SK pharmteco

White Paper

Evolution of an Adherent HEK293 Cell Line into a cGMP Serum-free Suspension Cell Line for Universal AAV, LVV, and Ad Vector Production

Shift To Suspension Cell Culture For Viral Vector Manufacturing

HEK293 cells, derived from human embryonic kidney cells, are widely used in viral vector production because of their general usability, including high efficiency of transfection and rapid growth rate. This cell line was established through transformation with fragmented DNA from adenovirus 5[1]. In the case of AAV, the constitutive expression of the early Ad virus protein, E1A, serves as a transactivator of the AAV P5 promoter, thus making HEK293 cells an excellent choice for generating recombinant adeno-associated virus (rAAV) when transfected with helper, AAV Rep/Cap, and ITR-GOI (gene of interest) plasmids. SK pharmteco holds a license to these cells, serving as the starting point for development of an optimal cell line for suspension-based production of AAV.

The initial gene therapies brought to market utilized viral vectors produced through adherent cell culture. This method is practical for treatments aimed at small patient groups, allowing production in systems like Corning's HYPERStacks® and Thermo Fisher's Nunc™ Cell Factory™. Adherent platforms continue to be effective for producing lentiviral vectors used in gene-modified cell therapies and certain AAV serotypes where high yields are not the primary concern[2]. Technological advancements, including fixed-bed bioreactors, have facilitated more efficient but expensive large-scale manufacturing using adherent cell culture methods[3].

As the demand for AAV vectors grows, primarily driven by numerous active clinical trials targeting prevalent diseases with larger patient populations, manufacturing capacities must expand to support this need. Scalable production platforms efficiently delivering high-quality viral products are crucial for success. Using scalable suspension cell culture with adapted HEK293 cells in chemically defined, animal-product-free media provides a practical approach for producing the numerous batches needed to meet clinical and commercial demands. As a result, forward-looking viral vector manufacturers are transitioning from adherent to suspension production[4].

In this white paper, learn more about:

- The complexities and challenges in scaling up viral vector production for cell and gene therapies.
- SK pharmteco's development of high-performing, flexible viral vector manufacturing platforms.
- Insights into innovative cell line development for AAV, LV and Ad production.
- Performance comparison of SK pharmteco's new cell line with industry competitors.
- Anticipating future developments in viral packaging cell line evaluation for lentiviral vectors and AAV, such as bioreactor and AMBR systems.

www.skpharmteco.com
info@skpt.com

Importance of Finding Optimal Cell Lines for Generation of cGMP Master Cell Banks

Optimizing suspension cell-culture processes for viral vector production requires consideration of several important factors, not the least of which are the target vector (including serotype, gene of interest [size, sequence], and therapeutic application, which guide the required production scale. Use of high-quality materials—plasmids, cell lines, media, transfection reagents are also essential. Ideally, these materials are also carefully designed for use in suspension-based transient transfection processes.

Manufacturing platforms for viral vector production developed using a design-of-experiment (DoE) approach can be particularly effective at decreasing development timelines and costs, so long as they are sufficiently flexible to accommodate the varying requirements of different serotypes and genes of interest.

One key to establishing a successful viral vector manufacturing platform is development of an optimal cell line for generation of a cGMP master cell bank. Such cell lines are adapted for suspension cell culture in serum-free conditions and engineered to afford higher titers of full viral capsids in higher purity with greater productivity[5,6]. Optimal clonal cell lines, derived from a single cell, produce large quantities of virus while also exhibiting optimal bioprocessing parameters (high cell density, rapid doubling time, high viability, lack of clumping, maintenance of titer for high passage numbers). Moreover, these cell lines ensure a uniform genetic background, which translates to predictable and reproducible cell behavior. This uniformity is crucial for maintaining consistent product quality and minimizing batch-to-batch variations. The use of clonal cells also facilitates regulatory compliance, as the defined and stable nature of these cell lines simplifies the validation and monitoring processes required for biomanufacturing.

Development Of A High-Performing Monoclonal Suspension HEK293 Cell Line For cGMP Cell Banking

As part of our efforts to develop an effective platform manufacturing process, SK pharmteco has created a high-performing, monoclonal, suspension HEK293 cell line for cGMP cell banking.

Generation of an optimal cell line for cGMP manufacture of Adeno-associated, lenti-, and Adenoviral vectors via suspension cell culture is a multi-step process. Historically, it has also been lengthy and labor-intensive, particularly for the separation and growth of single cells for the generation of a clonal cell line. SK pharmteco has benefited from progress on this front.

Adherent HEK293 cells were adapted for suspension cell culture in multiple commercially available serum-free media formulations. Single-cell printing technology was used to rapidly separate single clones for evaluation of their growth characteristics. The clones with optimal biological attributes (i.e., viral production, etc.) were then investigated at a small scale using a DoE approach. The most promising clones were further investigated by conducting larger-scale (200mL) transient transfection reactions and closely evaluating the characteristics of the resultant vectors. These initial screenings resulted in the selection of SKPT-HEK293 clone 4G9. Follow-on studies evaluating cell growth and viral productivity in single-use bioreactors and high-density perfusion culture also demonstrated very favorable performance for clone 4G9.

Generation of a Serum-free, Suspension Adapted Pool

First, suspension-adapted cells had to be obtained in chemically defined (serum- or animal product-free) media. This process can be achieved in various ways. One option is the selection of a high-producing AAV clonal line in adherent, serum-containing conditions before serum-free adaptation. We did not choose this route under the assumption that the phenotypic shift in adherent to serum-free suspension growth might negatively impact AAV production. This is the premise of the theory known as the “production gap” common to adherent vs suspension cell culture for viral vector production. Our approach is outlined in **Figure 1**, including stepwise depletion of FBS of adherent cells followed by suspension adaptation (Option #1), direct media exchange of adherent cells into serum-free suspension (Option #2), or suspension adaptation in 10% fetal bovine serum (FBS) followed by stepwise movement to serum-free media (Option #3). While all these methods were deemed successful paths, to achieve serum-free suspension adaptation, we found that Option #2 was the fastest and most ideal path forward.

Following successful adaptation into serum-free media and the subsequent creation of a small research cell bank, we evaluated cell performance in a panel of commercially available media feeds. To achieve this, cells were adapted by direct media exchange into roughly fifteen media formulations and evaluated for cell growth and transfection efficiency, as measured by cell count and GFP expression, respectively. We compared these critical quality attributes to AAVMAX Virus-Producing Cell 2.0 (VPC2.0 part of the AAV-MAX Helper-Free AAV Production System; Thermo Scientific). Moreover, as part of the media selection strategy, we were cognizant of the cost (USD/L) associated with each media formulation, another critical parameter used to select a media feed to move forward. The cost savings incurred at scale through this calculated media selection criterium are detailed on Page 22 of this document.

