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COMMUNICATION

Towards a scalable bioprocess for rAAV production using a HeLa stable cell line

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Abstract

The majority of recombinant adeno-associated viruses (rAAV) approved for clinical use or in clinical trials areproduced by transient transfection using the HEK293 cell line. However, this platform has several manufacturing bottlenecks at commercial scales namely, low product quality (full to empty capsid ratio <20% in most rAAV serotypes), lower productivities obtained after scale-up and the high cost of raw materials, in particular of Good Manufacturing Practice grade plasmid DNA required for transfection. The HeLa-based stable cell line rAAV production system provides a robust and scalable alternative to transient transfection systems. Nevertheless, the time required to generate the producer cell lines combined with the complexity of rAAV production and purification processes still pose several barriers to the use of this platform as a suitable alternative to the HEK293 transient transfection. In this work we streamlined the cell line development and bioprocessing for the HeLaS3-based production of rAAV. By exploring this optimized approach, producer cell lines were generated in 3-4 months, and presented rAAV2 volumetric production (bulk) > 3×10^{11} vg/mL and full to empty capsids ratio (>70%) at 2 L bioreactor scale. Moreover, the established downstream process, based on ion exchange and affinity-based chromatography, efficiently eliminated process related impurities, including the Adenovirus 5 helper virus required for production with a log reduction value of 9. Overall, we developed a time-efficient and robust rAAV bioprocess using a stable producer cell line achieving purified rAAV2 yields > 1×10^{11} vg/mL. This optimized platform may address manufacturing challenges for rAAV based medicines.

KEYWORDS

bioprocess development, cell line development, downstream processing, gene therapy, HeLaS3, recombinant adeno-associated viruses (rAAV), stable cell line

José Escandell and Filipa Moura contributed equally to this work.

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1 | INTRODUCTION

Over the last 30 years, numerous proof-of-concept experiments in animal models have shown the therapeutic effectiveness of the recombinant adeno-associated virus (rAAV) vector for gene transfer. This preclinical data provides the framework for a comprehensive rAAV-based gene therapy pipeline to address several human disorders. Indeed, the number of gene therapy clinical studies based on rAAV vectors has increased significantly in recent years. As an example, rAAV vectors were employed as a delivery system in 39% of all gene therapy clinical studies in the United Kingdom in 2021, with this number increasing to 90% in in vivo gene therapy clinical trials (Catapult report, 2021). However, the doses required for systemic treatments in clinical trials, such as those for Duchenne muscular dystrophy, are beyond the current capabilities of Chemistry, Manufacture, and Control (CMC) if the commercial production (reviewed in Escandell et al., 2022). The primary manufacturing bottleneck is the use of transient production platforms, mainly using the HEK293 cell line (reviewed in (Escandell et al., 2022). While this technology seems cost-effective in delivering the rAAV product quantity needed for phase I/II clinical trials and/or nonsystemic dosages, it isn't viable at large scale since it lacks robustness and due to high cost of raw materials, such as Good Manufacturing Practices grade plasmid DNA. Therefore, the manufacturing process still poses a challenge to the commercial feasibility of rAAV-based therapies.

An alternative strategy for the industrial production of rAAV vectors is the use of producer cell lines (PCL). Recently, Cevec Pharmaceuticals described a tetracycline based inducible system for rAAV production using the HEK293 cell line, partially solving some scalability issues (Coronel et al., 2021). Nonetheless, the HeLa cell line and its clonal HeLaS3 derivate still remains the widely used stable cell line production system for rAAV-based gene therapy (reviewed in (Merten, 2016)). First described by Clark et al (Clark et al., 1995), this production system has been already used at the 2000 L bioreactor scale, with the potential for scaling up to larger volumes if needed (Thorne et al., 2009). To generate a rAAV PCL, HeLaS3 cells are stably transfected with a plasmid that includes cis-acting sequences (Rep-binding sites [RBSs]) and encodes for nonstructural trans elements (AAV nonstructural proteins Rep78/68) and structural CAP proteins (Liu et al., 2000) and relies on the infection of a helper virus to induce production, usually wild type adenovirus5 (wtAd5). This process yields high-quality rAAV, presenting full to empty capsid ratios (a Critical Quality Attribute (CQA)) of >50% and titers $>1 \times 10^{10}$ viral genomes (vg/mL) (Martin et al., 2013). Human papillomavirus E6 and E7 oncogenes present in HeLa cells have been shown to have a beneficial impact on rAAV production and capsid filling (Cao et al., 2012). However, the presence of E6 and E7 raises safety concerns due to the possible encapsulation of oncogenic DNA into the rAAV capsid. Notably, several studies have demonstrated a good safety profile associated with HPV genes during rAAV production with HeLa cells (Martin et al., 2013; Tatalick et al., 2005).

