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Development of an HEK293 Suspension Cell Culture Medium, Transient Transfection Optimization Workflow, and Analytics for Batch rAAV Manufacturing

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Keywords: analytical method development | design of experiments (DOE) | media formulation | recombinant adeno-associated virus (rAAV) production | suspension HEK293 cell culture | transient transfection optimization

ABSTRACT

Recombinant adeno associated virus (rAAV) vectors have become popular delivery vehicles for in vivo gene therapies, but demand for rAAVs continues to outpace supply. Platform processes for rAAV production are being developed by many manufacturers, and transient chemical transfection of human embryonic kidney 293 (HEK293) cells is currently the most popular approach. However, the cutting edge nature of rAAV process development encourages manufacturers to keep cell culture media formulations, plasmid sequences, and other details proprietary, which creates hurdles for small companies and academic labs seeking to innovate in this space. To address this problem, we leveraged the resources of an academic-industry consortium (Advanced Mammalian Biomanufacturing Innovation Center, AMBIC) to develop an rAAV production system based on transient transfection of suspension HEK293 cells adapted to an in-house, chemically defined medium. We found that balancing iron and calcium levels in the medium were crucial for maintaining transfection efficiency and minimizing cell aggregation, respectively. A design of experiments approach was used to optimize the transient transfection process for batch rAAV production, and PEI:DNA ratio and cell density at transfection were the parameters with the strongest effects on vector genome (VG) titer. When the optimized transient process was transferred between two university sites, VG titers were within a twofold range. Analytical characterization showed that purified rAAV from the AMBIC process had comparable viral protein molecular weights versus vector derived from commercial processes, but differences in transducing unit (TU) titer were observed between vector preps. The developed media formulation, transient transfection process, and analytics for VG titer,

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capsid identity, and TU titer constitute a set of workflows that can be adopted by others to study fundamental problems that could improve product yield and quality in the nascent field of rAAV manufacturing.

1 | Introduction

Adeno-associated virus (AAV) vectors are used in nearly half of viral gene therapy clinical trials due to their wide serotype-dependent tissue tropism, nonpathogenicity, and low immunogenicity compared to other viral vectors (Au et al. 2022; Chancellor et al. 2023). Six recombinant AAV (rAAV) therapies have been approved by regulatory agencies (Alomari 2023; Chancellor et al. 2023), and hundreds of clinical trials for rAAV products are ongoing (Shen et al. 2022; Zhao et al. 2022). However, current manufacturing processes cannot keep up with demand for the increasing number of rAAV therapies, as existing rAAV production systems produce orders of magnitude fewer doses per batch than established processes for antibody production (Wright 2022).

The most common platform used to manufacture rAAV vectors is transient transfection of human embryonic kidney 293 (HEK293) cells (Ayuso et al. 2010; Fu et al. 2023b; Ou et al. 2024). In this system, DNA sequences required for rAAV production, the AAV replication (*rep*) and capsid (*cap*) genes, adenovirus helper genes, and a therapeutic gene cassette flanked by the native AAV inverted terminal repeats (ITRs), are supplied across three plasmids. This platform is adaptable to the manufacture of different therapies by changing plasmid DNA sequences encoding the capsid and therapeutic genome, and can be set up in a new facility upon procurement of a cell culture medium, a host cell line, and plasmid DNA (Coplan et al. 2024; Zhao et al. 2020). However, the variable nature of transient transfection compounded with differences between media formulations, HEK293 hosts, and plasmid sequences between platforms has made it difficult for the field to establish consistent and high-producing upstream processes. Inter-process comparisons are also complicated by differences in methods used to measure rAAV genome, capsid, and functional titers (François et al. 2018; Fu et al. 2019; Gimpel et al. 2021; Kontogiannis et al. 2024; Wang et al. 2020).

Formulation of a robust cell culture medium is a crucial first step in establishing a therapeutic protein production platform as it determines how effectively cells can grow and produce the protein of interest. Media should be free of serum or animal components for safety and reproducibility reasons, but the exact ingredients needed in a chemically defined formulation are cell line and product dependent (Ou et al. 2024; Petiot et al. 2015). Progress has been made to identify lipid components that are sufficient replacements for serum (Miki and Takagi 2015; Rodrigues et al. 2011) and define additional ingredient groups required to sustain mammalian cell cultures including amino acids, vitamins, and trace metals (Chaderjian et al. 2008; Kim et al. 2005; Shen et al. 2010; Takagi et al. 2017). However, commercial media formulations are often proprietary, making it challenging for small companies and academics to select ingredients for optimization studies (Cordova et al. 2023). As a result, many published reports investigating how media formulation affects HEK293 culture performance have been performed with non-chemically derived components such as serum and peptones, study the effect of single components without knowing their base levels, or provide blind comparisons of proprietary commercial media or feeds (Celebi et al. 2022; Liste-Calleja et al. 2014; Ou et al. 2024; Petiot et al. 2015; Shen et al. 2010). Several suppliers now offer media panel optimization services to find the best chemically defined media for a customer's cell line (Martin and Zatina 2021), but this approach obscures information that would be necessary for researchers to systematically explore novel formulation adjustments. Identification of a core group of ingredients that support robust cell growth, transient transfection efficiency, and rAAV yield would help more organizations establish industrially relevant processes.