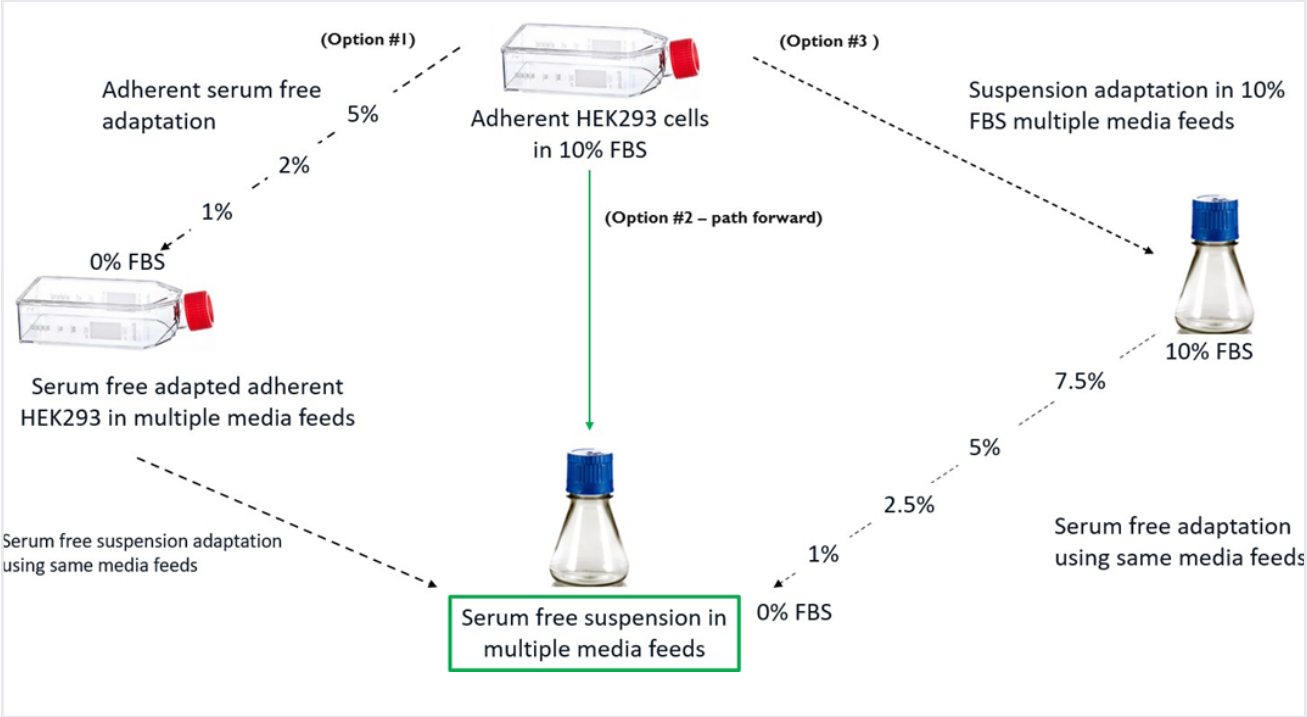

Figure 1. Serum-free adaptation to suspension culture. Process flow diagram to highlight three independent routes to achieve adherent culture to a chemically defined (Option #1), serum-free (Option #2), or suspension cell process (Option #3). Paths #1 and #3 involve the stepwise depletion of serum over time while Option #2 involves direct placement into suspension culture with

concurrent removal of serum.

Processes were run at a 30 mL scale using FectorVIR®-AAV (Polyplus®, now part of Sartorius) transfection reagent with GFP expression plasmids under the control of the strong CMV promoter. 48h post-transfection, cells were viewed with the EVOS M5000 cell imaging system (Thermo Scientific).

As shown in **Figure 2**, not all media formulations produced equal amounts of color. Several media formulations were able to support robust GFP expression, while others were not. Some of the Sartorius media formulations , notably HEK GM , are not designed to support protein production because of the presence of anti-clumping agents. Moreover, we also found that our cells did not grow well in the presence of anti-clump; hence the lack of GFP expression is not unexpected. In general, all the Thermo formulations supported GFP expression, however, when one considers the cost/L for each of these feeds, BalanCD media (FUJIFILM Irvine Scientific) provided optimal results from both a performance and cost perspective. At a cost of ~2x per liter a considerable 50-75% cost savings with BalanCD versus the Thermo media at larger scale is achievable. Given that the differences in GFP expression between the different media feeds were minimal, we decided to advance with BalanCD media to maximize the cost-benefit ratio.

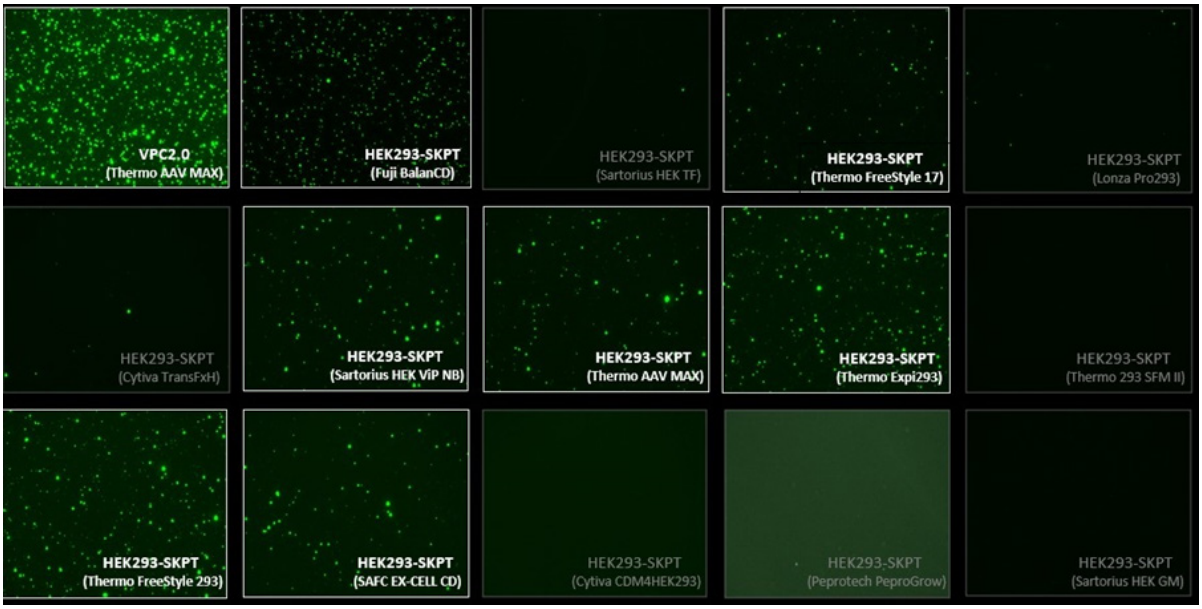

Figure 2. Media Selection Panel. Screening for virus production was performed using a CMV-GFP *cis*-plasmid as the packaging construct.

Transfection efficiency was measured on an EVOS 5000 microscope. Several media formulations exhibited GFP production and ultimately the best viral titers were observed in BalanCD basal media.

In the presence of BalanCD media, performance of the HEK293-SKPT pool was compared to the commercially available VPC2.0 cell line as well as two other competitor lines to assess doubling time and AAV production. The serum-free, suspension-adapted SKPT-pool demonstrated a ~27h doubling time, as compared to ~24h doubling expected for VPC2.0 cells (**Figure 3**, **left panel**). VPC2.0 doubling time, in our hands, was shown to be identical to the manufacturer's recommendation. Estimation of AAV8 VG/mL titers using digital PCR (Qiagen) from 30mL scale bulk harvests of a transient, triple-transfection, demonstrate favorable and comparable performance to VPC2.0 cells (**Figure 3**, **right panel**). For a list of conditions used during the triple-transfection process, please refer to **Table 1**.

Estimation of median VG/mL titers showed ~1e12VG/mL for VPC2.0 versus ~2e11 VG/mL for the HEK293-SKPT suspension pool, falling within the range of other competitor cell lines. The SK pharmteco pool functioned effectively and the creation of a small research cell bank was generated with concomitant mycoplasma and sterility testing after freezing in liquid nitrogen. Based on these results, where the SK pharmteco pool performed nearly as well as the VPC2.0 cell line, suggested that isolating a unique subclone and screening for higher performance, from within the pool, was warranted.

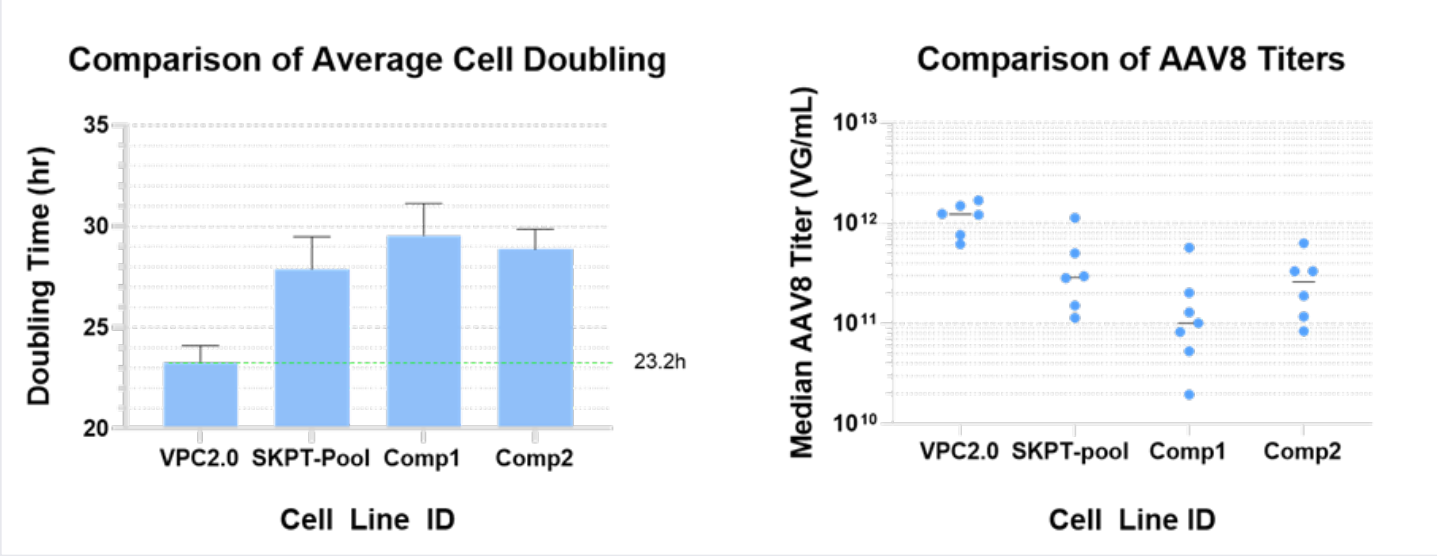

Figure 3. Evaluation of HEK293-SKPT Pool versus competitors. A) Average doubling time was calculated over a 10-passage interval (~5 weeks duration) and B) Determination of AAV8 titers (VG/mL) at 72 hours post-transfection comparing the HEK293-SKPT suspension adapted pool versus VPC2.0 cells and other competitors.

