Development of Highly Productive, Rhabdovirus-Free Sf9 Insect Cell Line for Large-Scale AAV Production for cardiovascular Gene Therapies

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Introductions

Adeno-associated virus (AAV) has become a safe and effective modality for in-vivo delivery of gene therapy (GT) into patients suffering genetic diseases. Recent approvals of AAV-based gene therapies, with dozens more in the late-stage development, highlight the increasing demand for efficient and cost-effective large-scale AAV manufacturing with high productivity and consistent quality across scales.

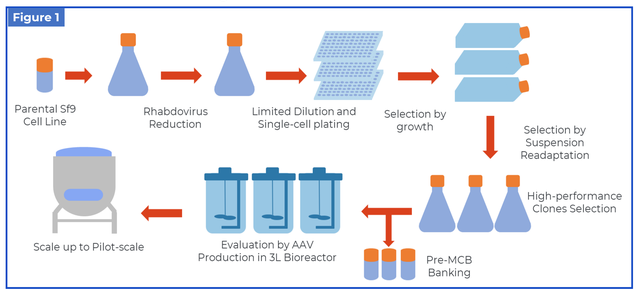
Sf9-based recombinant baculovirus (rBV) expression system in Sf9 cells remains as one of well-established production platforms for AAV manufacturing with several advantages over HEK293 platform using the triple transient transfection. While the HEK293 platform can rapidly supply AAV vectors for research and early-stage clinical trials, Sf9/rBV boasts high packaging efficiency (vector genome yield) and reliable scalability suitable for large-scale AAV manufacturing to deliver therapies to larger patient population.

Since the identification of a novel invertebrate Rhabdovirus (SfRV) in ATCC Sf9 cell line by the FDA in 2014, demonstrating adequate clearance of rhabdovirus from Sf9/BEVS has been challenging and costly, especially for early stage AAV GT products. In this work, we report the successful development of the new generation of Sf9 cell line that is SfRV-free at Tenaya Therapeutics, which can critically improve the safety and purity of AAV produced in Sf9/rBV system.

Materials and Methods

The overall workflow for screening and development of SfRV-free Sf9 insect cell line is summarized in the Figure 1.

Figure 1



ATCC-derived Sf9 Parental Cell Line was thawed and passaged until recovery in regular condition before adaptation to culture conditions for SfRV reduction. The culture was routinely monitored for growth and viability, as well as log-reduction of SfRV signal by performing reverse-transcriptase (RT) PCR-based assay on cell pellet and spent media. After 4 weeks of SfRV reduction, the resulting culture was single-cell plated to 96-well plate, while the spent media - determined RV-free after no RV signal was detected – was used to promote the growth of clones.

Total 222 wells were identified with presence of a single-cell. After several rounds of screening for growth in 96-, 48-, 24-, 12-, and 6-well plates (6 weeks), 21 clones were transferred to T-flask and then readapted to suspension culture in shake-flasks.

Elimination of SfRV confirmed in clonal cultures

Levels of SfRV present in cultures were measured over RV-reduction period and in clonal cultures. Separate measurement of SfRV genomes in cell pellet and supernatant demonstrated that extracellular SfRV reduction occurs more rapidly – SfRV signal in supernatant was reduced to below limit of detection by PCR-based method. This allowed us to safely utilize the saved spent media from RV-reduction pool culture for faster propagation of single-cell clones.

Meanwhile, SfRV signal was observed to persist in cell pellet despite the prolonged period in RV-reducing condition. However, upon reaching 1E-3 RV genome per cell level, it was determined that limited dilution would generate RV-free clones with high success rate (Table 1). Final RV-elimination was confirmed via next generation sequencing (Table 2).

Table 1

RV-Titer by ddPCR	RV-Genome/Cell
Parental Cell Line	418
1 Week RV Reduction	0.45
4 Week RV Reduction	1.25E-03
Single-Cell Clones	BLOD
8 Week Post Suspension Readaption	BLOD
At Pre-MCB Banking*	BLOD

Table 2

Clones	% To Total Reads*
Sf9 Control	0.406
RV-free Clone A	< 0.000001
RV-free Clone B	< 0.000001
RV-free Clone C	0.000000

*For each clone, >4.5e7 reads of 150bp length collected using Illumina NextSeq 550 were mapped to published SfRV sequence (NC_025382)

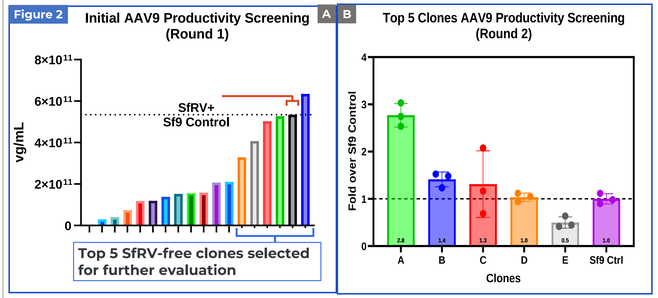
Top SfRV-Clones Show Improved Productivitity

Of 21 clones that were readapted to suspension culture, 18 clones that demonstrated stable growth rate was evaluated for their AAV productivity using AAV9:GFP construct. Using each clone, recombinant baculovirus (rBV) encoding AAV9 Rep/Cap and ITR-flanked EGFP were produced.

Each clone was then co-infected with corresponding rBVs for AAV production in shake-flask scale (Figure 2A).