Baseline transfection processes can be established for media screening, but it is advantageous to optimize rAAV transient transfection parameters after a final medium formulation is selected. Continuous variables such as viable cell density (VCD) at transfection and parameters for plasmid DNA complexation have been studied most frequently, and there are reports investigating discrete variables such as the choice of host cell line, media, or transfection reagent (Chen et al. 2024; Coplan et al. 2024; Fu et al. 2023a; Grieger et al. 2016; Gu et al. 2018; Guan et al. 2022; Zhao et al. 2020). Optimization of continuous process parameters such as plasmid ratios is often done using a multi-staged design of experiments (DOE) approach that first identifies high impact parameters and then hones their ranges to maximize titer and refine other product quality attributes (Mandenius and Brundin 2008; Politis et al. 2017). This approach has been successfully employed for optimization of PEI-mediated triple transfection batch processes and has enabled 1.2- to 17-fold vector genome (VG) titer increases depending on the rAAV serotype and production platform (Coplan et al. 2024; Zhao et al. 2020). Mechanistic modeling approaches have also been applied to optimize rAAV production. These studies have shown that the offset timing of *rep* and *cap* expression contributes to the high percentage of empty capsids in transiently transfected batch cultures, which reveals additional upstream process parameters to optimize (Nguyen et al. 2021; Nguyen et al. 2024; Srinivasan et al. 2024).

The selection of culture mode is also important when choosing a workflow for process optimization. Most studies published to date employ batch cultures grown in shake flasks or bioreactors as the base system for transient transfection optimization (Chen et al. 2024; Coplan et al. 2024; Fu et al. 2023a; Grieger et al. 2016; Gu et al. 2018; Guan et al. 2022; Zhao et al. 2020), but some recent studies have used perfusion culture (Deng et al. 2025; Mendes et al. 2022; Nguyen et al. 2024; Park et al. 2024a). While some published batch culture workflows used media exchange or top off steps post-transfection (Grieger et al. 2016; Guan et al. 2022) and glucose or sodium butyrate containing feeds (Coplan et al. 2024; Zhao et al. 2020), all of

these cell culture systems have a single transfection step followed by ~72 h of rAAV production before harvest. The relatively short duration of batch culture allows for efficient screening of transfection parameters, culture conditions, and culture media, but the maximum VCDs achieved by these systems are limited. Culturing cells in perfusion enables the introduction of fresh nutrients throughout rAAV production, subsequently extending batch duration and increasing the maximum VCD achieved. rAAV VG titers have been shown to increase up to 3.4-fold in perfusion cultures versus batch cultures (Deng et al. 2025; Mendes et al. 2022; Nguyen et al. 2024; Park et al. 2024a). Interestingly, some of these VG increases have been achieved after multiple transfections were performed during culture periods of up to 15 days (Nguyen et al. 2024). These results show that while many of the same transfection parameters must be optimized for perfusion and batch processes, there are distinct differences in how parameters for cell culture and media parameters should be optimized for each culture format. While all the aforementioned batch and perfusion culture studies have provided valuable information regarding process optimization frameworks, they can be difficult to draw general conclusions from because the culture media and cells used have been proprietary.

The ability to interpret results of process and media development studies is also dependent on the robustness of analytical characterization methods. Many published optimization studies use VG titer as the primary or only output for comparing conditions because it is the dose determining metric for rAAVs and is straightforward to measure by quantitative or digital polymerase chain reaction (qPCR or dPCR) (Chen et al. 2024; Grieger et al. 2016; Gu et al. 2018; Guan et al. 2022). Digital droplet (ddPCR) and qPCR assays have also been used in multiplex format to study genome integrity to monitor levels of full versus partially full capsid populations (Eisenhut et al. 2024; Fu et al. 2023a; Furuta-Hanawa et al. 2019; Zanker et al. 2022). In addition, characterization of rAAV capsid populations for attributes including total capsid titer (Leibiger et al. 2024b), full/empty ratio (Heldt et al. 2023; Suk Lee et al. 2024; Wagner et al. 2023), and capsid protein (VP) ratio (Onishi et al. 2023) is crucial for evaluating process performance during development studies. Analysis of vector functional titer by transduction is important but is more complex, as it requires selection of a permissive in vitro system and a therapeutic genome or protein analysis technique with a sufficient detection range (Green and Lee 2021; Wright 2020). Methods based on DNA detection such as the gold standard $TCID_{50}$ assay are more established (Duong et al. 2023), but measuring protein expression as the output of a functional titer assay is a more direct indicator of product efficacy versus quantifying genomes transferred into target cells (François et al. 2018; McColl-Carboni et al. 2024). Developing a protein-expression-based assay that is adaptable for different transgenes and does not require expensive equipment would aid product developers in setting transducing unit (TU) titer benchmarks for their manufacturing platforms.