Cell Printing and Clonal Expansion

After banking the suspension-adapted pool, we focused on establishing clonality using single-cell printing technology. Utilizing the F.SIGHT™ system from Cytena, we employed single-cell dispensing combined with whole-well imaging. This method facilitates accurate single-cell assays, essential for monoclonal cell line development and other critical applications in cell and gene therapy.

For subclone evaluation, single cells were printed into individual wells of 96-well plates and monitored using high-content imaging on the CloneSelect® (Molecular Devices) imager for monoclonality verification, colony outgrowth, and expansion. Specifically, the HEK293-SKPT pool was placed into a chamber that enables the generation of a suspension of separate cells. Single cells were isolated and dispensed one per well into a 96-well plate, through a funnel (up to 100 cells every five minutes). The movement of individual cells was tracked, and the locations of the individual cells in each well were known exactly (**Figure 4**). The growth characteristics of the cells were then monitored over time.

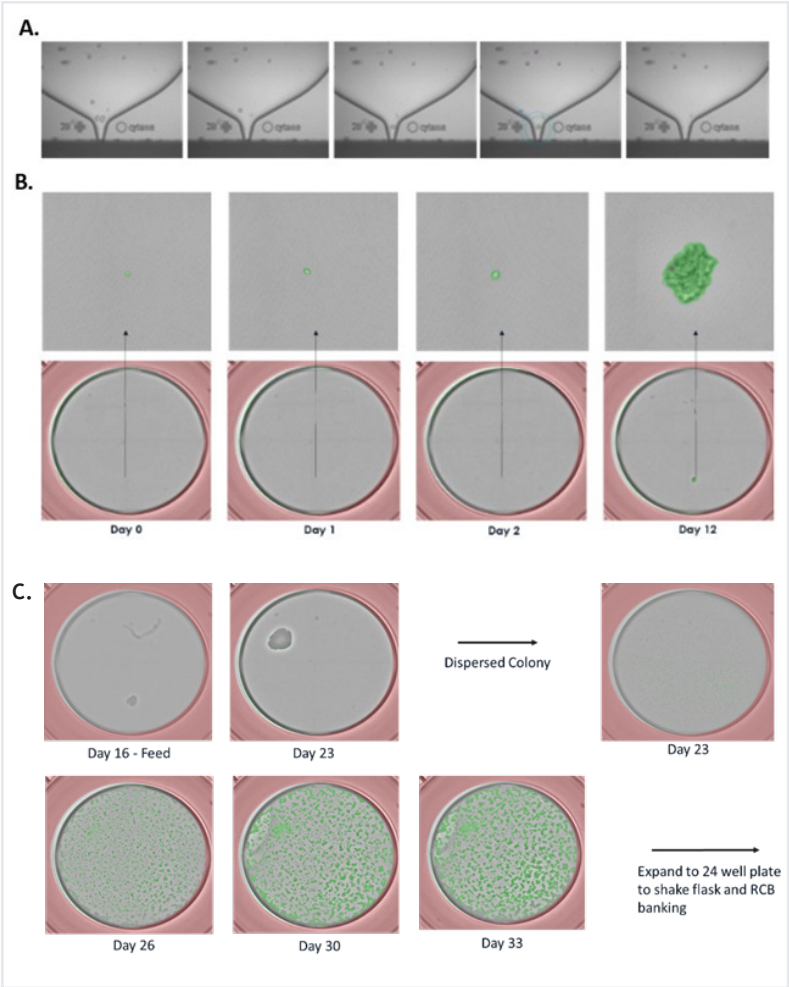

Figure 4. Single-cell printing of a mixed population of serum-free suspension HEK293-SKPT BalanCD cells using the Cytena F.Sight. A. First three images: cells as they approach the nozzle for printing. Fourth image: identification of a single cell and well identification for deposition. Fifth image: post-deposition image verification of single cell clonality. B. Growth expansion of a HEK293-SKPT subclone (4G9) starting from a single cell through days post-printing. C. Continued expansion with cell pellet dispersion and transfer to a sterile, non-tissue culture treated 24-well plate.

All subclones printed into the 96-well plates were expanded to 24-well plates and ultimately, shake flasks, after which, research cell banks were frozen. To achieve successful expansion and outgrowth of single cells, we optimized several parameters pertaining to media formulation and proprietary plating conditions. For the promising subclones derived from the serum-free, suspension-adapted HEK293-SKPT pool, clusters were observed by day 12 (**Figure 4b, right panel**), and colonies sufficiently large enough to require dispersion were seen by day 23 (**Figure 4C**). Expansion to shake flasks occurred after approximately one month. Fast growth (~24-26 hour doubling time) and high cell viabilities (96-99%) were observed at seeding densities between 0.4-0.8e6 cells/mL (data not shown). This property is crucial, as any increase in doubling time dramatically lengthens the process time at a large scale.

Approximately 100 subclones were expanded to research cell banks prior to evaluation. Surviving clones were evaluated for AAV production, cell growth, and clumping as an initial screen. Clones were further evaluated for LV production in a secondary screen. Selected clones (3 independent clones) that ranked highest were further evaluated for their ability to support Adenovirus. Based on the results of this screening effort and the identification of several leads that met all initial selection criteria (growth, biophysical properties, expandability, and virus production), one clone, HEK293-SKPT-4G9 (referred to as 4G9), emerged as the leading candidate.

Performance Testing of Lead Clone HEK293-SKPT-4G9

Effective cell-line development requires a deep understanding of the growth characteristics of the selected cell population. Understandably, cell lines that exhibit faster growth and can maintain high viability at increased cell densities are desirable when trying to expand to large-scale manufacturing. To confirm the results of the initial screening efforts that identified 4G9, we examined cell morphology, growth kinetics, and ability to support production of six common, clinically relevant AAV serotypes. As shown in **Figure 5A**, 4G9 grew as a uniform, single-cell suspension culture. At 10x magnification (**Figure 5A, left panel**) very little clumping was observed and at increased magnification (20x), the cells show exhibited a distinctly round morphology (**Figure 5A, right panel**). Monitoring cell growth over 10 passages, we estimated doubling time of ~27 hours (**Figure 5B**). Terminal growth curves demonstrated that 4G9 achieved an appreciable viable cell density within 10 days of seeding at 0.4e6 cells/mL (**Figure 5C**). Transfection parameters (defined in **Table 1**) were used for these reactions and AAV titers were measured as output of the conditions.

Parameter	Description
GOI	*cis*-ITR-CMV-GFP-IRES-fLuc (V2)
Volume	30 mL
Cell Density	2.0 x e6 cells/mL
DNA Quantity	45 µg
DNA Concentration	1.0 µg/ 1 e6 cells
Plasmid Ratio	pHelper : Rep2Cap8 : *cis*-ITR-CMV-GFP-IRES-fLuc
Transfection Reagent	FectoVIR®-AAV (Polyplus, now part of Sartorius)
Complexation Volume/Media	5% Gibco™ Opti-MEM™ (Thermo Fisher Scientific)
Complexation Time	15 min

Table 1. Representative transfection parameters for production of multiple AAV serotypes using the HEK293-SKPT pool and VPC2.0 cells at a 30-mL scale. To scale up to a larger volume, numbers were multiplied by a constant factor (i.e.., 30mL to 150mL = 5x increase in DNA, cells, and media).

It should be noted that numerous other process parameters required for optimal triple transfection were evaluated during a small-scale DoE study, such as the plasmid ratio, DNA:transfection reagent ratio, DNA:transfection reagent complexation time, ratio of DNA-to-cell number, and other factors known to influence the efficiency and productivity of transfection reactions (data not shown). At the conclusion of these experiments, we applied optimal process parameters for triple transfection and demonstrated excellent comparability of an AAV serotype panel (AAV1, 2, 5, 6, 8, and 9) between clone 4G9 and VPC2.0 cells (**Figure 5D**).

Scalability of transfection processes is essential to enable production of the larger quantities of viral vectors that will be required for many candidate gene therapies advancing through the clinic. Adequate performance (as measured by dPCR of harvest lysates) at very small-scale shake flasks (20-30mL), without purification, does not necessarily correlate to acceptable performance at large scale, even for suspension-adapted cell lines. Increasing production output to a level that allows for purification and subsequent viral analytics can portend expected outcomes at much larger scales.