2 | GENERATION OF RAAV PCL USING THE HELAS3 CELL LINE

One of the drawbacks of establishing HeLaS3 production system as an industry standard is the need for a robust cell line development methodology to generate stable cell lines rapidly and consistently (reviewed in Merten, 2016). In this study, two distinct approaches were evaluated to optimize the generation of rAAV PCL in the HeLaS3 production system. To stablish the PCLs, we used a single plasmid carrying all information required to generate rAAV2 as previously described by Clark et al. (1995). This plasmid encoded for REP2 gene, CAP2 gene and Green Fluorescence Protein (GFP) as transgene. In Strategy 1, we adapted the stable cell line generation process commonly used to generate monoclonal antibodies PCLs using CHO cell line (Sehgal et al., 2018) to generate rAAV PCLs in HeLaS3 based production system (Figure S1). Briefly, the plasmid was transfected to HeLaS3 cells by nucleofection, followed by a 4-week selection process using an appropriate selection agent which in our case was blasticidin at 10 µg/mL. Cells were then seeded at low cell concentration (0.3 cells/well) in 96-well plates to isolate single-cell clones. After cells reached confluency of 50%, cells were infected with wtAd5 at a multiplicity of infection (MOI) of 12.5 viruses/cell. Four days postinfection, rAAV titer (intracellular and extracellular) was determined by quantitative polymerase chain reaction (qPCR). Although we screen 300 wells for rAAV productivity, none of them achieved titers above 10^8 vg/mL, value that is close to limit of detection of the technique (data not shown). We hypothesized that by continuing the selection pressure for an extended period of time, we selected cells with partial plasmid integrations, generating cell populations with the resistance gene but lacking essential genes for rAAV2 production. Therefore, to overcome the selection pressure of high producing stable cell clones, cells were seeded in a 96 well plate (Strategy 2) at a cell concentration of 5000 cells/well with blasticidin (10 µg/mL) 3 days posttransfection (Figure 1a). Following this aproach, potential producer clones could be screened, identified, and isolated sooner in the stable cell generation process. Notably, by using this system, we observed that 92% of the masterwells tested positive for rAAV2 production with 11% demonstrating titers $>1 \times 10^{10}$ vg/mL (Figure 1b).

Regulatory agencies require demonstration of PCL clonality before their use in biotherapeutics manufacture. Since masterwells produced by strategy 2 do not have single-cell origin, another round of single-cell cloning was needed. High producers masterwells were seeded at low cell density (0.3 cells/well) and imaged for clonality report (see example in Figure S2). Clones isolated from these outgrowths were evaluated for their ability to produce rAAV2, achieving titers greater than 1×10^{10} vg/mL and 1×10^5 vg/cell (Figure 1c). In addition, to demonstrate the robustness of the stable cell generation process herein developed, three other rAAV2 PCLs (each expressing a different transgene) were generated and were observed to display similar rAAV2 titer (Figure S3). One of the difficulties of the HeLaS3 manufacturing platform was that selecting PCL was a time-consuming and cumbersome process. However, the

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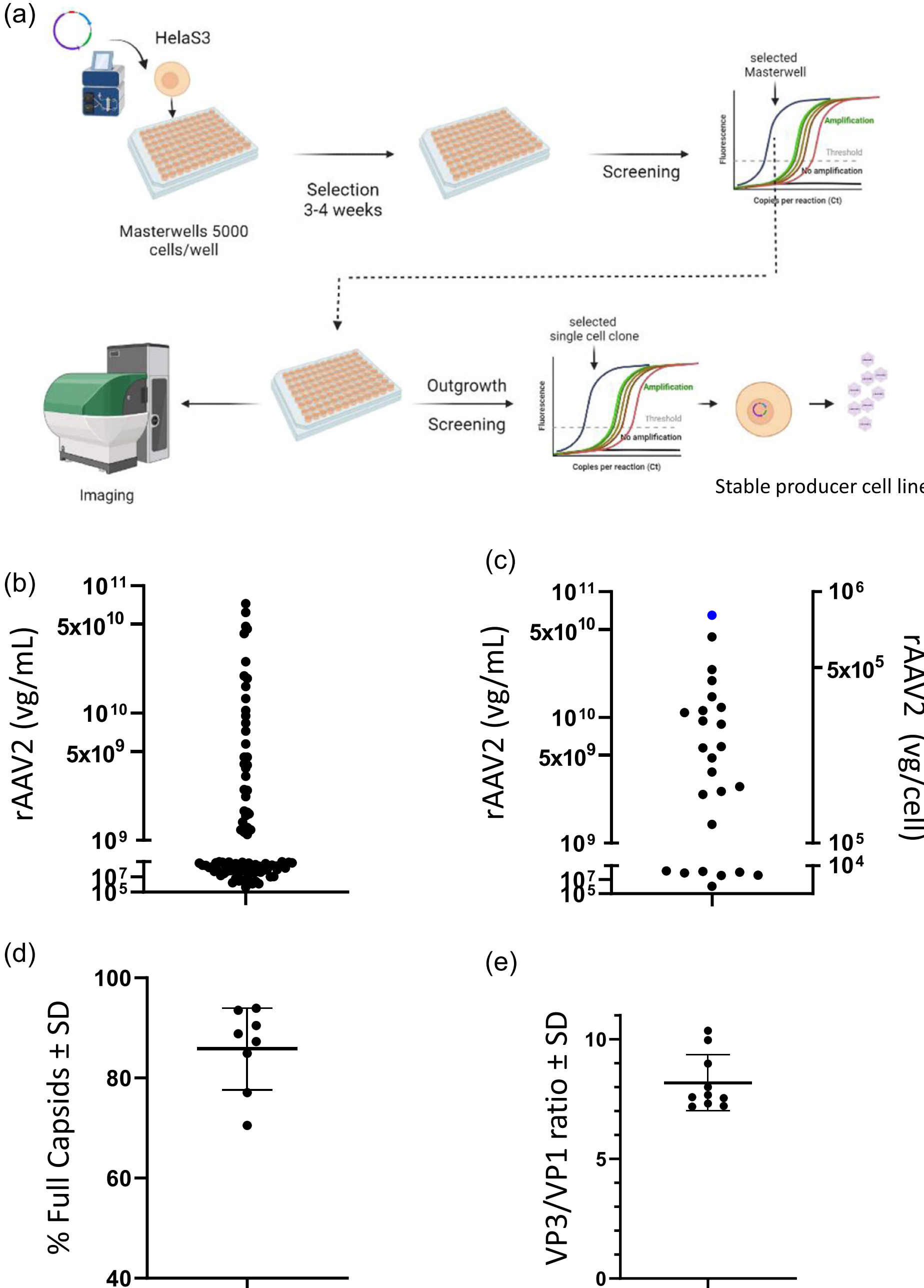