In this study, we developed an upstream platform for batch rAAV production using an easy to procure suspension HEK293 cell line that was adapted into a chemically defined medium with a formulation developed in-house. rAAV was produced using an optimized transient transfection process based on PEI-mediated plasmid DNA transfer, and vectors were characterized by qPCR for VG titer, ddPCR for genome integrity, liquid chromatography-mass spectrometry (LC-MS) for capsid identity, and in-vitro transduction coupled with flow cytometry for TU titer. Media, process, and analytics development were done at three different universities, and the resources of the Advanced Mammalian Biomanufacturing Innovation Center (AMBIC), an industry-university cooperative research center (IUCRC), were leveraged during the project. We found that balancing iron and calcium levels in the medium were crucial to maintaining transfection efficiency and minimizing cell aggregation, respectively. Transient transfection parameters with the highest impact on VG titer were PEI:DNA ratio and cell density at transfection. Purified rAAV from the AMBIC (in-house) process had viral capsid proteins of comparable molecular mass versus vector derived from commercial processes, but differences in TU titer were observed between vector preps. The developed system including the media, process, and analytics can be adopted by others to study how improvements can be made to rAAV manufacturing process.

2 | Materials and Methods

2.1 | Plasmid Preparation

The plasmids needed for triple transient rAAV transfection, pAdDeltaF6 (pHelper; Addgene 112867—a gift from James Wilson), pAAV2/2 (pRepCap; Addgene 104963—a gift from Melina Fan), and pAAV-CMV-GFP (pGOI; Addgene 67634—a gift from Connie Cepko; Xiong et al. 2015), were purchased from Addgene. Plasmid carrying *Escherichia coli* were grown in Luria-Bertani broth supplemented with 100 μg/mL ampicillin or carbenicillin and cells were harvested for plasmid preparation. Plasmids were extracted and purified with the ZymoPURE II Plasmid Maxiprep Kit (Zymo Research) or EndoFree Gigaprep kit (Qiagen) and final DNA concentrations was measured by A260 absorbance on a Nanodrop OneC (ThermoFisher) or DS-11 FX+ (DeNovix).

2.2 | Cell Culture

HEK293s cells (ATCC 1573.3) adapted to BalanCD HEK293 (BalanCD; Irvine Scientific), AMBIC 1.1 (Millipore Sigma), or AMBIC 1.293 (Millipore Sigma) medium supplemented with 4 mM GlutaMax (ThermoFisher), HEK293 suspension adapted cells (Mass Biologics) in BalanCD medium with 4 mM GlutaMax, and Expi293F cells (ThermoFisher A14527) in Expi293 medium (ThermoFisher) were cultured in 125 mL flat-bottom shake flasks. Cells were cultured in a 20–30 mL working volume and passaged to 0.2×10^6–0.5×10^6 cells/mL every 3–4 days based on counts taken using trypan blue exclusion. The details of culturing and cell counting methods used during each stage of process development are listed in Supporting Information S1: Table S1. HEK293s, HEK293, and Expi293 cells were used in the in-house process, the baseline commercial system for process development, and the commercial process for analytical internal standard generation, respectively.

2.3 | Adaptation of HEK293 to AMBIC 1.1 Medium

HEK293s cells were thawed in BalanCD medium and cultured for two 3-day passages. Cells then underwent two 3-day passages in a 75%–25% v/v (BalanCD-AMBIC 1.1) media mixture. The cells were subsequently cultured in a 50%–50% v/v mixture for two 3-day passages, followed by continued passaging in 100% AMBIC 1.1 medium. Cells were banked in AMBIC 1.1 medium with 10% dimethyl sulfoxide (DMSO) for use in subsequent experiments.

2.4 | Reformulation of AMBIC 1.1 for HEK293 Growth and Transfection

AMBIC 1.1 medium (IA 87093 C), which is an edited version of AMBIC 1.0 previously developed and published for CHO culture (Cordova et al. 2023) was manufactured by Millipore Sigma's imMEDIAte ADVANTAGE® small volume custom media service. To ensure solubility, Ferric Ammonium Citrate was used in AMBIC 1.1 instead of the Ferrous Sulfate used in AMBIC 1.0. The rest of the changes made to AMBIC 1.0 to formulate AMBIC 1.1 include combining sources of nutrients, especially trace metals. Vanadium, selenium, zinc, magnesium, asparagine and tocopherol sources were changed into single-source salts rather than multiple salts for the main nutrient. The list of all nutrients that were removed from AMBIC 1.0 are available in Supporting Information S1: File S2, along with their CAS numbers.