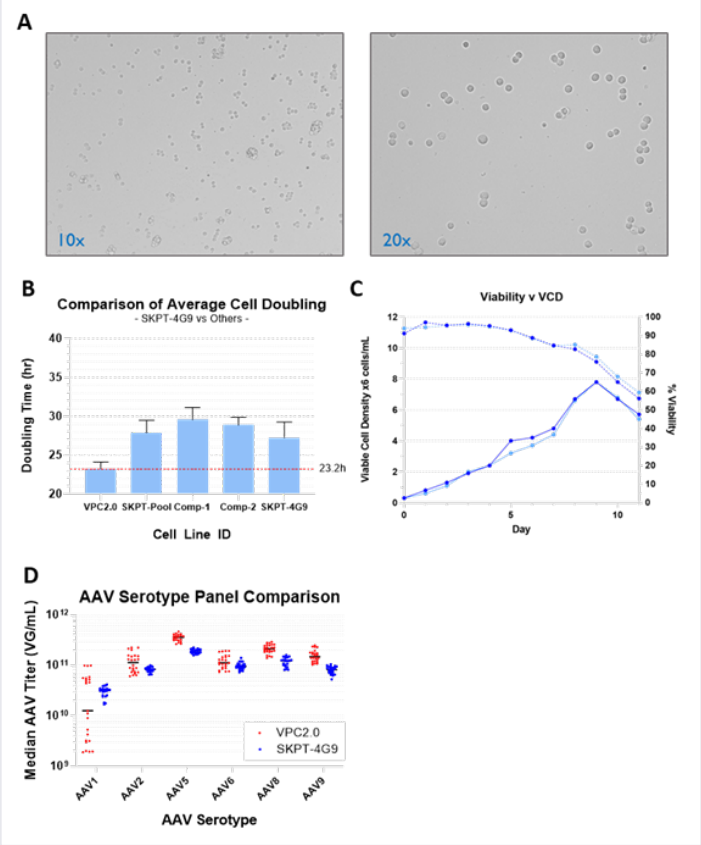

Figure 5. HEK293-SKPT clonal pool and subclone 4G9 growth characteristics. A. Bright-field microscopy of clone 4G9 at 10x (left panel) and 20x (right panel) magnification. B. Comparison of cell doubling time across a panel of cell lines. C. 4G9 expansion monitoring viable cell density and viability over a 12-day period. D. Transient, triple-transfection of AAV serotype panel comparison between cell lines. dPCR estimation of VG/mL titers at 72h post-transfection.

Towards that end, we generated AAV vector at 150mL scale to provide sufficient virus for AKTA-based purification and subsequent downstream analytical characterization. Typical process parameters for plasmid DNA:transfection reagent complexation and transfection were applied when performing transient transfection at the 150mL scale (referenced in **Table 1**). We chose AAV9 as the target virus, based on its excellent production characteristics. HEK293-SKPT subclone 4G9 was compared to its predecessor parental HEK293-SKPT pool and VPC2.0 cells (control). Two triple-transfection plasmid ratios were previously identified during small-scale DoE studies that demonstrated the highest yield of VG/mL titers and were, hence, compared in this small study.

Harvest, by detergent/salt lysis, was performed three days post-transfection. The virus was first purified using AKTA-based chromatography by loading the clarified harvest supernatant onto the POROS® Capture Select high-bind AAV9 affinity resin (Thermo Scientific). Eluted virus was buffer exchanged into no salt, pH>10 buffer, and subjected to anion-exchange chromatography (AEX) polishing using the Mustang® Q membrane (Pall Corporation). Separation of viral sub-species (empty, full, other) was achieved using an increasing salt step gradient.

Chromatograms obtained for AAV9 virus produced by HEK293-SKPT subclone 4G9 and the VPC2.0 control cells after the AEX polishing step are shown in Figure 6A&B. For both the HEK-SKPT pool and subclone 4G9, we observed one distinct peak, and depending on the ratio used for transfection, we noted several distinct peaks for VPC2.0 cells. All viruses, irrespective of the cell line used or plasmid ratio tested, showed A260 (**red**)>A280 (**blue**) traces, which is indicative of “full” AAV particles. For VPC2.0 cells, there was a very minor peak (#3 on right of chromatogram) identified where A260<A280 which is indicative of “empty” particles. Nonetheless, at the plasmid ratios tested, both yielded excellent full particle distribution in the AEX chromatograms.

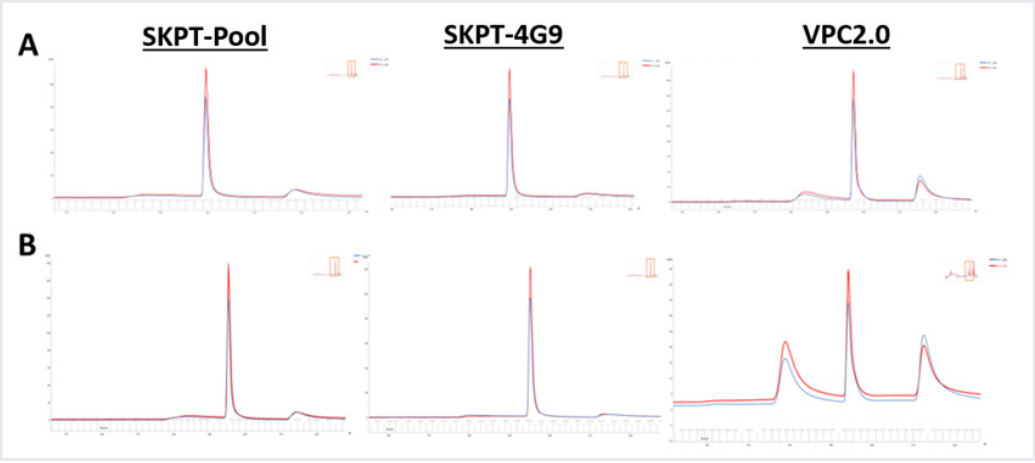

Figure 6. Anion-exchange Chromatogram of AKTA-affinity purified AAV9 virus with Analytical Ultra-Centrifugation. Two plasmid molar ratios were compared between the three cell lines and 2 plasmid molar ratios. A) Ratio #1 and B) Ratio #2 pHelper : Rep2Cap9: *cis*.

The fractions containing virus were pooled and virus titers were determined using digital PCR analysis of primer/probe sets targeting the gene-of-interest region of DNAse-resistant particles (**Figure 7**). The results obtained for the two different plasmid ratios are shown in left (Ratio #1) and right (Ratio #2) panels. All cells produced titers >1e11 VG/mL at harvest. Total mass balance across bulk harvest, affinity capture, and anion exchange demonstrated comparable yields across the three cell lines. Peak performance was noted for Ratio #1. Minimal loss of virus was observed across all downstream purification steps.

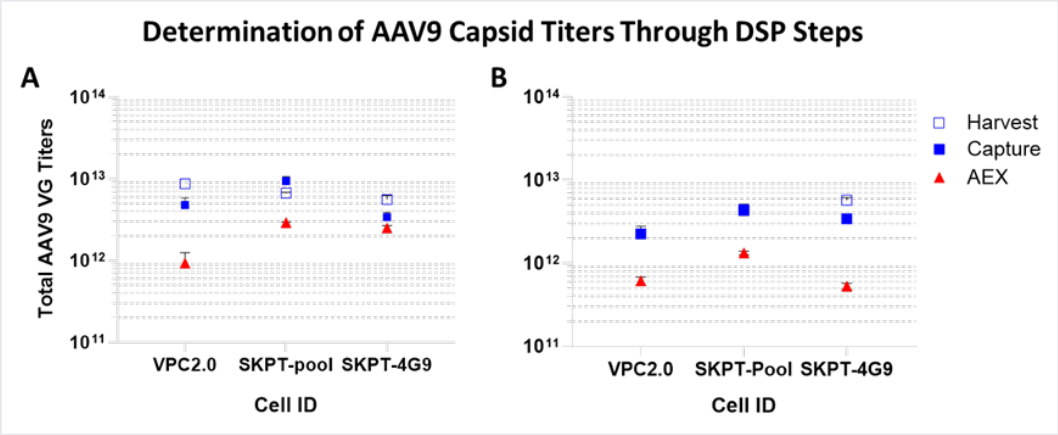

Figure 7: AAV9 Mass Balance Through Downstream Purification. Titers of transient triple transfection, 72h, post-harvest were determined at the molar plasmid ratios A) Ratio #1 and B) Ratio #2 (pHelper:Rep2Cap9:*cis*) by dPCR. Total mass balance at each process step (harvest, affinity, anion exchange) was determined by multiplying the dPCR titer x total volume.

