Cell Line and Process Development Strategies to Establish HEK293-Based Recombinant Adeno-Associated Virus Production Platforms

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Recombinant adeno associated virus (rAAV) vectors have become popular delivery vehicles for *in vivo* gene therapies, but demand for rAAVs continues to outpace supply due to low process yields and manufacturing capacity limitations. Transient transfection of human embryonic kidney 293 (HEK293) cells is the current gold standard rAAV manufacturing process, but this system produces orders of magnitude fewer doses per batch than established processes for antibody production and are not readily scalable to produce vector for large patient populations. Stable producer cell lines (PCLs) can be cultured at much larger scales than transient processes, making stable platforms attractive for manufacturing of AAVs, particularly those with higher demand or dosing requirements. However, generation of rAAV PCLs is difficult because sequences encoding AAV replication (*Rep*) genes, AAV capsid (*Cap*) genes, and helper adenovirus (AdV) genes must be integrated into the host cell under the control of inducible promoters.

In this work, we established rAAV production, analytical characterization, and cell line development workflows to support the design and screening of inducible gene constructs that can be used for the generation of PCLs. Initial studies focused on establishing a traditional, triple transient transfection platform. The plasmids for this system encode the necessary rAAV production genes across three constructs (pRepCap, pHelper, pGenome) containing constitutively expressed, wild type viral gene sequences. This enabled us to optimize transient process parameters, set benchmarks for expected titer ranges, and develop analytical methods for vector genome (VG) and capsid particle (CP) titer measurements.

We then developed recombinase mediated cassette exchange (RMCE) and PiggyBac (PB) transposase mediated stable gene insertion workflows for the design and testing of inducible gene cassettes expressing fluorescent reporter genes. The RMCE system was used to screen 12 doxycycline (dox) inducible cassette configurations because it enables stable integration at a predefined genetic locus, reducing biological variability between experimental conditions. We observed that promoter driving expression of the reverse tetracycline transactivator (rtTA) affected the leakiness, fold induction, and maximum induced fluorescence of inducible gene cassettes more than the tet responsive promoter or rtTA variants themselves. The top performing cassette supported induction of a fluorescent reporter by 420-fold and displayed minimal leak in RMCE pools, and similar behavior was observed when the same sequences were integrated in PB pools.

The RMCE system was applied for a second screen to study rAAV production gene cytotoxicity that informed the design of inducible rAAV production plasmids. No decreases in cell density nor viability were observed for any of the cultures during transfection, selection, or routine passaging to generate RMCE pools expressing these viral genes, demonstrating tight control of the inducible gene circuit. However, we observed that stable dox induction of two production genes, AAV replication gene *Rep78* and adenovirus helper gene *E4-34K*, suppressed cell growth and caused viability to decline over the three days in culture. This result motivated the screening of rAAV production gene cassettes designed to modulate cytotoxic protein expression. A four-plasmid, transient inducible rAAV production system was used to enable better elucidation of the functions of the *Rep* and *Cap* gene sequences, as they have nested readings frames in the native three-plasmid system, and to test if the inducible constructs could support rAAV synthesis at appreciable levels.

VG and CP titer both increased when *Rep78* expression was modulated with an internal ribosome entry site (IRES), and this expression change also had a positive effect on cell culture health. However, the changes to helper plasmid design did not affect cell density or viability during rAAV production and had variable effects on VG and CP titers that depending on the *Cap* sequence that was co-transfected. The maximum VG and CP titers reached in our transient inducible study were 4.6×10^{10} VG/mL and 1.4×10^{12} CP/mL, which were 49% and 74% of those from cultures transfected with wild type sequences, respectively. The rAAV production constructs drive high enough titer for translation to a stable expression context and serve as a baseline for future cassette design improvements using the transient inducible and RMCE systems.