The same service was used for production of "AMBIC 1.1-Deleted," a base formulation without calcium chloride, calcium pantothenate, iron(III) citrate, and sodium citrate that was used during HEK293 media development. AMBIC 1.1-Deleted was spiked with stock solutions of removed components and other ingredients of interest during media optimization studies. Stock solutions of 100 mM calcium chloride (C4901-500G, Sigma Aldrich),100 mM calcium pantothenate (P022-100GM, Caisson Labs), and 100 mM sodium chloride (S5886-500G, Sigma-Aldrich) were used during the development of AMBIC 1.293A, an updated version of AMBIC 1.1-Deleted medium supplemented with calcium at levels optimized for HEK293 growth. Solutions of 110 mM iron(III) EDTA (EDFS-100G, Sigma Aldrich) and 0.5 M EDTA (E0307, Teknova) were used in spiking studies to develop the final AMBIC 1.293 formulation. All media formulations tested during development were supplemented with 4 mM GlutaMax (ThermoFisher). Osmolarity of media formulations was measured using a 3250 Single-Sample Osmometer (Advanced Instruments). HEK293s cells were cultured for ≥ 3 passages in new media formulations before transfection efficiency experiments were performed. During each passage, cells were seeded at 0.5×10^6 cells/mL and were cultured for 3–4 days.

2.5 | DOE Design and Model Fitting

DOE design and model fitting for the screening DOE (sDOE) and optimization DOE (opDOE) studies were performed in MODDE® (V13.0, Sartorius, Sweden). Partial least squares (PLS) analysis was used to estimate coefficient terms in the models, and logarithmic transformation of response data sets was performed as needed to ensure all response data distributions were normal to improve predictive capability. Non-significant model terms ($p > 0.05$) were then removed using the MODDE® auto tuner function until no further improvement in model fit was observed. Optimal setpoints for the sDOE and opDOE were determined by performing a target optimization to maximize VG titer and transfection efficiency. The acceptance limit was set to 1% probability of failure, resolution was set to 16 discrete blocks, and 50,000 simulations were performed in each block per response.

2.6 | Transfections for eGFP Expression or rAAV2-eGFP Production

HEK293 cells were chemically transfected after ≥ 3 passages in their respective culture medium to express enhanced green fluorescent protein (eGFP) during media development studies or to produce rAAV2-eGFP. Detailed transfection parameters used during media development at Site A, in-house process optimization at Site B, in-house process transfer at at Site C, and rAAV reference material production using a commercial process at Site C are listed in Supporting Information S1: Table S2. In brief: a chemical transfection reagent was added to plasmid DNA pre-diluted in complexation medium, reagent-DNA complexes were formed during the subsequent incubation step, and complexes were added dropwise to flasks that were then cultured for 3 days. Three plasmid transfection was used to produce rAAV using pRepCap, pGOI, and pHelper at all sites. eGFP was expressed during media development at Site A with only pGOI added to the same total DNA mass as all three plasmids used for rAAV production. Cell counts were taken daily using trypan blue exclusion and transfection efficiency was measured by microscopy or flow cytometry. Following the termination of rAAV production, cultures were harvested by chemical (Site A) or mechanical lysis (Site B, Site C). Detailed harvest steps used during each stage of platform development are listed in Supporting Information S1: Table S3.

2.7 | rAAV VG Titer Measurement by qPCR

rAAV lysate samples were treated with DNaseI for removal of residual DNA and then proteinase K for capsid digestion to release VGs before qPCR analysis (Supporting Information S1: Table S4). Samples were then diluted in molecular biology grade water for qPCR. When standard curves were generated from rAAV reference material, vector samples were was treated identically to the lysate samples. If plasmid DNA (pDNA) was used for standard curve generation, linearized or supercoiled pDNA was serially diluted in molecular biology grade water. Primers used for qPCR targeted the eGFP transgene. Regression analysises for plots of the $\log_{10}$ standard concentration versus quantification cycle (Cq) were performed to generate standard curves, and regression equations were then used to determine copies of rAAV per sample.

2.8 | rAAV Three-Dimensional Genome Integrity Analysis by ddPCR

rAAV lysate samples were processed with the Site C protocol for DNaseI digestion and proteinase K treatment (Supporting Information S1: Table S4). Samples were diluted in molecular biology grade water before ddPCR analysis such that reactions contained less than 4000 positive droplets to minimize fractionation of multiple genomes per droplet (Zanker et al. 2022). Multiplexed ddPCR was performed on a QX ONE system (Bio-Rad) using 2x ddPCR Supermix for Probes without dUTP (Bio-Rad). Reactions were prepared with 900 nM primer, 250 nM probes, and 3 μL diluted rAAV sample and run using the default direct quantification (DQ) protocol with the exception of changing the primer annealing temperature to 55°C. The primer/probe assays targeted the cytomegalovirus (CMV) promoter, eGFP, and the human β-globin polyadenylation tail (polyA tail) with PrimeTime qPCR probe assays containing Cy5, FAM, and HEX probes, respectively (Supporting Information S1: Table S5).

2.9 | Purification of rAAV2-eGFP for Analytical Characterization

Purification of rAAV2 capsids was performed using Capto™ AVB (Cytiva) affinity resin with a Tricorn 5/50 (Cytiva) column (1 mL column volume) and AKTA™ Pure (Cytiva) chromatography system. Equilibration was performed with with 20 column volumes (CV) of equilibration buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 7.5). A total of 50 mL of 0.22 μM filtered clarified cell culture lysate was then loaded, the column was washed with 10 CV of equilibration buffer, and rAAV2 was eluted with 20 CV of elution buffer (0.1 M Glycine-HCl, pH 2.6). All steps were performed at a flow rate of 1 mL/min. Elution pool neutralization was immediately performed by 10% v/v addition of 1 M Tris-HCl, pH 8.7. The elution pool was measured for VG titer using the Site C qPCR method (Supporting Information S1: Table S4) and was concentrated using an Amicon Ultra-15 spin filter with a 10 kilodalton cutoff (Millipore Sigma).