To confirm the full particle distribution noted in Figure 6, the predominant peak fraction(s) were pooled and further analyzed by several orthogonal assays including sedimentation velocity analytical ultra-centrifugation (SV-AUC), mass photometry, A260/A280 ratio, and dPCR/ELISA. SV-AUC analysis of pooled fractions indicated that the bulk of particles from the predominant peak fraction(s) were indeed full particles (Figure 8A&B). These results were confirmed using an alternative technique, mass photometry9 (Refyn) (Figure 8C). Slightly better full particles were produced using the Ratio #1 vs Ratio #2 for clone 4G9 with close to 80% full particles observed.

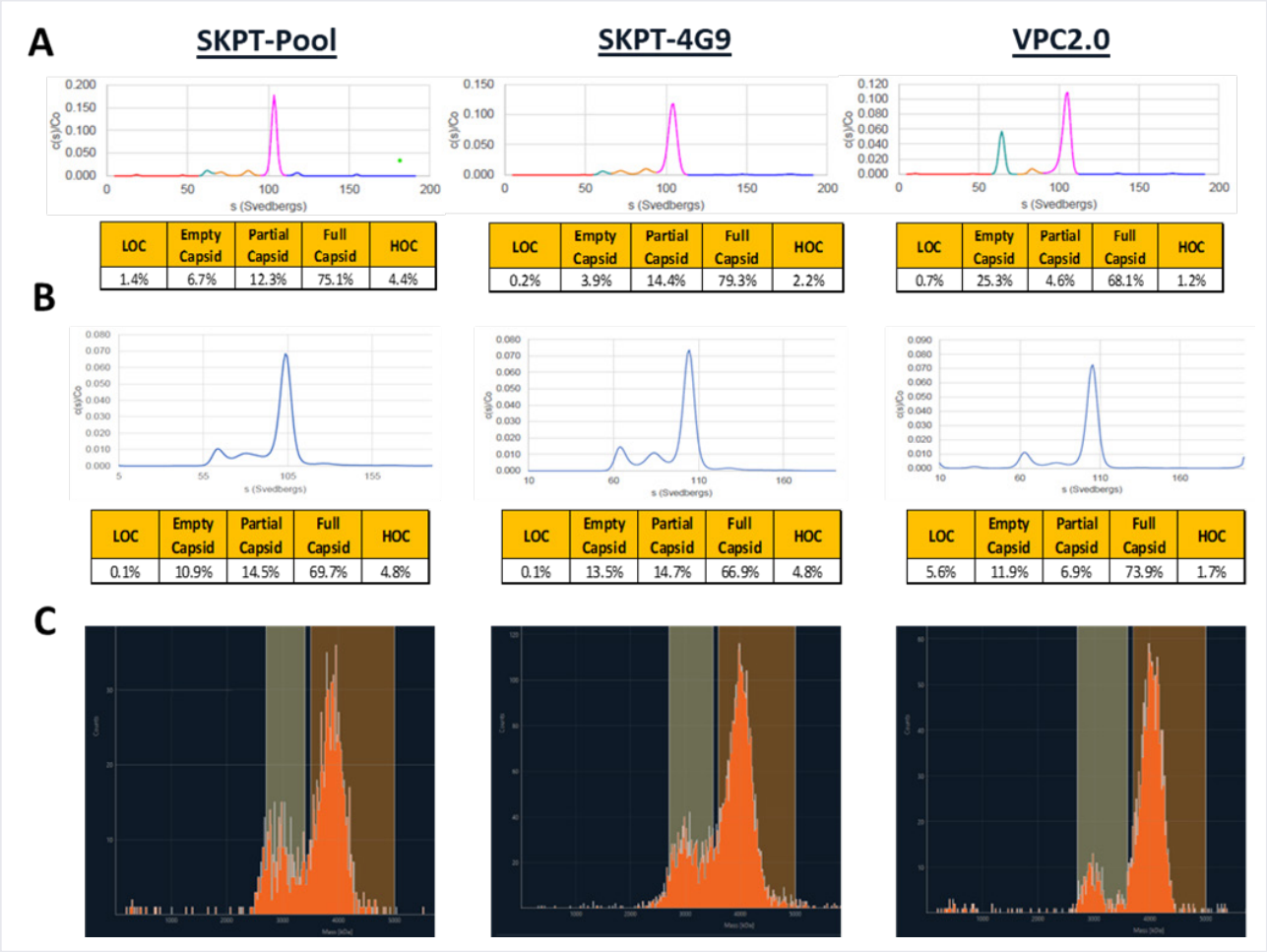

Figure 8: Analytical characterization of AAV9 from pooled AEX peak fraction. A) Sedimentation-Velocity Analytical Ultracentrifugation (SV-AUC) of peak fraction from triple transfection molar ratios A) Ratio #1 and B) Ratio #2. C) Mass photometry trace for the three cell lines using Ratio #1 for virus production.

Since the absorbance values were provided as a read-out on the AKTA system (chromatography in general), this information was used to predict full particle percentages quickly and efficiently. Using data sourced through the literature, it was possible to generate a standard curve plotting A260/A280 vs full particle (%). Once this curve was generated it was used for all further downstream predictions using absorbance ratios (**Figure 9A**). SDS-PAGE gel analysis revealed that neither the plasmid ratios nor the cell line impacted the stoichiometric incorporation of capsid bands VP1, VP2, or VP3 between the purified viral preps (**Figure 9B**). The results from SV-AUC, A260/A280, mass photometry, and other orthogonal methods measuring dPCR/ELISA or dPCR/stunner, were compiled to show which method accurately predicted full particles percentages. Not surprisingly, we found that absorbance, SV-AUC, and mass photometry showed excellent correlation at estimating full particle percentages, while dPCR over

ELISA/Stunner methods that measure the ration of dPCR titer over capsid quantification did not offer much predictive value (**Figure 9C**). Similar results were reported by Wehrle et al8. Rank order indicated the following full particles percentages at Ratio #1, 4G9>HEK293-SKPT-pool>VPC2.0 and at Ratio #2, VPC2.0>HEK293-SKPT-pool>4G9. It is worth noting that regardless of the cell line used, all cells produced excellent amounts of full particles. Other species of virus comprising empty and partially filled represented a very minor fraction (<15%) of the total peak fraction. To confirm that the virus was infectious, AAV2 packaged with a pCMV-Green Fluorescence Protein (GFP) *cis* plasmid were transduced on HEK293 adherent cells. 72h post-transduction, imaging using the EVOS7000 (Thermo) was performed (**Figure 9D**). 4 fields per view at 20x magnification were stitched together to create the image for each condition.

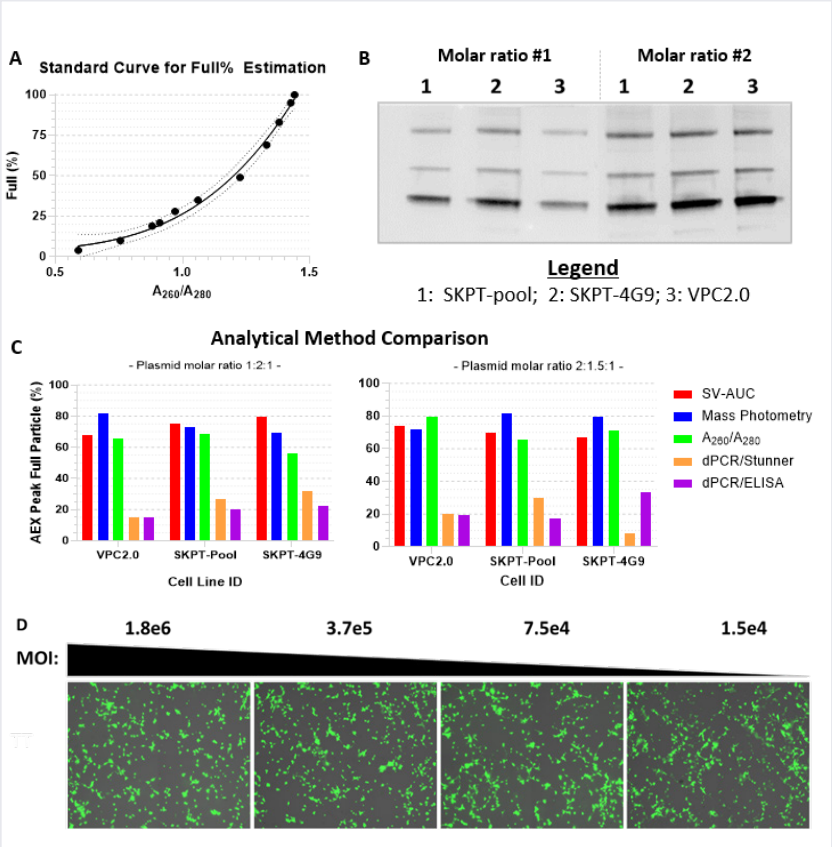

Figure 9: AAV9 Viral Analytics. A) Standard curve generation of Full (%) vs A260/A280 used for all calculations. B) SDS-PAGE analysis of purified AAV9 virus at the indicated plasmid ratios used during transfection. Bands indicated (top to bottom) VP1, VP2, VP3. C) Compilation of Full (%) estimation from the orthogonal method listed. Stunner (Unchained Labs) measured total intact protein capsids like ELISA (reported as VPs). D) AAV2 cell transduction assay on adherent HEK293 cells at the indicated multiplicity of infection (MOI) = VG titer added per cell.