FIGURE 1 Generation of rAAV2 producer cell lines (PCLs). (a) Schematic representation of the workflow used (Strategy 2) to generate rAAV2 PCLs. HeLaS3 cells were transfected with triple play plasmid encoding REP2, CAP2 and the gene of interest. The cells were then seeded at high density (5000 cells/well) and selected with appropriate selection markers until cell outgrowth is detected. (b) 95 masterswells were screened for rAAV production by qPCR in DNAse-treated samples. The graph represents masterswells' rAAV2 titer. (c) Selected masterwell (H3) underwent another round of single-cell cloning as shown in Figure 1a. Thirty single-cell clones were screened for rAAV production. The graph represents the titer (vg/mL left Y axis and vg/cell right Y axis) for rAAV2. Clone H32D2 was chosen to continue the experiments, highlighted by the blue dot. (d) Graph represents full to empty capsid ratio (percentage) of Clone H32D2 in different production conditions. The percentage of full to empty capsid ratio was calculated by dividing vg/Total particles (TP)x100. vg was calculated either by qPCR or ddPCR. TP was calculated by ELISA. (e) VP3/VP1 Critical quality attribute analysis by capillary electrophoresis. 1×10^{11} vg of purified rAAV2 were labeled with 3-(2-Furoyl) quinoline-2-Carboxaldehyde dye and injected in several runs into a CESI Plus 8000 equipment. VP1 and VP3 peaks were integrated, and ratio was calculated by dividing VP3/VP1 normalized areas. Graphs represent VP3/VP1 ratio mean ± SD. ELISA, enzyme-linked immunosorbent assay; qPCR, quantitative polymerase chain reaction; rAAV, recombinant adeno-associated virus; SD, standard deviation.

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clone selection approach outlined here may be performed in under 4 months, solving this disadvantage.

The development of a PCL for recombinant viral vector manufacturing requires detailed characterization of individual clones combined with a comprehensive evaluation of vector quality. We performed the characterization of the rAAV2 produced by the selected HeLaS3 clone by assessing two CQAs, the ratio of full to empty capsids and viral protein (VP)3/VP1 ratio. The full to empty capsid ratio was higher than 70% in all experiments evaluated (Figure 1d), which is greater than previously observed values for other production systems including transient transfection methodologies (reviewed in (Merten, 2016). In the HeLaS3-based production system, wtAd5 E1A proteins and other adenoviral factors are responsible for the activation of endogenous rAAV promoters, triggering a biological response that stimulates the production of high-quality rAAV. This is also true for other adenoviral-based production systems, such as the non-replicative adenovirus production system (Su et al., 2022). Nonetheless, the ratio of full to empty capsids ratio on the platform reported by Su et al reached up to 20%, which is lower than our observations. Purified rAAV2 was also analyzed by capillary electrophoresis to estimate the VP3/VP1 ratio (Figures 1e and S4). The average of VP3/VP1 ratio of 8 was obtained which is consistent with previously reported VP3/VP1 ratio for rAAV2 particles using HeLaS3 based production system (Fernandes et al., 2022; Zhang et al., 2021) and close to wtAAV2 value of 10 (Oyama et al., 2021).

Another essential component in cell line development, besides single-cell origin and in-depth product analysis, is clone stability. Stability was assessed for clone H32D2, which was cultured over 90 generations and tested for rAAV2 production every 5-10 generations (Figure S5). This clone maintained population doubling times (PDT) below 30 h and viabilities above 95% through all generations (Figure 2a). Importantly, we did not see any reduction in rAAV production along passages. All these results demonstrate the robustness of this production system in terms of product quality and cell line stability.

3 | CRITICAL PROCESS PARAMETERS FOR HELAS3 PRODUCTION PLATFORM

A typical feature of viral-based processes is the cell density effect (CDE), a multicausal phenomenon describing the decline of viral vectors production yield with the increase in producer cell concentration (Dill et al., 2019). In fact, cell concentration at time of infection (CCI) is one of the main parameters influencing viral vector production. Therefore, we investigated the impact of CCI on virus titer. Clone H32D2 was seeded at 0.2, 1, and 2×10^6 cell/mL and infected with wtAd5. The highest titers were obtained when cells were infected at the concentration of 1×10^6 cell/mL, achieving $8 \times 10^{10} \pm 2.64 \times 10^{10}$ vg/mL (Figure 2b). At cell concentration of 2×10^6 cell/mL, titers were significantly lower ($1.7 \times 10^{10} \pm 2.17 \times 10^9$ vg/mL and $8 \times 10^3 \pm 1.13 \times 10^4$ vg/cell) thus demonstrating CDE (Figure 2c). Regardless of the cell concentration, full to empty capsid ratios were comparable across all three conditions (Figure 2d). Overall, the best CCI to obtain the highest titer while maintaining vector quality was 1×10^6 cell/mL.