2.10 | Intact Capsid Protein Identity Analysis by LC-MS

A total of 1.5×10^{11} VG of rAAV2-eGFP were treated with 10% v/v acetic acid for 15 min and then centrifuged at 16,260g for 5 min (Jin et al. 2017; Zhang et al. 2021). LC-MS was performed using a Waters BioAccord with ACQUITY Premier instrument and Agilent ZORBAX RRHD 300 Å StableBond C18 column (2.1 × 100 mm, 1.8 μm). Method parameters are summarized in Supporting Information S1: Table S6. The FASTA sequences from UniProt (uniprot.org) corresponding to AAV2 VP1, VP2, and VP3 proteins were defined in the analysis method.

2.11 | TU Titer by in Vitro Transduction and Flow Cytometry

A total of 500 μL of HEK293s cells (ATCC 1573.3) seeded at 0.3×10^6 cells/mL in AMBIC 1.293 medium were aliquoted into each well of a 96 deep well plate (96 DWP; Biotix) to seed 150,000 cells per well. Purified rAAV2-eGFP vector stocks produced with the in-house process, produced internally with the Expi293 (commercial) system, or purchased from Addgene (Addgene # 105530-AAV2) were diluted and added to DWP cultures in triplicate at eight multiplicities of infection (MOIs) ranging from 15 to 65,000 VG/cell based on qPCR titration with the Site C protocol. Non-transduced controls were cultured in parallel. Cells were incubated at 37°C, 350 rpm (25 mm throw), 5% CO_2, and 80% relative humidity for 72 h before samples were taken for flow cytometry analysis.

Flow cytometry data was acquired on an Accuri C6 Plus flow cytometer (BD) equipped for detection of eGFP (488 nm laser, 510/15 emission). The gating scheme to isolate eGFP+ cells was set using untransduced HEK293s host cells (Supporting Information S1: Figure S1). Data analysis for ≥ 10,000 single cell events per well was performed using BD CSampler™ Plus Software v1.0.27.1 and Python v3.9.17 with FlowKit v1.0.1. The ratio of VGs to transducing units (VG/TU ratio) was calculated by taking the inverse of the slope in the linear region of the data for the eGFP positive (eGFP+) cell fraction (out of 1) versus MOI. The linear region for each vector preparation was defined as the MOI range in which $R^2 > 0.95$ on a plot of eGFP+ cell fraction versus MOI and median fluorescence of GFP+ cells versus MOI varied by less than twofold to minimize inclusion of cells transduced by multiple rAAVs in the calculation of VG/TU ratio (Supporting Information S1: Figure S2).

3 | Results and Discussion

3.1 | Initial Assessment of HEK293s Growth and Transfection in AMBIC 1.1 Medium

Cells banked in BalanCD, a widely used cell culture medium for rAAV production, were adapted to AMBIC 1.1, a chemically defined cell culture medium. AMBIC 1.1 and its predecesor AMBIC 1.0 were developed for stable mAb production in suspension CHO cells (Cordova et al. 2023), and AMBIC 1.1 was used as a starting point for definition of a cell culture medium for transient production of rAAV in HEK293s. Throughout the adaptation from BalanCD to AMBIC 1.1, cell aggregation remained severe and large clumps of > 30 cells were observed (Supporting Information S1: Figure S3A). Although cell growth was slower in AMBIC 1.1 than in BalanCD, cells remained viable, allowing us to attempt chemical transfection of HEK293s to express eGFP. Fluorescence was observed in cells cultured in BalanCD, but not in the cells cultured in AMBIC 1.1 (Supporting Information S1: Figure S4).

3.2 | Identification of Media Components Causing Aggregation and Transfection Inhibition

We first sought to determine the cause of aggregation. Cells can aggregate for many reasons, including when adapting from serum containing to chemically defined media, via ion-mediated adhesion of cell surface proteins, and due to shear stress (Alberts et al. 2002; Cruz et al. 1998; Zhao et al. 2007).