Cis-Plasmid evaluation in HEK293-SKPT-4G9

The size of the *cis*-plasmid is a crucial factor in adeno-associated virus (AAV) packaging due to the limited packaging capacity of AAV vectors. AAVs can efficiently package approximately 4.7 to 5.0 kilobases (kb) of single-stranded DNA. Exceeding this limit can significantly impact the efficiency of viral packaging, transduction efficiency, and overall vector yield. Similarly, oversized genomes can lead to rearrangements or deletions during the packaging process which compromises the integrity of the packaged genome, thus further reducing the functional efficacy of the AAV vector yields[10,11].

To mitigate these issues, it is critical to optimize the size of the transgene and regulatory elements within the *cis* plasmid to stay within the optimal packaging range for AAV vectors. However, even at *cis* plasmids less than optimal (4.7kb), heterogeneity can still exist. Packaging heterogeneity refers to the variability in the composition and size of the DNA encapsulated within the AAV capsids12. This heterogeneity can affect the overall quality and consistency of the AAV vectors produced. Several critical factors contribute to this phenomenon including: 1) Homologous recombination when that occurs due when packaging sequences contain repetitive or homologous sequences; 2) Replication and encapsidation mechanism[13] during AAV production can that introduces variability, especially when the *cis*-plasmid is larger and still within the acceptable range, and 3) Production conditions14, mainly the type of producer cells used and the quality of plasmid DNA.

To address this, we created a series of *cis*-plasmids with packaging variability ranging from 2.1 kb (scAAV) and 4.24-4.55kb (ssAAV). Since promoters have complex sequence structure (GC-rich, hairpin structures, etc.) which can impact packaging, we examined a panel of diverse promoters and transgene sequences (**Figure 10A**).

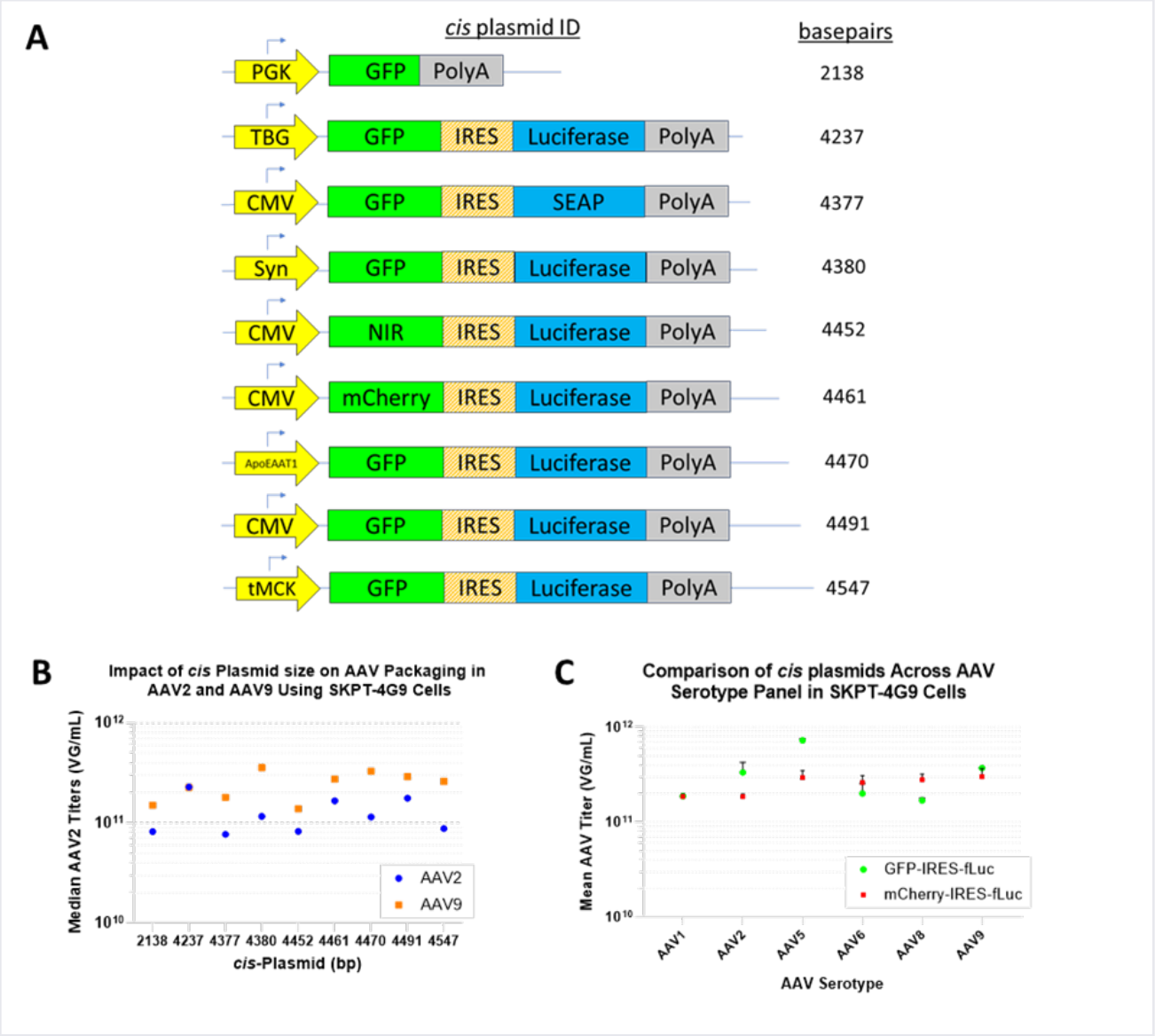

Figure 10. *cis*-Plasmid Comparison. A) Schematic diagram representing the *cis* plasmids used in this study with the corresponding base pairs. The first plasmid (PGK-GFP; 2138bp) is an scAAV construct. B) Median AAV titers of 72h harvest of triple transfected cells comparing all the plasmids outlined in (A) generated in AAV2 and AAV9 serotypes. C) Mean AAV titers of two *cis* plasmids produced in a panel of AAV serotypes.

Comparison Of Lentiviral Vector Production Between HEK293-SKPT-4G9 AND VPC1.0 Cell Lines

Concurrent with our screening efforts to identify HEK293-SKPT-4G9 as a clonal cell line for AAV production, we were simultaneously evaluating the ability of clonal lines to support lentivirus production. To simplify “platform” processes, we used FectoVIR as the transfection reagent of choice for these studies. Disappointingly, while the VPC1.0 cell line (Thermo) was able to support LVV production with FectoVIR, we found that 4G9 and other lead clones had much lower production levels (data not shown). To resolve this issue, we evaluated the Mirus VirusGEN® transfection reagents as an alternative. The Mirus platform is a combination of proprietary polymer/lipid formulations that cooperate with proprietary viral enhancers: RevIT™ for AAV or LV enhancer for LVV. When used in combination with VirusGEN® transfection platform, they can significantly improve virus production. We evaluated mini-DoE style experiments to optimize Mirus reagents to achieve optimal LV production (data not shown). Towards that end, we varied total DNA (ug/mL), the presence or absence of enhancers (RevIT™ or LV) and compared that to FectoVIR + Lentivirus enhancer. All conditions were tested using a fixed mass Ratio #3 representing LV trans-plasmid: gag-pol: VSV-G env: Rev, respectively. It was encouraging to note that, in the presence of LV enhancer, we rescued LVV production in 4G9 cells using a combination of either FectoVIR + or VirusGEN® + enhancer using a p24-antigen associated ELISA as a read-out.

To confirm these results, we cloned a trans-plasmid encoding a CD19 chimeric antigen receptor (CAR) under the control of the strong, ubiquitous viral promoter (SFFV) flanked by a WPRE regulatory element. This virus was crudely purified using a precipitation method (Lenti-X; Takara) and resuspended in PBS. To determine infectious titer, SupT1 cells, a human T lymphoblastic lymphoma cell line that is permissive to lentiviral infection, were transduced with a serial dilution of virus and transducing units (TUs) were determined as described in Kandell et al15. CD19 CAR-T expression was determined by a flow-based assay using the CD19 CAR-T detection reagent (Miltenyi).