The HeLaS3 production platform requires only two biological components, namely the producer cell line and the wtAd5 as helper virus. To have a robust platform, these components need to be well characterized. Indeed, one of the main factors regulating rAAV production in HeLaS3 production system is the MOI of the helper virus. Therefore, we aimed at optimizing infection conditions in our HeLaS3 production system. First, we assessed the infectivity capacity of wtAd5 virus stock in HeLaS3 cells. We used various wtAd5 virus stock dilutions to infect clone H32D2 in production media. After 24 h, cells were fixed and stained for wtAd5 Hexon 5 protein as readout of wtAd5 infected cells (Figure 3a). We determined that wtAd5 virus stock concentration was $6.3 \pm 0.6 \times 10^9$ Infectious Units (IU)/mL. Next, we investigated the best wtAd5 MOI to maximize rAAV2 production (Figure 3b). We infected clone H32D2 with wtAd5 at MOI ranging from 0.1 to 50 (IU/cell) in small-scale 20 mL shake flasks. Results showed that the optimal MOI for rAAV production was 0.5–3 IU/cell with titers $>3 \times 10^{11}$ vg/mL. This MOI range is 10-fold lower than the values described in (Liu et al., 2000). The referred publication describes a different rAAV bioprocess in the presence of serum which confirms that MOI is a critical process parameter that needs to be optimized for each production conditions.

Production of rAAV2 in clone H32D2 was done in 2 L stirred-tank bioreactor (STB). HelaS3 rAAV2 PCL was infected with wtAd5 using previously optimized conditions, that is, 1×10^6 cell/mL and MOI = 1 IU/cell. Cell viability decreased throughout time reaching 10% at 72 h postinfection (hpi), and rAAV2 titer achieved maximum value of 3.3×10^{11} vg/mL at 48 hpi and remained constant at 72 hpi (Figure 4a) with a full to empty capsids ratio of 87%. These results demonstrate the scalability of the production platform herein developed as high rAAV2 titers and quality were obtained.

4 | DOWNSTREAM PROCESSING EFFICIENTLY ELIMINATES WTAD5

The development of a HeLaS3 stable cell line-based production platform is further hampered by the need of helper viruses, to trigger rAAV2 production. Helper viruses (normally wtAd5) are considered a process-related impurity and thus must be removed during purification, significantly impacting downstream processing (DSP). In this study, we developed a DSP scheme to effectively remove wtAd5 during rAAV2 purification. Since we determined that rAAV2 were mainly located intracellularly (>80%), cell harvest was carried out by centrifugation, avoiding the need for concentration steps. Centrifugation was followed by a lysis and clarification step. Mustang Q membrane adsorber was used as the first purification step since it is established that this ion exchange chromatography (IEX) membrane is able to efficiently retain wtAd5 (Kawka et al., 2022). To optimize rAAV2 recovery while maximizing wtAd5 binding to the membrane, the ionic strength of the loading buffer (containing NaCl