Since our cells were already in chemically defined media and not experiencing excessive shear in non-baffled shake flask cultures, we investigated whether cell adhesion was the primary cause of cell clumping. We chose to examine how calcium levels affect cell growth because members of the cadherin superfamily have been shown to be upregulated in suspension HEK293 cells (Malm et al. 2020), which may cause high levels of calcium in culture medium to induce cell aggregation and differentiation (Liu et al. 2010; Tu and Bikle 2013; Zhao et al. 2007). Transcriptomics analyses have shown that the *cadherin 23* gene is upregulated in high producer cells during rAAV production, further motivating the need to tune calcium levels in culture media for vector manufacturing (Tworig et al. 2024). HEK293 cells grown in BalanCD media with 110 μM calcium did not clump significantly, but spiking calcium levels to 510 μM suppressed the final VCD by 75% and viability by 42% over a 6-day culture relative to the base condition (Figure 1A,B). Adding EGTA to the culture for selective calcium chelation (Feng et al. 2007; Neely et al. 1976) minimized the impact of the calcium spike, causing only a 24% final VCD decrease relative to the base BalanCD culture (Figure 1A) and minimized formation of cell aggregates (Supporting Information S1: Figure S3B). AMBIC 1.1 contains 822 μM of calcium, so it was apparent that calcium levels in a new AMBIC HEK medium would need to be significantly decreased.

Although aggregation can contribute to transfection repression, the total inhibition observed when transfecting HEK293s in AMBIC 1.1 (Supporting Information S1: Figure S4) suggested that there was at least one inhibitory media component in AMBIC 1.1. We took a particular interest in iron(III) citrate, which has been shown to inhibit chemical transfection in CHO cells (Capella Roca et al. 2020; Eberhardy et al. 2009) and is present at 130 μM in AMBIC 1.1. This inhibitory effect can be alleviated by the addition of EDTA, a compound that has an higher affinity for iron than citrate (Bertheussen 1993; Eberhardy et al. 2009). We observed that adding increasing amounts of EDTA up to 300 μM was positively correlated with transfection efficiency but had a slight negative effect on viability for cultures in AMBIC 1.1 (Figure 2A,B). A 300 μM EDTA spike restored transfection efficiency to 28%, which was close to the 31% transfection efficiency observed in the BalanCD control condition (B-) without added EDTA or iron(III) citrate (Figure 2A). There appeared to be an upper limit to the amount

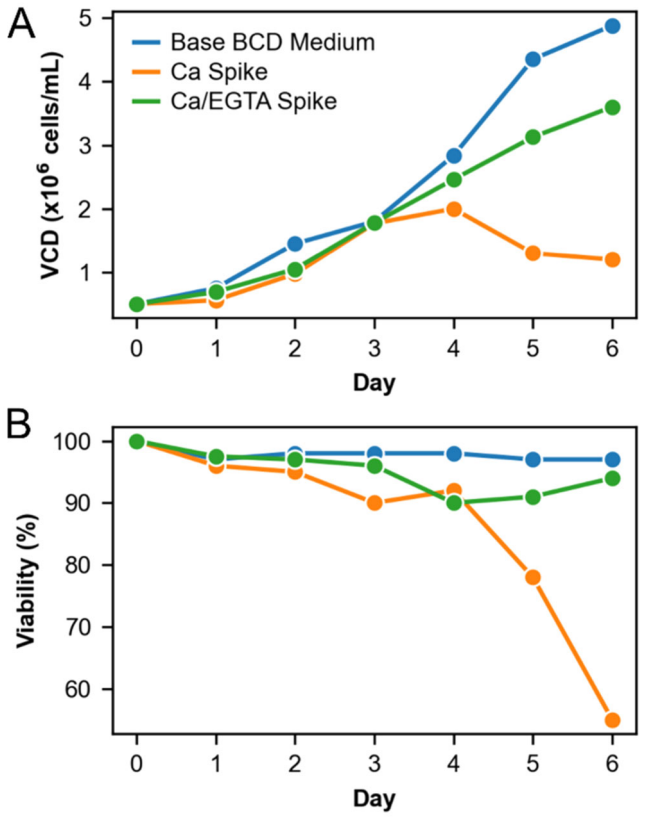

FIGURE 1 | (A) VCD and (B) viability curves for HEK293s singlet cultures grown for one passage in base BalanCD HEK293 (BCD) medium (blue), BCD medium spiked with 400 μM $CaCl_2$ (orange), and BCD medium spiked with 400 μM $CaCl_2$ and 400 μM EGTA (green).

