Simplifying Progress

The Titer Gap – A Holistic Approach to Understanding High and Low HEK293 Producer Cell Lines in AAV Production Processes

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Introduction

Despite the latest developments in the field of Adeno-Associated Virus (AAV)-based gene therapy, there are still essential aspects in AAV biology and production that are not well understood. There is an ongoing effort to identify host cell characteristics that are beneficial for product yield in viral vector production processes. Since there are various HEK293 subclones that all carry different genetic modifications, the comparison between a cell line that is known to produce high viral titers and a cell line that generates less viral vectors can give insights into which cellular factors play a role in the various steps of AAV production.

Methods

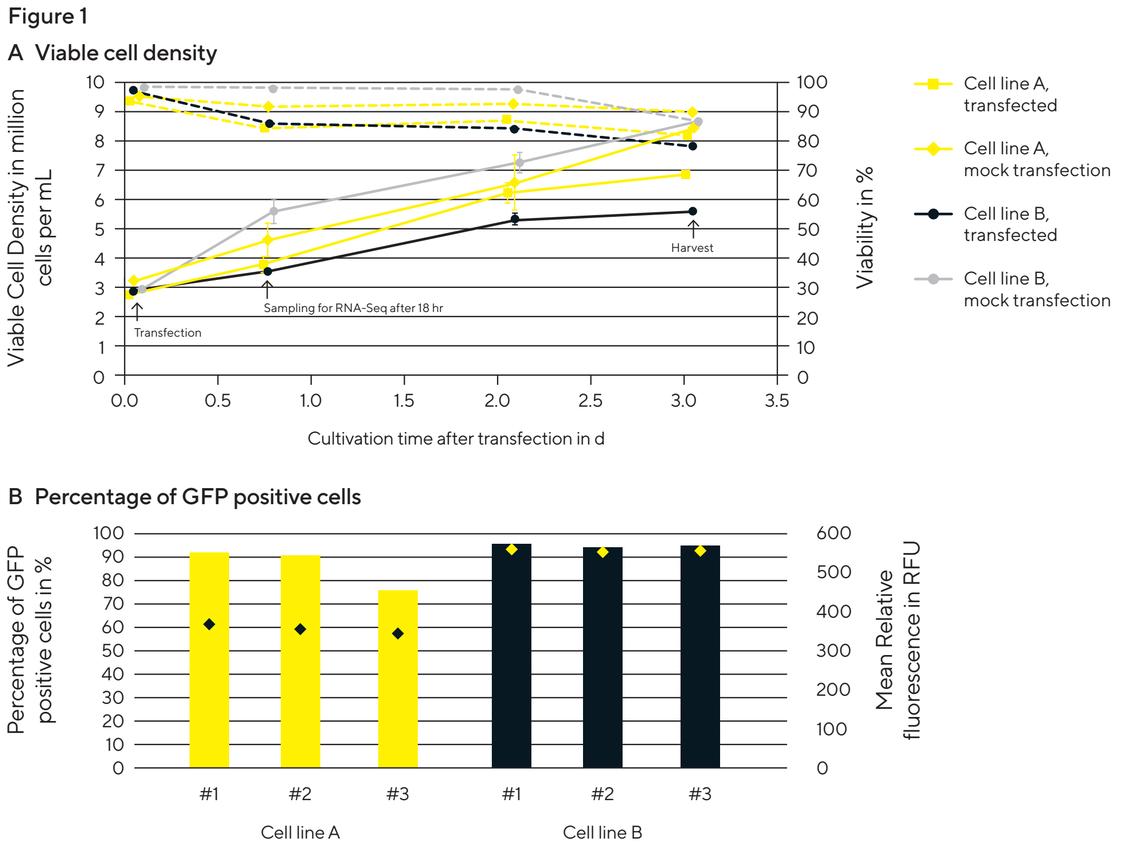
Two commercially available HEK293 cell lines (cell line A and B) were cultivated in 125 mL plain shake flasks using HEK ViP NB medium (Sartorius Xell GmbH). Both cell lines were transfected in triplicates with a two-plasmid system (PlasmidFactory) with eGFP as a transgene and using PEI-MAX (Polysciences) as transfection reagent. Non-transfected cultures were treated the same way as transfected cultures but received equal amounts of PBS instead of plasmid DNA and PEI-MAX. During the production phase, daily samples of cell culture supernatant were collected and analyzed regarding different nutrients and metabolites via HPLC. Produced AAV vectors of both cell lines were quantified via ddPCR (Bio-Rad) and ELISA (Progen). RNA-Seq was done on a NextSeq system (Illumina).