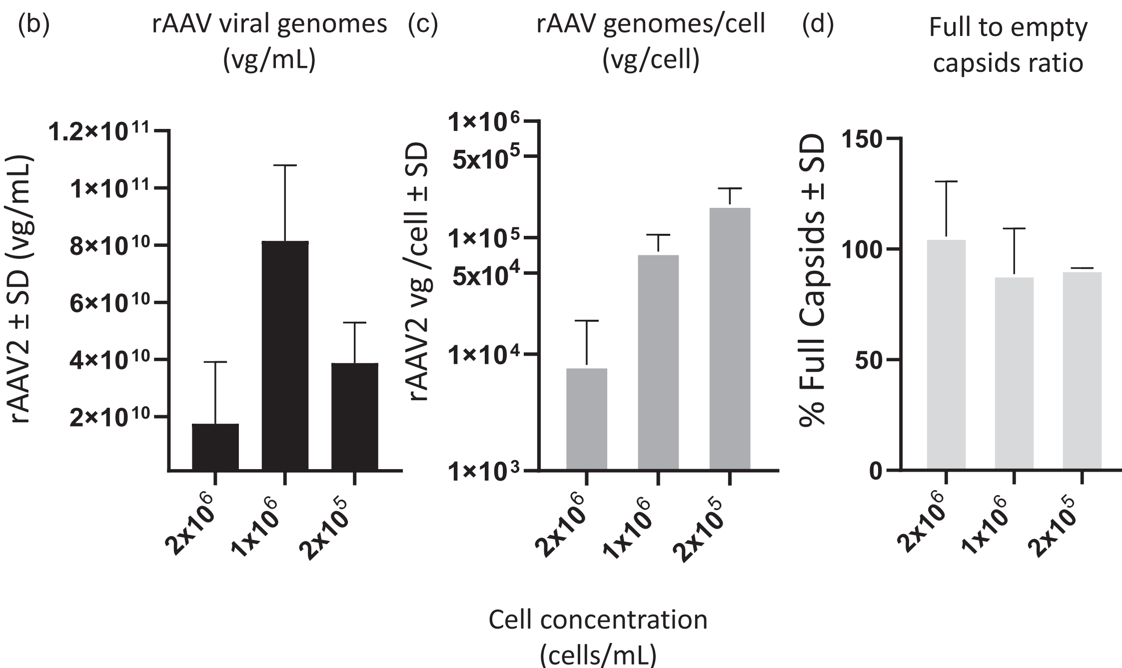

FIGURE 2 (a) HeLaS3 single cell clones' productivity remains stable throughout 90 generations. Graph representing PDT (h), cell viability (%) and rAAV2 titer (vg/mL). PDT was calculated with the following formula PDT (h) = ln (2)/μ where μ = (ln cell concentration$_2$ − ln cell concentration$_1$)/(time of measurement$_2$ (h)/time of measurement$_1$(h)). Impact of cell concentration upon infection on H32D2 clone parameters that determine productivity and vector quality Clone H32D2 was seeded at different cell concentrations and infected with wtAd5. Cell harvest was performed at 4 days postinfection. rAAV2 titer (vg/mL in (b) vg/cell in (c)) was determined by qPCR of DNAse treated samples. vg/cell was calculated by the following formula (rAAV2 vg/mL × cell concentration). (d) Percentage of full to empty capsids ratio was determined by dividing vg/TP. Standard deviation (SD) represents biological replicates of $n = 3$. PDT, population doubling times; qPCR, quantitative polymerase chain reaction; rAAV, recombinant adeno-associated virus.

concentrations ranging 200-400 mM) was evaluated. To mimic the production conditions, purified rAAV2 samples were spiked with wtAd5 (at a concentration of 10^9 vg/mL) before loading on the Mustang Q membrane, in five independent runs (see a representative chromatogram in Figure S6a). We determined that wtAd5 clearance was independent of the ionic strength in the range evaluated, achieving a 4-log reduction value (LRV) (Figure 4b). For all the evaluated conditions, rAAV2 recovery was close to 100%. Based on these results, the 350 mM NaCl condition was selected as it presents the highest recovery value, and it is also the most suitable for the next DSP stage. The second chromatographic step evaluated was the AVB affinity resin. This step was first assessed individually, that is, without having the Mustang IEX step in the purification train. The rAAV2 recovery obtained was higher than 60% with a 5 LRV wtAd5

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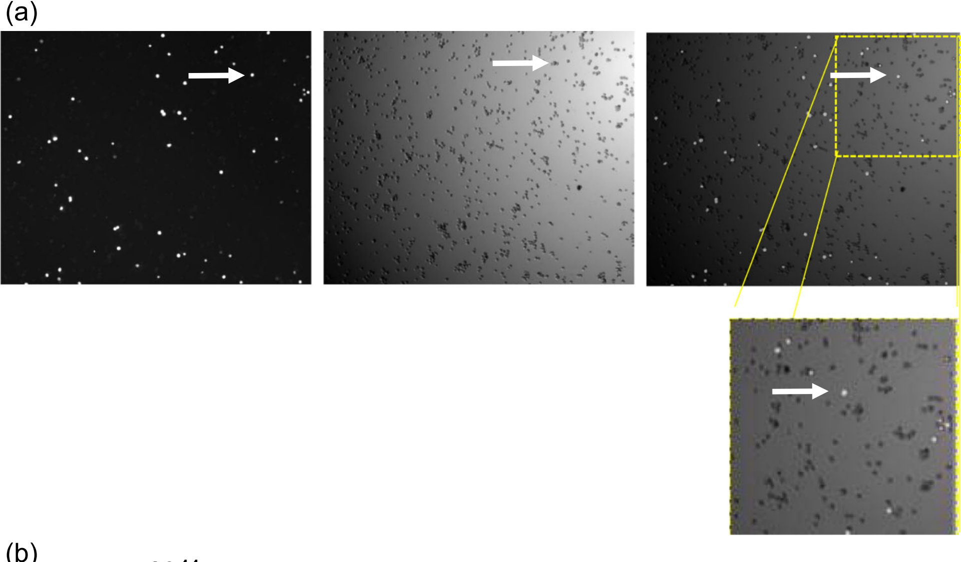