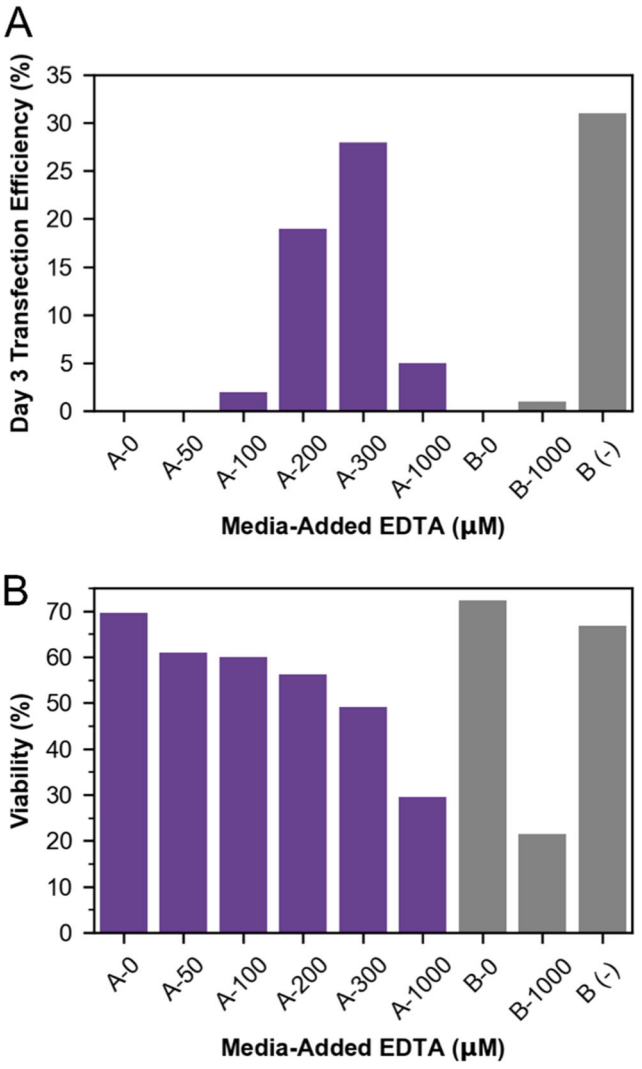

FIGURE 2 | (A) Day 3 transfection efficiency and (B) viability after transfection of HEK293s singlet cultures with an eGFP expressing plasmid. Conditions are labeled by the culture medium (AMBIC 1.1, A [purple bars], BalanCD HEK293; B [grey bars]) and added EDTA concentration (0–1000 μM). All AMBIC 1.1 medium conditions contained 130 μM of iron(III) citrate and BalanCD medium conditions B-0 and B-1000 were spiked with 130 μM of iron(III) citrate. The B (−) control was not spiked with EDTA nor with iron(III) citrate.

of EDTA that can be used to rescue transfection efficiency in AMBIC 1.1, as adding 1000 μM of EDTA reduced transfection effiiciency to 5% and caused a 40% drop in viability versus the AMBIC 1.1 0 μM EDTA condition (Figure 2A,B). Similar effects were observed in BalanCD medium spiked with iron citrate, as a transfection efficiency of 0% was observed in the 130 μM iron citrate/0 μM EDTA condition, and a 1% transfection efficiency was observed in the 130 μM iron citrate/1000 μM EDTA condition. Additionally, the 130 μM iron citrate/1000 μM EDTA culture in BalanCD medium experienced a 50% drop in viability versus the 130 μM iron citrate/0 μM EDTA BalanCD condition (Figure 2A,B). Iron(III) has also been shown to decrease the viability of cultured HEp-2 cells at high concentrations (Terpiłowska and Siwicki 2017).

3.3 | Development of AMBIC 1.293 Medium

The above findings about the negative effects of calcium and iron citrate motivated the formulation of "AMBIC 1.1-Deleted," a modified AMBIC 1.1 medium completely lacking calcium chloride (previously 778 μM), calcium pantothenate (previously 22 μM), ferric ammonium citrate (previously 130 μM), and sodium citrate (previously 112 μM). The AMBIC 1.1-Deleted medium was used as a base for developing a formulation suitable for HEK293 growth and transfection. First, calcium salt was added to AMBIC 1.1-Deleted to a total concentration of 110 μM, the same level present in BalanCD medium (Gorfein et al. 2015). The calcium was supplied by a blend of 78 μM calcium chloride and 22 μM calcium pantothenate, or by 110 μM calcium chloride alone. Growth and viability curves over four passages for the AMBIC calcium chloride and pantothenate blend generally trended with those for BalanCD media (Figure 3). However, peak VCD and viability declined for the calcium chloride only condition during the third and fourth passages, with passage 4 terminal VCD and viability being 4.1- and 2.1-fold lower than they were at the end of passage 1, respectively (Figure 3). We then formulated AMBIC 1.293 A, a calcium modified version of AMBIC 1.1-Deleted, with 78 μM calcium chloride and 22 μM calcium pantothenate. The need for pantothenic acid, an important coenzyme precursor (Schnellbaecher et al. 2019), is aligned with literature, as it has been shown to have a positive effect on cell growth and monoclonal antibody product quality in CHO cultures (Gangwar et al. 2021; Zhang et al. 2013).

Subsequent experimentation focused on determining appropriate iron supplementation to develop a final, transfection permissive AMBIC HEK medium. Three iron salts, iron(III) nitrate, iron(III) chloride and iron(III) EDTA, were tested for their solubility at 113 μM, which was done to match the concentration of iron(III) citrate in AMBIC 1.1. Only iron(III) EDTA (FeEDTA) was soluble at 113 μM. Transfection of eGFP in AMBIC 1.293A supplemented with 113 μM FeEDTA showed a < 1% transfection efficiency, which was a slight improvement over the total inhibition observed in AMBIC 1.1 (Supporting Information S1: Figure S5). However, we suspected that 113 μM FeEDTA still provided iron in excess, as transfection efficiency in AMBIC 1.293A without iron was 21.5%, which was close to the 22.6% transfection efficiency observed in BalanCD (Supporting Information S1: Figure S5), but cells did not grow well post-transfection in the absence of iron.