At each corresponding dilution factor, the CAR-T expression on the surface of cells was plotted as (% +ve), (**Figure 11A, right axis**) and the formula shown in Figure 11B was applied to determine the Transducing Units/mL (TU/mL) (Figure 11A, left axis). The TU/mL titers obtained from flow were compared to those obtained by p24-antigen associated ELISA (Cell Biolabs) and plotted in Figure 11C. Lastly, virus copy number from SupT1 transduced cells was determined. Briefly, genomic DNA was collected and subjected to dPCR comparing the signal obtained from Viral Gene Copies to Housekeeping (GAPDH) Copies (Hs.PT.39a.22214847, IDT Validated Set). That ratio was divided by 2 (ploidy background for SupT1) (**Figure 11D**).

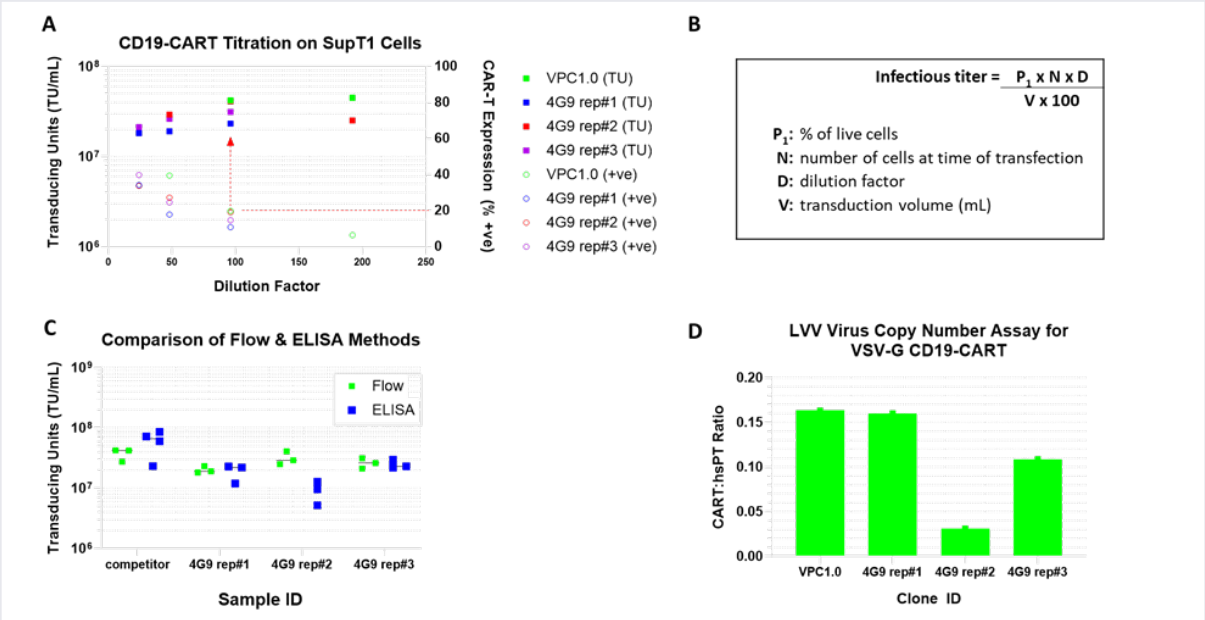

Figure 11. Lentiviral production comparison between HEK293-SKPT-4G9 and VPC1.0 (Thermo) cells. A) Cells were transduced with purified LVV generated in VPC1.0 or 4G9 cells and transduced on SupT1 cells at the indicated dilution factor. CAR-T Expression, as a percentage of total cells determined by flow cytometry (right axis). Calculated TU/mL titer (left axis) based on % positive expression. B) Calculation used to determine transducing unit/mL titers. C) Comparison of flow and ELISA-based methods to determine transducing unit titers. D) Virus copy number (VCN) assay to monitor integrated copies of lentivirus from SupT1 transduced cells.

Comparison Of Adenoviral Vector Production Between HEK293-SKPT-4G9 and EXPI293 Cells

Our existing cell line for Adenovirus utilizes Expi293F cells (Thermo). To understand the adenoviral production capability of HEK293-SKPT-4G9 versus Expi293F cells, we ran a head-to-head comparison for Ad virus production in a set of four 2-L single-use bioreactors. Cell culture samples were collected daily and analyzed on the BioProfile FLEX2 (Nova Biomedical) and Vi-CELL BLU (Beckman Coulter). The following performance metrics were summarized and reported: viability (**Figure 12A**), viable cell density (**Figure 12B**), and titers, as measured by ddPCR (**Figure 12C**), at time of harvest.

All (4) vessels were infected on day 3 with Helper Virus (target VCD of 0.50 E+06 vc/mL) and a MOI of 100 or 200. Cell count and metabolite sampling (data not shown) were performed daily. 48 hours post-infection, the bulk reactor contents were harvested. A single 500 mL aliquot from the presumed lead condition (SKPT-4G9, 200 MOI) was transferred to Downstream for purification development. When compared to Expi293F cells, our HEK293-SKPT-4G9 demonstrated similar VCDs and Ad Vector titers.

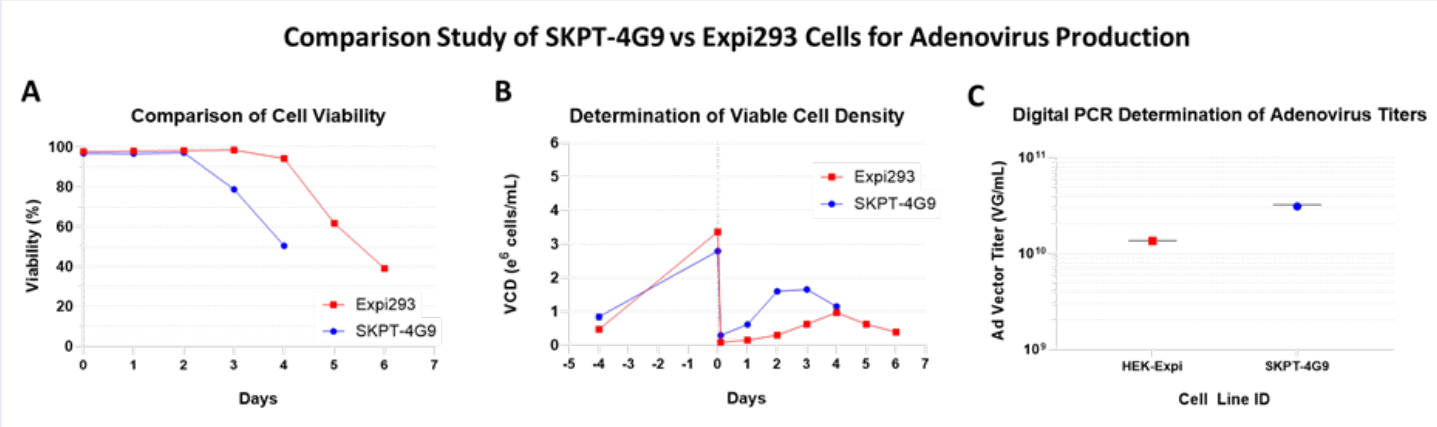

Figure 12: Adenovirus production comparison between HEK293-SKPT-4G9 and Expi293 cells (Thermo). A) Measurement of viability throughout the Ad virus production process. B) Viable cell density measurements starting 4 days prior to Ad virus production at the start, Day 0 (dotted line), and throughout the process. C) Estimation of Ad virus titers following downstream purification.

Developing An Optimal Viral Vector Manufacturing Platform Starting With A cGMP Cell Line

Large-scale viral vector manufacturing is crucial for the successful commercialization of promising gene therapies currently advancing through clinical trials. Achieving this goal presents numerous challenges. SK pharmteco developed a flexible, high-performing adeno-associated virus (AAV) manufacturing platform. This platform was based on a comprehensive evaluation of plasmid production, plasmid-transfection reagent complexation, triple transient transfection, and alternative plasmid formats to enhance performance.

In optimizing the transfection process, SK pharmteco employed a Design of Experiments (DoE) approach and state-of-the-art technology and created a high-performing, stable cell line for AAV, LV, and Ad vector production in suspension cell culture. Our viral vector platforms for manufacturing leverage this new cell line, which demonstrated performance on par with leading commercially available alternatives. Combined with our in-depth understanding of viral production, purification, and a flexible platform approach, our new cell line enables clients and partners to rapidly develop cost-efficient viral vector manufacturing solutions, producing target virus species in high titers with favorable full-to-empty ratios and minimal undesired virus species.

After extensive data evaluation, the HEK293-SKPT-4G9 subclone was selected as the lead subclone for all platform development and commercial manufacturing at SK pharmteco. A cGMP master cell bank with a full cell line history report has been completed. We continue to evaluate the line's performance in bioreactors and AMBR systems, as well as its application in viral packaging cell line development for lentiviral vectors (LVV) and AAV. These studies will be the focus of future white papers.