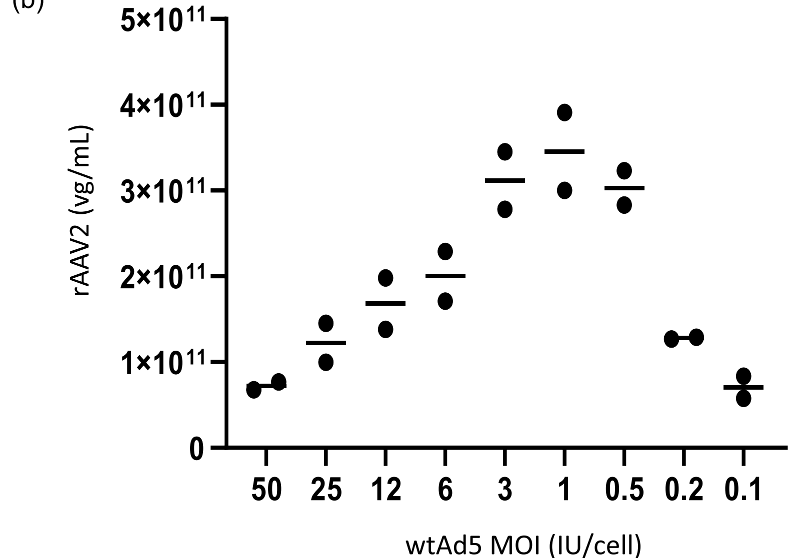

FIGURE 3 Helper virus MOI optimization to maximize rAAV titer. (a) Three serial dilutions of wtAd5 viral stock were used to infect HeLaS3 H32D2 clone in production conditions. After 24 h, cells were fixed with methanol and stained with Hexon5 antibody followed by incubation with Alexa488 secondary antibody. Green arrows represent wtAd5 infected (Hexon5 expressing) cells. 8000 cells (4 experimental conditions) were quantified to determine wtAd5 infectivity. (b) H32D2 clone was infected with wtAd5 at MOIs ranging from 50 to 0.1. After 4 days cells were harvested and rAAV2 titer was quantified by qPCR in DNAse-treated samples. MOI, multiplicity of infection; qPCR, quantitative polymerase chain reaction.

removal (see a representative chromatogram in Figure S6b). Based on the LRV measured for the individual chromatographic steps we could determine a theoretical value of 9 LRV for wtAd5 clearance.

The proof of concept for the complete DSP train was then evaluated using material generated in a computer-controlled 2 L STB, using cell pellets from the harvested culture bulk containing 3×10^{14} vg of rAAV2 and 10^{11} IU of wtAd5. After cell lysis, clarified bulk lysate was loaded onto the Mustang Q membrane adsorber, followed by AVB-based chromatography. AVB eluate was concentrated and formulated using a TFF membrane cassette (see scheme in Figure 4c and detailed protocol in Extended material and methods). Overall rAAV2 recovery was 32% (with a production yield $>10^{11}$ vg/mL) while wtAd5 infectious titer was reduced to levels below the limit of quantification of the assay used (~1×10^3 IU/mL). Notably, the full to empty capsid ratio of rAAV2 produced in the bioreactor (crude extract) was 86 ± 5% (measured by vg/TP ratio). This value is considerably higher than the ones obtained using transient transfection platforms (reviewed in (Merten, 2016)). Therefore, the HeLaS3 platform facilitates further DSP steps to purify full capsids. While rAAV2 is mainly located intracellularly, other serotypes are located extracellularly, impacting the DSP strategy. This DSP train has the potential to be adapted to other serotypes, applying small modifications depending on intra or extracellular virus' distribution and scale of production. Moreover, the removal of process-related impurities was also efficiently achieved: host cell DNA presented a 3 LRV and the final rAAV2

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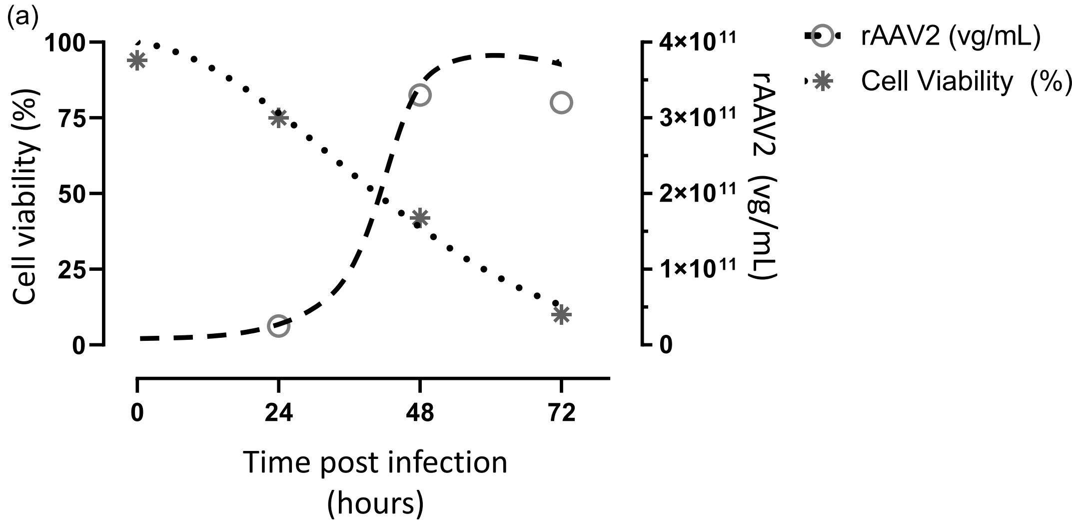

(b)

NaCl (mM)	rAAV2 recovery (%)	WtAd5 (LRV)
200	84	4
250	100	4
300	99	4
350	102	4
400	97	4

(c)

