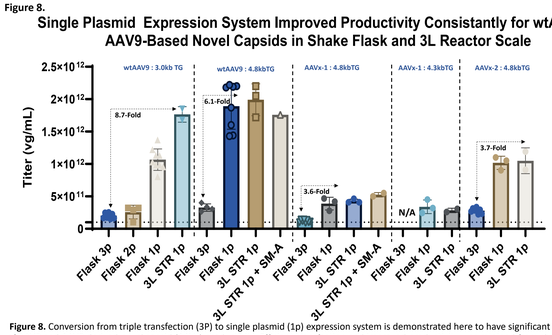


Figure 8. Conversion from triple transfection (3P) to single plasmid (1p) expression system is demonstrated here to have significant improvement of AAV9 and AAV9 derived novel capsid (AAVx) productivity. Efficient transfection and improved productivity was retained from shake flask scale through 3L Stir Tank reactor consistently achieving ~6-fold improvement in wtAAV9 with 4.8kb Cassette and >3.5-fold improvement in 2 different AAVx capsids. All productions in this figure utilize Tenaya C20 HEK293 and Com-2 +Enhancer transfection unless other-wise indicated for 3L wtAAV9 and AAVx-1 with 4.8kb cassette where SM-A 1uM was evaluated in place of the commercial enhancer.

Figure 9.

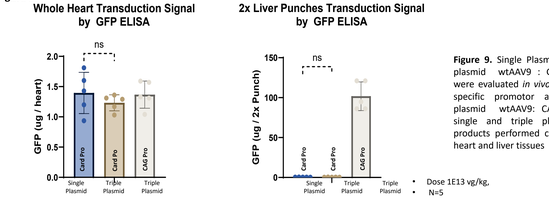


Figure 9. Single Plasmid and triple plasmid wtAAV9 : GFP products were evaluated *in vivo* with cardiac specific promotor against triple plasmid wtAAV9: CAG.GFP. Both single and triple plasmid AAV9 products performed comparably in heart and liver tissues

- Dose 1E13 vg/kg,
- N=5

CONCLUSION

- Demonstrated comparability with Tenaya's C20 clonal producer cell line to VPC 2.0 utilizing commercially available medium, transfection reagents and enhancers
- Our proprietary Single Plasmid AAV expression configuration has demonstrated consistent and robust improvement in productivity over triple and dual plasmid systems for AAV9 and AAV9-base novel capsids
- AAV products produced by single plasmid transfection are demonstrated here to be comparable in murine *in vivo* animal models
- Tenaya PD has also identified significantly cheaper alternatives to transfection complexation reagents and productivity enhancers-with the ability to be produced or formulated for GMP use.
  - Top in-house productivity enhancer (SM-A), at a fraction of the cost, has demonstrated to be scalable to 3L reactor and comparable to commercial reagents
- Additional investigation and tailoring of Tenaya's novel transection cocktails will be pursued to further mature our economical and high-quality HEK293 AAV manufacturing platform

ACKNOWLEDGEMENTS

Thank you to all Tenaya collaborators.

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- Process Development Team where construct builds, screening productions, many characterization assays and scale-up took place

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