Identification of universal parameters that play a critical role in rAAV production using a transient two-plasmid packaging system in the adherent HEK293T cell line.

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Adeno-associated virus (AAV) is the vector of choice for gene therapy due to its relatively good safety profile and persistence. Consequently, the number of AAV clinical trials are expanding rapidly. To meet the increasing demand, challenges in manufacturing of recombinant AAV (rAAV) viral vectors such as low yields and low full:empty capsid ratios in unpurified material need to be tackled.

In this work, we studied the effect of critical process parameters that determine the yield and quality of rAAV production. We started from a baseline rAAV production protocol based on the adherent human embryonic kidney (HEK)293T cell line in combination with a two-plasmid transient transfection system in which *rep* and *cap* genes and Ad helper genes are on the same plasmid. As this protocol routinely yields decent yields and full:empty ratios, we considered it a good starting point for identifying the process parameters that lead to superior rAAV production yields. We evaluated the effect of plasmid quantification, confluency at the time of transfection, total plasmid DNA (pDNA) amount, DNA:PEI ratio, and plasmid ratio on vg titer, VP titer and % full for AAV2, AAV5, and AAV9. In addition, we measured the intracellular average copy number of the different plasmids 16h after transfection in HEK293T cells to allow monitoring of the effect of aforementioned parameters, both on transfection efficiency as on AAV production. In an effort to obtain more reproducible transfection conditions, we found that determining pDNA concentrations using the Invitrogen Qubit HS assay was more reliable than using a Nanodrop spectrophotometer. Most likely, this is due to the tendency of traditional UV absorbance-based methods to overestimate sample concentrations due to the presence of contaminants. We found that there is an optimum in terms of amount of total pDNA and the DNA:PEI ratio at which maximum transfection efficiency is obtained, which correlated well with intracellular pDNA copy numbers and the % full particles. Moreover, an inverse relationship between total vg and % full particles was observed, meaning that parameters that increase total vg concomitantly decrease % full and vice versa. Independent of the serotype, the best results for rAAV production were obtained by decreasing the helper:production plasmid ratio, with a gradual decrease in total vg though concomitant increase in % full.

In summary, we found that UV absorbance-based methods for pDNA quantification do not form a reliable basis for rAAV production process development, and that monitoring of intracellular pDNA copy numbers allow for better insight into the transfection and production process. Moreover, we observed an inverse relationship between total rAAV particle yield and percentage full. The driving force behind this relationship is yet unclear and the subject of follow-up research.