5 top-producing clones were selected from the initial round of screening for further evaluation. The following triplicate experiment, also using AAV9:EGFP, further narrowed down the list to top 3 clones (SfRV-free Clone A, B, and C; Figure 2B.). **Clone A demonstrated specific vg titer close to 3-fold level compared to Sf9 Control.**

Figure 2



SfRV Clones Do Not Impact Purity and in vitro Transduction Efficiency

The top 3 clones were evaluated alongside SfRV-positive Sf9 control cell line (ATCC-derived) for their performance in 3L-scale benchtop bioreactor production for AAV9:GFP and AAV9-based therapeutic vector. In 3L-bioreactor, all **top 3 SfRV-free clones exhibited equal or higher AAV9 productivity** compared to the control. In addition, respective shake-flask satellite cultures performed very similarly to the bioreactor culture, demonstrating robust potential for their scalability (Figure 3A).

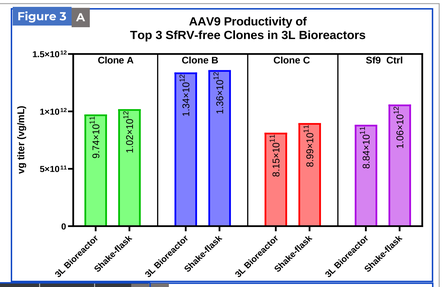
Several key quality criteria were evaluated for the AAV9:EGFP produced in each clone. %vg-to-cp ratio, showed no significant difference across the clones. Moreover, consistent AAV capsid protein ratio (VP1:VP2:VP3) was also observed consistent across the clones (Figure 3B).

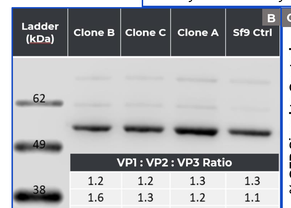
In-vitro GFP transduction levels in iPSC-derived cardiomyocytes were measured to assess the rAAV's potency against target tissue. While Clone B demonstrated lower transduction, **Clone A and Clone C showed no difference to the Sf9 control cell in GFP expression transduced** by AAV they respectively produced (Figure 3C).

To further assess the SfRV-free clones' capability as a potential platform cell line, an AAV9-based therapeutic (4.8kb) product was produced at 3L-scale where **Clone A's productivity outperformed SfRV-positive Sf9 Ctrl by 3-fold**, **reaching nearly 2e12 vg/mL titer** (Figure 4A).

Resulting materials were further processed through downstream purification via affinity capture and step-elution. The chromatogram exhibits each clone's peak size closely correlating to the respective vg titer. Meanwhile, the 4 peaks demonstrated similar geometry as well as their A260/A280 ratio, suggesting comparable full-empty ratio across all clones (Figure 4B; arbitrarily positioned across x-axis for display).

Figure 3





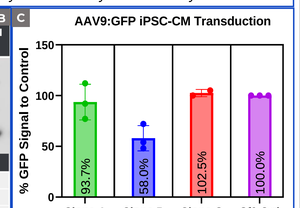
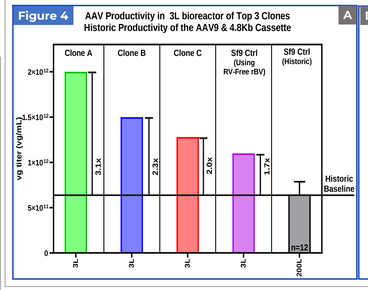
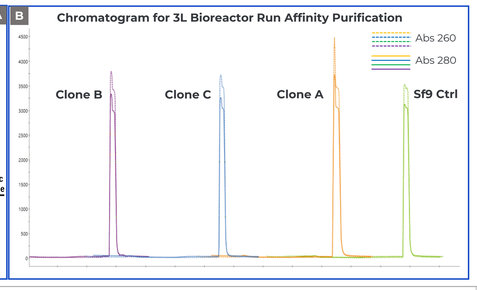


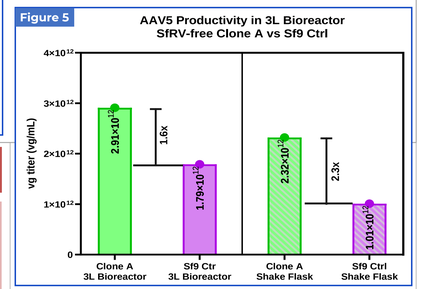
Figure 4





Clone A, determined as our top pick, was further evaluated for its capability to produce alternative AAV serotype. In production for AAV5-based construct, Clone A exhibited 1.6-2.3 fold improvement compared to the control, **reaching nearly 3e12 vg/mL in upstream titer**.

Figure 5



Conclusions

- **SfRV-free Sf9 clones were successfully generated and verified by both PCR- and NGS-based assays.**
- **After initial screening based on the growth and AAV9 productivity, several top-producing SfRV-free clones were selected for scale-up into 3L-bioreactors.**
- **Using both AAV9:EGFP and AAV9 w/ therapeutic cassette, up to 3-fold increase in vg titer compared to our historic upstream titer was confirmed, and the top-performing Clone A was identified.**
- **In both vg-cp ratio and GFP expression in in-vitro transduction assay, Clone A showed no difference compared to the RV-positive Sf9 Control.**
- **Performance of the Clone A was further evaluated in AAV5 productivity, where it produced up to nearly 3e12 vg/mL.**

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