Results

Growth, transfection and AAV production of two HEK293 cell lines

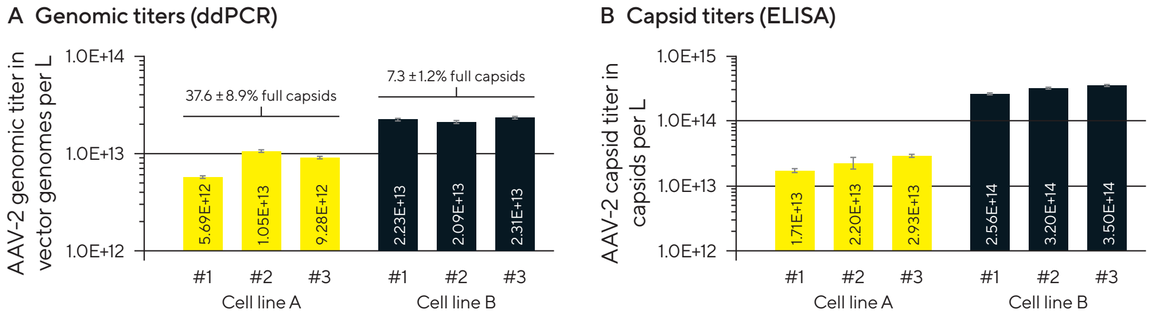
Transfected cultures showed slower growth and a drop in viability compared to non-transfected (mock) cultures, especially in cell line B. This detrimental effect of the transfection is most likely caused by the known cytotoxic effect of PEI-MAX and the additional metabolic stress on the cell, caused by replication and synthesis of the viral genome and proteins. Genomic titer as well as total capsid titer were considerably higher in cell line B. Interestingly, this cell line showed a low packaging ratio of 7.3 ±1.2%, while the low-producing cell line A reached a ratio of 37.6 ± 8.9%.

Figure 1



Note: (1A) Viable cell density and viability throughout the three-day production phase. The values shown are the mean values of three biological replicates. The error bars indicate the standard deviation with n=3. (1B) Transfection efficiency (bars) and mean relative fluorescence of GFP positive cells (diamonds) for all three biological replicates of cell line A and B 72 hr after transfection. The values were determined through flow cytometry measurement of GFP (GOI, expressed as byproduct during AAV production).