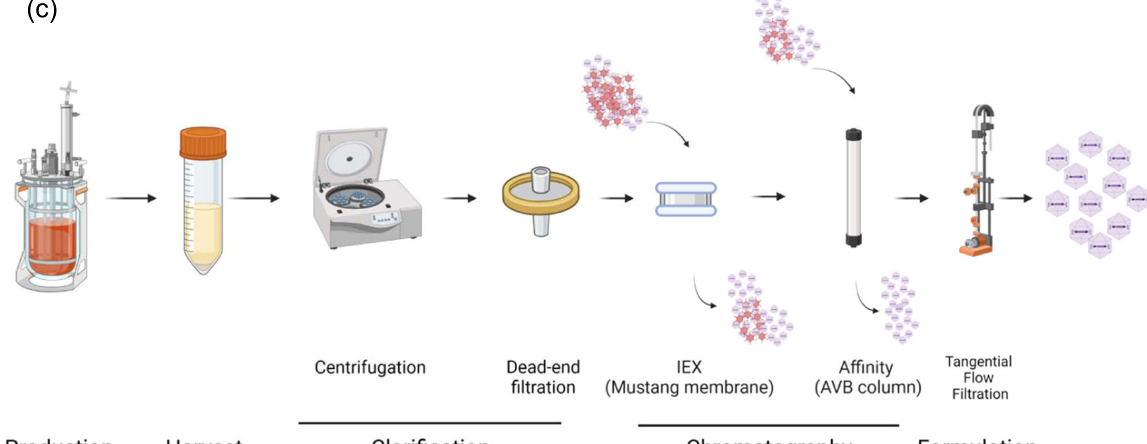

FIGURE 4 rAAV2 bioprocess with high volumetric productivities and expected purity (a) Production of rAAV2 at bioreactor scale (1 L). From a 3-day culture (N-1 seeded at 0.5×10^6 cells/mL) a STR was seeded at 1×10^6 cells/mL. Cells were then infected with wtAd5 (MOI 1) to trigger rAAV2 production. Samples were collected every 24 h to measure percentage of cell viability (black stars) and rAAV2 productivity (open circles). **B)** wtAd5 and rAAV2 spiked sample was purified with different ionic strengths ranging from 200 to 400 mM NaCl using a IEX Mustang Q membrane adsorber. rAAV2 particles were recovered in the flow through. The table represents log reduction value (LRV) of wtAd5 and rAAV2 recovery (percentage). **C**) Schematic representation of the of DSP workflow. rAAV2 was produced in stirred tank bioreactor with Clone H32D2. The DSP consisted in a clarification step (centrifugation and filtration) followed by AEX (Mustang Q) and affinity chromatography (Capto AVB column) and tangential flow filtration.

purity was 99.6% (Figure S7a). We also measured, by multi angle dynamic light scattering (MADLS), the particle size in the final formulation. Analysis showed the presence of a single peak corresponding to a 20 nm particle size (Figure S7b) which is concomitant with rAAV2 particle size (Colomb-Delsuc et al., 2022) and suggests that rAAV2 was not aggregated in final formulation.

Risk reduction for a wider range of potential adventitious agents may be improved by combining purification and inactivation

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processes. For relevant viruses, the process should have sufficient clearance capability to provide an adequate degree of product safety, and the incorporation of an inactivation step is recommended by ICH guidelines. Because of the well-established differential thermostabilities of AAV and wtAd5, heat inactivation easily provides 6 LRV of clearance (Thorne et al., 2008). In addition, nanofiltration of wtAd5 provides an additional 6-7 LRV (reviewed in (Merten, 2016). within summary, combining our downstream process along with international council harmonization, we calculate a theoretical 20-21 LRV for wtAd5 clearance, which would retrieve the necessary safety profile for the purified product.

Overall, this work describes a time-efficient and robust method for the development of producer cell lines to produce rAAV2, which could potentially be used to other serotypes. Additionally, it describes a scalable bioprocess to produce rAAV2 vectors where high titers of AAV2 with required purity and quality were obtained. This platform can help overcome bottlenecks in CMC, such as high cost of raw materials or low productivities when scale up is needed and shows potential to streamline the development of commercial rAAV-based gene therapy products.

5 | MATERIAL AND METHODS

5.1 | Cell lines

HeLaS3 (ATCC CCL-2.2) cell line were routinely maintained as adherent monolayer culture in Dulbecco's Modified Eagle Medium (DMEM) (10-013-CV, Corning) supplemented with 10% (V/V) Fetal Bovine Serum (FBS) (1027016, Gibco) in T-flasks in a 5% CO_2 atmosphere, at 37°C. When adapted to suspension, cells were routinely sub-cultured at 0.2×10^6 cell/mL every 3–4 days when cell concentration reached $2–3 \times 10^6$ cell/mL in EX-CELL HeLa Serum-Free Medium (14591 C, Sigma-Aldrich) with 6 mM L-glutamine. Cells were maintained in a 5% CO_2 atmosphere, at 37°C and with agitation rate of 125 rpm (25 mm orbital diameter).

5.2 | Clone generation process

HeLaS3 transfected cells with appropriate plasmids (detailed transfection protocol in Extended material and methods section) were seeded a cell concentration of 5000 cells/well (masterwells) in 96-well plates using DMEM with 4 mM L-Glutamine, 10% (V/V) FBS with appropriate selection marker. When wells reached 80% confluency, approximately 12 days after seeding, the masterwells were tested for rAAV2 production as described below. The wells that showed high levels of rAAV2 production, were selected to generate single-cell clones. The selected masterwells were seeded at a cell density of 0.3 cell/well in DMEM with 10% (V/V) FBS, 4 mM L-Glutamine, and appropriate selection marker in a 96-well plate. Single-cell clones were further tested for rAAV2 production as described below.

