# ON THE AAVENUE TO SUCCESS: ADVANCES IN TECHNOLOGIES FOR AAV PRODUCTION

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Key Words:       rAAV, HEK293, gene therapy, RNA sequencing.

Recombinant adeno-associated virus (rAAV) has become one of the predominant viral vectors used in gene therapy applications. Along this way to success, production processes have evolved from adherent to suspension culture and new analytical tools for product characterization have been developed. Still, major bottlenecks exist in transient process scalability, low transfection efficiency and titers, low packaging ratio of viral capsids and dose-dependent immune response, to only name a few. To overcome these challenges, our work aimed at gaining insight into different process steps – many of them so far poorly understood – from transient transfection, plasmid uptake and processing, to cellular response caused by virus gene expression and finally capsid assembly. A range of analytical tools including ddPCR, ELISA, SEC-MALS, AAV genome sequencing, and total RNA sequencing using Illumina technology was applied to characterize production of several rAAV serotypes in different HEK293 suspension cell lines.

rAAV production via transient transfection is still industry standard but has proven to be an inherently ineffective process. Our results show that only 10-20% of added DNA ends up in the cytosol, and an even lower amount in the nucleus, where replication of viral genomes and packaging of capsids takes place. To further improve transfection and AAV production, we investigated different media components and enhancer-like molecules, among them HDAC inhibitors, achieving up to five-fold increase in viral titer.

The efficacy and safety of AAV-based therapies also depends to a significant extent on the quality of the vector preparations. However, a uniform standard for this has yet to be found. Here, AAV genome sequencing and SEC MALS analysis were used to identify serotype-specific differences in AAV aggregation levels as well as residual DNA impurities. More than 3% of mispackaged sequences were found, including resistance markers for the amplification of plasmids in *E. coli* as well as HEK293 host cell DNA. Among the packaged vector sequences, truncated vector genomes, and fusion products were identified. Our results indicate the potential for truncated GOI plasmids as well as optimized production and purification processes along with in-depth analytics for future developments of safe gene therapy products.

To increase understanding of cellular mechanisms during rAAV production, we used Illumina sequencing to compare the transcriptomes of two different commercial HEK293 cell lines, one being a low producer (cell line A) and one being a high producer (cell line B). On average, 69 million reads per sample were sequenced. Of these, 94.8 % were mapped to the human reference genome (GRCh38) for the lower producing cell line A, while 88.4 % were mapped for the high producer cell line B. For the latter, a higher percentage of extrachromosomal reads - mostly viral transcripts - was found. Further analysis of differential expression showed that from a total of 61,544 annotated genes (coding and non-coding), 11,250 were differentially regulated in cell line B 18 h after transfection for rAAV production, when compared to the mock-transfected culture. Cell line A differentially expressed only 4,973 genes at this time point. Comparison of cell line A and B, both after transfection, revealed 8,195 differentially regulated genes. Among these, cell line B showed downregulation in pathways involved in e.g., cell killing, innate immune response and cellular defense response. Furthermore, more than 50 differentially regulated transcription factors were identified using bioinformatic workflows. Our findings highlight how this approach can be used to rationally optimize cell line development and viral vector production, with more potent transient cell lines as well as stable AAV producer cell lines on the rise.