Figure 2

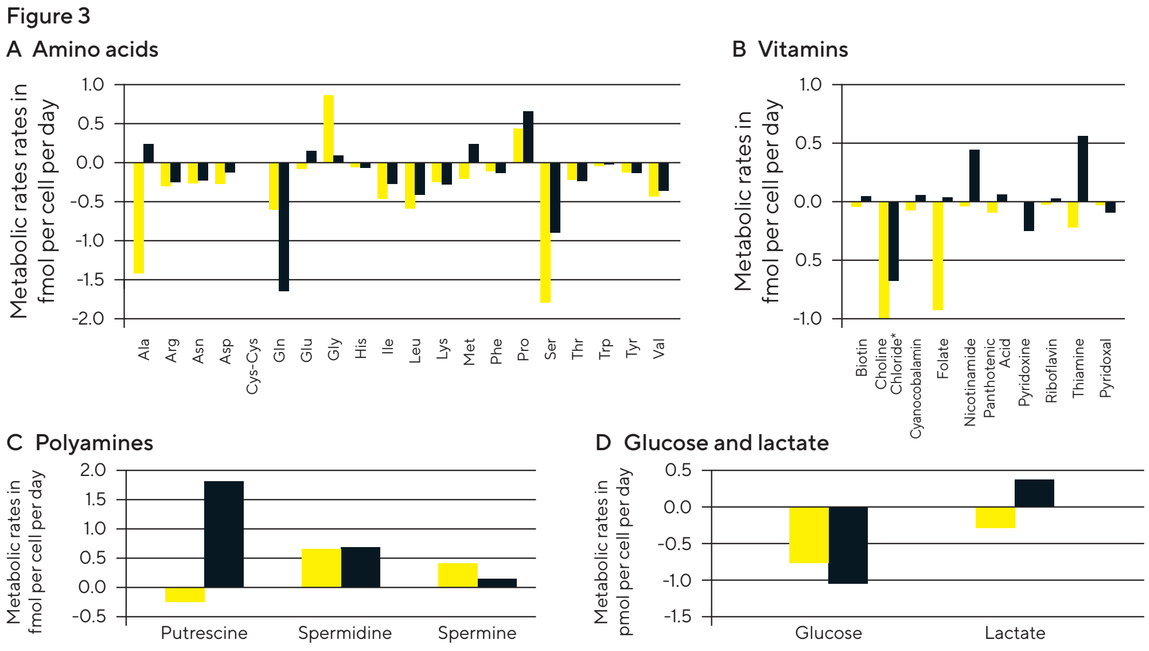


Note: Genomic (2A) and capsid titer (2B) for all three replicates of cell line A and B 72 hr after transfection, measured via ddPCR and ELISA, respectively. The error bars indicate the 95%-Poisson confidence interval for the ddPCR results and the mean deviation from technical duplicates for the ELISA results. Packaging ratio is given as a mean value from three biological replicates including the standard deviation.

Spent media analysis and metabolic rates

Cell line A metabolizes alanine, while cell line B shows a small build-up throughout the production phase. Alanine is mainly used as a precursor of pyruvate and plays a role in the mitochondrial energy metabolism. Another prominent difference is the metabolization of choline chloride and folate by cell line A. Folate comes into play in the synthesis of nucleic acids and in other central metabolic pathways. It is reasonable to assume that it also has an impact on the synthesis of viral DNA and RNA. Choline serves as a precursor for numerous cellular components like membranes and signaling molecules. Cell line A performed a so-called lactate switch, which is characteristic for immortal cell lines under glucose limiting conditions.

Figure 3

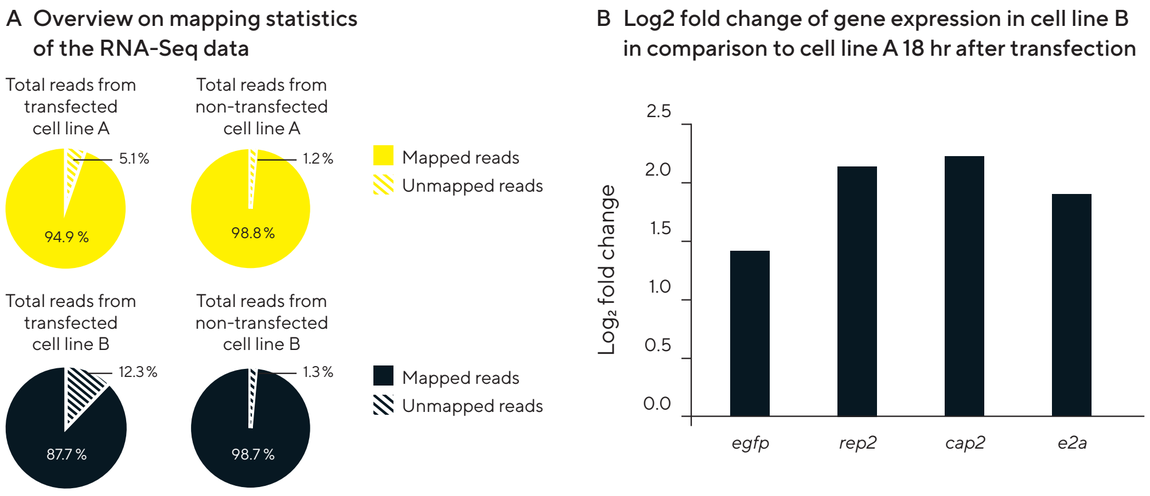


Note: Metabolic rates of cell line A (yellow) and cell line B (black) of different nutrients: Amino acids (3A), vitamins (3B), polyamine (3C) and glucose and lactate (3D). The values are based on HPLC analysis of spent media samples from cell culture supernatant from 48 hr and 72 hr after transfection and the corresponding viable cell density measurements.
* The consumption rate of cell line A for choline chloride is off scale, with a value of -48.96 fmol per cell per day.

Differential expression levels revealed in RNA-Seq.