5.3 | Production of AAV2 in bioreactor

AAV2 production stirred tank bioreactor was performed in a BIOSTAT B-CDU 2 L vessel (Sartorius) using HeLaS3-rAAV2 producer (clone H32D2) cell line. Bioreactor headplate comprised multiple ports for temperature, pH, and pO_2 sensors, as well as for additions (such as culture media, cells, and wtAd5) and sampling/harvesting the cell culture. The bioreactor was equipped with a single Rushton impeller, and gas was supplied through a ring sparger at a flow rate of 0.01 vessel volume per minute (VVM). Partial pressure of oxygen (pO_2) was maintained at the setpoint of 50% by varying agitation rate between 70 and 270 rpm and the percentage of O_2 in the gas mixture between 0% and 100%. The culture pH was maintained at 7.30 by addition of 1 M $HNaCO_3$. Bioreactor was operated at a working volume of 1 L, inoculated at a cell concentration of 1×10^6cell/mL, and infected immediately with wtAd5 at an MOI of 1 IU/cell.

5.4 | Downstream processing

HeLaS3-rAAV2 producer (clone H32D2) cells were harvested at 72 hpi by centrifugation at 300 g for 5 min at 20°C. Cell pellets were resuspended in lysis buffer (described in supplementary methods) and incubated for 1 h at 37°C with 150 Units/mL of Benzonase (Merck) with mild agitation. The lysate was clarified by a centrifugation step at 4000 *g* for 5 min followed by filtration using a 0.8/0.2 µm Sartopore 2 XLG (5445307 GV –LX—C (300 mL scale) or 5445307GS —FF (1 L scale), Sartorius Stedim Biotech GmbH). The first purification step consisted of anion-exchange chromatography using a Mustang Q membrane adsorber Sartoscale 25 or XT5 capsule, depending on the scale (MSTGXT25Q16 or XT5MSTGQPM6, Pall Corporation). The flow through was collected and loaded onto the second purification step, carried out using a HiTrap Capto AVB column (17372211, Cytiva), 1 mL or 3 × 1 mL, depending on the scale. The elution fraction containing the rAAV2 was collected. Both chromatographic steps were performed in an ÄKTA Avant 25 system (Cytiva). The rAAV2 fraction was further concentrated and formulated by tangential flow filtration using a Sartocon Slice 50 Eco 100 kDa (3D91466850MLLPU, Sartorius). Detailed protocol in Extended material and methods.

5.5 | rAAV2 total particles and packaged vector genomes quantification

Cells were lysed using a solution of 0.5% Sodium-Deoxycholate, 2 mM of Magnesium chloride with 60 Units of Benzonase (101695001, Merck) and incubated for 1 h 30 min at 37°C. For total particles quantification an ELISA specific for the AAV2 serotype was performed (PRATV, Progen). To quantify viral genomes cells extracts were then treated with 60 U/mL DNAse (PROMM6101, Promega) for 30 min at 37°C followed by Proteinase K digestion treatment for

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30 min at 37°C. Proteinase K was inactivated by heat for 20 min at 95°C. The qPCR was performed in Light Cycler 480® (Roche) using SYBR Green detection reagent (04707516001, Roche). Digital PCR was performed in the QX200 AutoDG Droplet Digital PCR system (Bio-Rad) using the detection reagent kit for probes (1863024, Bio-Rad). In both systems primers for BGH polyA sequence (Fw: 5′- TCTAGTTGCCAGCCATCTGTTGT-3'; Rv: 5′-TGGGAGTGGCAC CTTCCA-3′) were used for rAAV2 quantification.

5.6 | Multiattribute measurement of rAAV purified product

To measure purity, 10^{11} vg of purified rAAV2 were labeled with the ATTO-TAG™ FQ Amine-Derivatization Kit (A2334, Life Technologies) following the protocol described elsewhere (Fernandes et al., 2022) and injected in the automated capillary electrophoresis equipment CESI 8000 Plus system (SCIEX). Detection was performed with a laser-induced fluorescence (LIF) detector module containing a 488 nm excitation and 600 nm emission filters. Particle size was determined by MALDS using the Zetasizer Ultra equipment (Malvern Panalytical). To measure DNA content, the Quant-iT PicoGreen dsDNA Quantification kit (P7589, Thermofisher) was used following the manufacturer's instructions.

5.7 | wtAd5 infectious units' quantification

To quantify the wtAd5 infectious particles, AdEasy Viral titer kit (972500, Agilent) was used according to the manufacturer's instructions. Briefly, HeLaS3 cells adapted to suspension were used to titrate the wtAd5 stock. wtAd5 stock was produced at iBET from ATCC source (VR-1516). Cells were infected in production media and after 24 h were fixed overnight with methanol at −20°C. To detect wtAd5 infected cells, cells were incubated with primary mouse anti-wtAd5 hexon protein antibody. Detection was carried out by either a secondary antibody conjugated with horseradish peroxidase followed by Diaminobenzidine substrate detection or with Alexa 488-conjugated goat anti-mouse antibody. Images were acquired in a Cytation 3 equipment (Agilent Biotek).

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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