At-Scale Cost Savings of HEK-293T-4G9

Reducing the Cost of Goods Sold (CoGS) in viral manufacturing is essential for the long-term adoption of advanced therapeutics by healthcare providers and their payers. Several key areas within the viral manufacturing process offer significant opportunities for COGS reduction, including enhanced media feeds and supplementation, improved transfection reagents, novel lysis buffers, and alternative high-fidelity plasmids. By optimizing these raw materials, it is possible to achieve a cost savings of approximately 75% at manufacturing scale, which can translate into millions of dollars in annual savings (**Figure 13**).

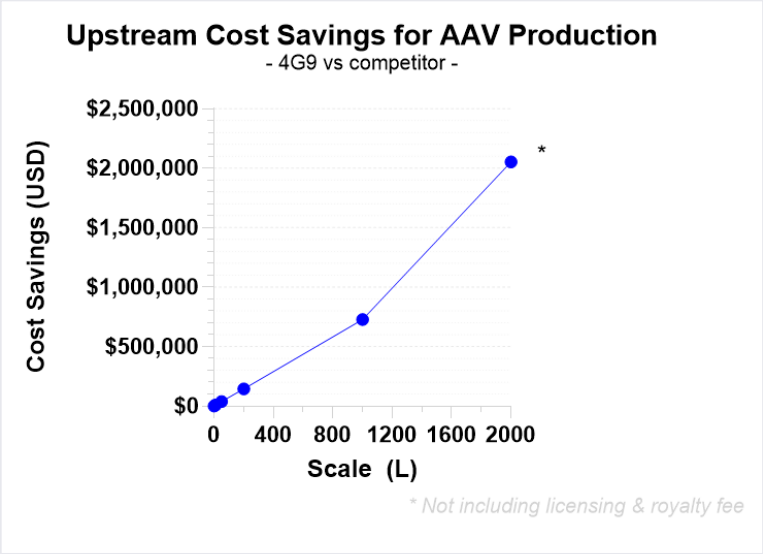

Figure 13. Cost Comparison Analysis. By implementing a platform process developed at SK pharmteco, with a focus on optimizing transfection reagents and custom media formulations, significant reductions in Cost of Goods Sold (CoGS) for large-scale manufacturing are achieved. When combined with SK pharmteco-manufactured and sourced plasmids, even higher cost savings are realized.

Although outside the scope of this report, SK pharmteco is actively exploring additional avenues to reduce manufacturing costs, such as sourcing alternative reagents, bulk purchasing, implementing stringent supply chain controls, and managing vendor relationships. Strategic facility design and the adoption of single-use technologies and disposable equipment can further reduce cleaning and validation costs while minimizing cross-contamination risks. By integrating lean manufacturing principles to minimize waste, applying Quality by Design (QbD) to address quality issues early, and harmonizing processes across sites through a dedicated regulatory team, a right-first-time approach can lead to substantial per-batch savings, significantly impacting overall COGS. The culmination of these cost-reduction efforts will ultimately expand advanced therapy administration beyond first-world health-care systems to enable global access.

Contact us for all your manufacturing needs and discuss these learning points:

- **Flexible viral vectors Manufacturing Platforms:** Learn about SK pharmteco's development of high-performing, and flexible viral vectors manufacturing platforms.
- **Optimization through DoE:** Explore the use of Design of Experiments (DoE) in optimizing the transfection process.
- **Innovative Cell Line Development:** Gain insights into the creation and application of stable, inducible packaging cell lines for AAV production.
- **Performance Comparison:** Review the performance of SK pharmteco's new cell line compared to leading commercially available cell lines.
- **Molecular Biology:** Comprehensive knowledge and capabilities to clone plasmids, promoters, and other regulatory motifs. Expertise in NGS assays for plasmid and viral vector characterization.
- **Future Studies:** Anticipate future developments in viral packaging cell line evaluation for both lentiviral vectors and AAV will be discussed in upcoming white papers.

References

1. Graham, F. L., Smiley, J., Russell, W.C., and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virolo 36, 59-74.
2. Denis Kole, Lenore Giannunzio, Cassie-Marie Peigné, and Timothy Cripe, "Round table discussion: Successful suspension-based viral vector manufacturing scale-up, from process development to clinic," Webinar, September 7, 2022 https://www.insights.bio/cell-and-gene-therapy-insights/webinars/350/Round-table-discussion-Suc- cessful-suspension-based-viral-vector-manufacturing-scale-up-from-process-development-to-clinics.
3. Renald Gilbert, "Key considerations for the use of suspension culture systems for viral vector manufacturing," Cell & Gene Therapy Insights 2020; 6(1), 143-148.
4. Ramin Baghirzade, "Adherent versus suspension-based platforms: what is the near future of viral vector manufacturing?" Cell & Gene Therapy Insights 2021; 7(11), 1365–1371.
5. Joshua C Grieger, Stephen M Soltys, and Richard Jude Samulski, "Production of Recombinant Adeno-associated Virus Vectors Using Suspension HEK293 Cells and Continuous Harvest of Vector From the Culture Media for CGMP
6. FIX and FLT1 Clinical Vector," Mol Ther . 2016 Feb;24(2):287-297.
7. Bingnan Gu et al., "Establishment of a scalable manufacturing platform for in-silico-derived ancestral adeno-associated virus vectors." Cell Gene Therapy Insights 2018; 4(S1), 753-769.
8. FUJIFILM Irvine Scientific poster with info on using supplemental BalanCD media feed.
9. Werle AK, Powers TW, Zobel JF, Wappelhorst CN, Jarrold MF, Lyktey NA, Sloan CDK, Wolf AJ, Adams-Hall S, Baldus P, Runnels HA. Comparison of analytical techniques to quantitate the capsid content of adeno-associated viral vectors. Mol Ther Methods Clin Dev. 2021 Sep 1;23:254-262.
10. Wagner C, Fuchsberger FF, Innthaler B, Lemmerer M, Birner-Gruenberger R. Quantification of Empty, Partially Filled and Full Adeno-Associated Virus Vectors Using Mass Photometry. Int J Mol Sci. 2023 Jul 3;24(13):11033.
11. Grieger JC, Samulski RJ. Packaging capacity of adeno-associated virus serotypes: impact of larger genomes on infectivity and postentry steps. J Virol. 2005 Aug;79(15):9933-44. doi: 10.1128/JVI.79.15.9933-9944.2005. PMID: 16014954; PMCID: PMC1181570.
12. Dong B, Nakai H, Xiao W. Characterization of genome integrity for oversized recombinant AAV vector. Mol Ther. 2010 Jan;18(1):87-92.
13. Barnes LF, Draper BE, Chen YT, Powers TW, Jarrold MF. Quantitative analysis of genome packaging in recombinant AAV vectors by charge detection mass spectrometry. Mol Ther Methods Clin Dev. 2021 Aug 26;23:87-97.
14. Bennett A, Mietzsch M, Agbandje-McKenna M. Understanding capsid assembly and genome packaging for adeno-associated viruses. Future Virol. 2017 Jun;12(6):283-297. doi: 10.2217/fvl-2017-0011. Epub 2017 Jun 8. PMID: 36776482; PMCID: PMC9910337.
15. Ibreljic N, Draper BE, Lawton CW. Recombinant AAV genome size effect on viral vector production, purification, and thermostability. Mol Ther Methods Clin Dev. 2024 Jan 17;32(1):101188.
16. Kandell J, Milian S, Snyder R, Lakshmipathy U. Universal ddPCR-based assay for the determination of lentivirus infectious titer and lenti-modified cell vector copy number. Mol Ther Methods Clin Dev. 2023 Sep 23;31:101120.

About SK pharmteco

Built on 80 years of experience, SK pharmteco is a trusted partner specializing in the manufacture of Active Pharmaceutical Ingredients (APIs), advanced intermediates, Cell and Gene Therapy technologies, registered starting materials, and analytical services for the pharmaceutical industry worldwide.

For your Cell and Gene Therapy needs, SK pharmteco operates two facilities on both sides of the Atlantic: one in the South of Paris, France and one in the Philadelphia, PA area. Together, they offer a full range of services throughout the advanced therapies process, from R&D to manufacturing and commercialization, all with a global presence.

Scan or click the QR code to learn more about our R&D offerings

Contact Us

www.skpharmteco.com

info@skpt.com

Authors

Brian Tomkowicz, PhD
Sr. Director, Research & Development

Marguerite Campbell
Associate Director, Research & Development

Kelly Geosits
Associate Scientist, Research & Development

Ngan Trinh
Associate Scientist, Research & Development

Srivanya Tummala
Associate Principal Scientist, Research & Development

Khan Umaer, PhD
Research Scientist, Research & Development

SK pharmteco