Initial analysis of the obtained transcriptome data from both transfected cell lines revealed differences in gene expression 18 hr after transfection. The large data set obtained through RNA-seq will now be further analyzed with regards to differential gene expression in various pathways, e.g. associated with anti-viral response and central metabolism. Comparison of mock vs. transfected for each cell and transfected cell line A vs. transfected cell line B will help to gain a better understanding of AAV production processes within the cells.

Figure 4

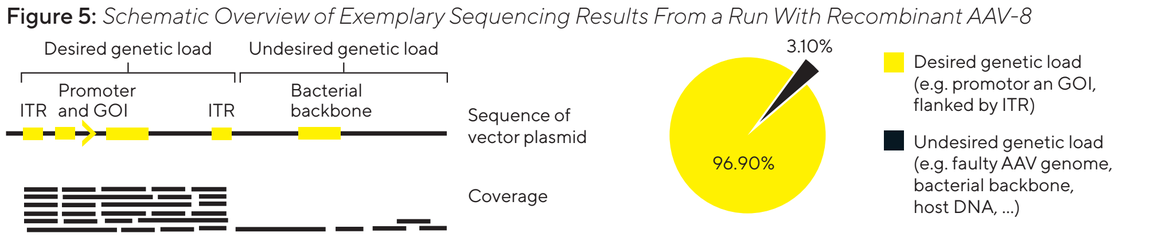


Note: (4A) For differential expression analysis, samples of ten million cells per shake flask were collected 18 hr after transfection and lysed with TRI Reagent (Zymo Research). Total RNA was extracted and sequenced on a NextSeq system (Illumina) with 2 × 75 nt paired end sequencing. The reads were mapped onto the human reference genome (GRCh38) using Bowtie 2 using the very sensitive local preset (see references 2 and 3 for further information). The increased percentage of unmapped reads in transfected cells is caused by the transcription of the viral genes. Unmapped reads were exported and mapped onto the sequence of the ITR plasmid and the helper plasmid with Geneious Prime (Biomatters Ltd.). The remainder of unmapped reads are most likely due to genetic differences in HEK293 cells and the human reference genome and sequencing artifacts. (4B) Log2 fold change was calculated based on mean coverage of the shown genes.

Summary | Conclusion

- **AAV production slows growth:** Cellular growth is inhibited by an increased AAV production, which in turn correlates well with the relative mean fluorescence of the reporter gene used in this study .
- **Protein is only half the battle:** As the protein production and, therefore the capsid titer, increases in the process, the genomic titer must also increase, or else the packaging ratio decreases, resulting in a lower quality AAV product.
- **Interplay of cellular factors and media:** The cell lines differ in metabolic rates of specific nutrients and media components, but no fundamental differences could be found that could be solely correlated with the production of AAV-2 or the necessary biomolecules.
- **Knowledge is power:** Understanding the factors which make one cell line a higher AAV producer will enable future cell line and process development efforts. Our RNA-Seq approach allows a broad insight into cellular pathways associated with AAV production.
- **Outlook | Take a look inside:** In addition to the genomic and capsid titer, the product quality gains a lot of relevance when it comes to clinical trials – here, we show a method that enables assessing the genomic load of the AAV particles and quantifies the amount of vector particles that carry an incorrect genetic load, which potentially leads to undesired immune effects.

Figure 5: *Schematic Overview of Exemplary Sequencing Results From a Run With Recombinant AAV-8*



Note: Sequencing was carried out using an Oxford Nanopore MinION sequencer. The produced AAVs were lysed and the extracted DNA was isolated using a NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel), following the protocol previously published by Radukic & Brandt *et al.* (2020). Around 400 ng of vector DNA was sequenced and mapped onto the sequence of the vector plasmid. Sequence of vector plasmid is simplified and only shows a selection of annotated sequences. Coverage means the number of non end-gap characters at each position.

References

(1) Marco T Radukic, David Brandt, Markus Haak, Kristian M Müller, Jörn Kalinowski, Nanopore sequencing of native adeno-associated virus (AAV) single-stranded DNA using a transposase-based rapid protocol. NAR Genomics and Bioinformatics, Volume 2, Issue 4 (2020)
(2) The Galaxy Community, The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2022 update, Nucleic Acids Research, 2022; doi:10.1093/nar/gkac247 | (3) Geneious Prime 2022.1.1. (https://www.geneious.com), Biomatters Ltd.