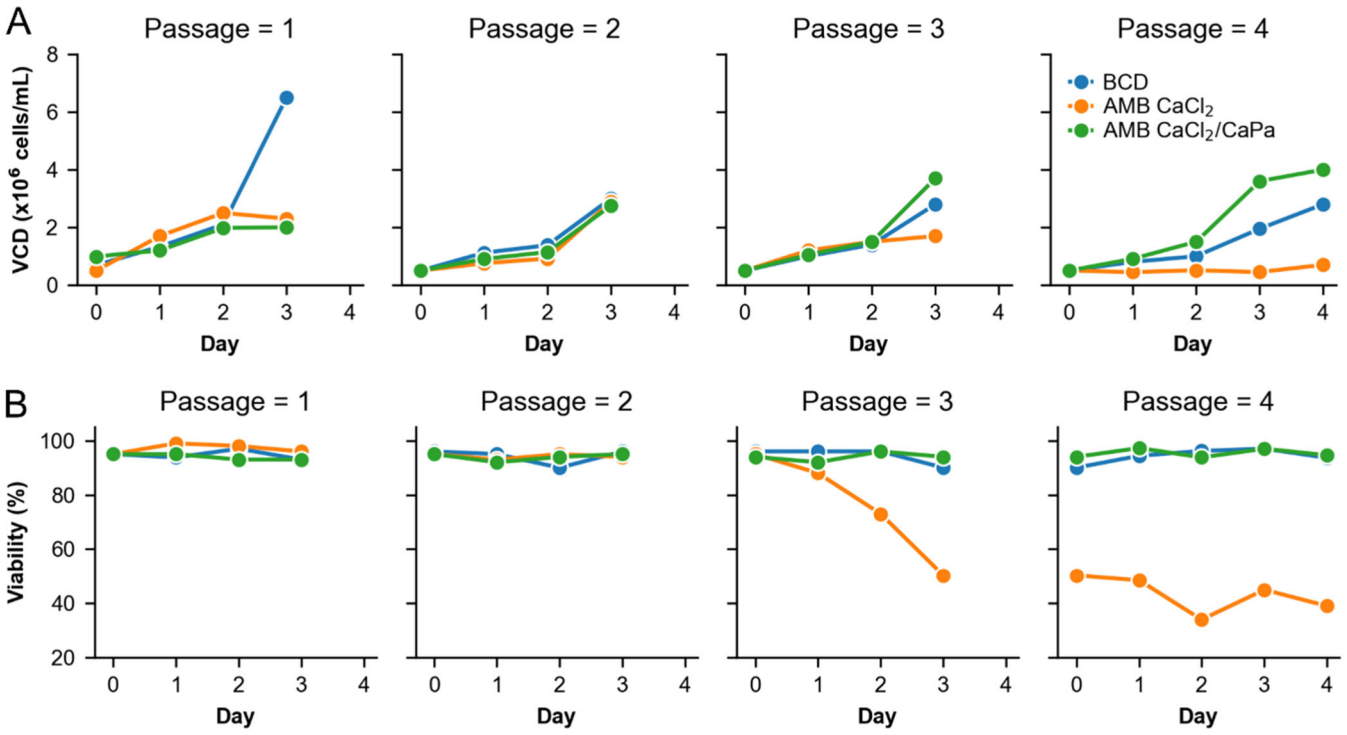

FIGURE 3 | (A) VCD and (B) viability curves for singlet cultures over four passages in BalanCD (BCD) medium (blue), AMBIC 1.1-Deleted medium (AMB) with $CaCl_2$ as the sole calcium source (orange) and AMBIC 1.1-Deleted medium with a $CaCl_2$ and calcium pantothenate blend (green).

After verifying that there were not appreciable osmolality differences between AMBIC 1.1, AMBIC 1.1-Deleted with and without added components, AMBIC 1.293A, and BalanCD media (Supporting Information S1: Table S7) that could be affecting transfection efficiency, a four passage growth screen was performed on cultures grown in AMBIC 1.293A with FeEDTA concentrations ranging from 15 to 113 μM (Supporting Information S1: Figure S6). Cultures grown in AMBIC 1.293 A media containing 15, 30, 60, 90, or 113 μM FeEDTA reached VCDs equivalent to or higher than cultures grown in BalanCD medium (Supporting Information S1: Figure S6A). Viabilities for the BalanCD culture and most AMBIC 1.293A cultures fluctuated from 90% to 100% over the four passages, with the exception of the 15 μM FeEDTA condition that had a 88% viability at the end of Passage 3 (Supporting Information S1: Figure S6B).

AMBIC 1.293A formulations with base FeEDTA concentrations of 30 or 60 μM with 0–14 μM EDTA spikes to chelate excess iron were then tested for growth over three passages and transfection efficiency during the third passage. Terminal Passage 3 VCD for all conditions was close to or greater than the VCD at the end of Passage 1 (Supporting Information S1: Figure S7A), and all cultures maintained viabilities of 90%–100% during Passage 3 (Supporting Information S1: Figure S7B). However, transfection efficiency for most conditions was 0.15- to 0.55-fold lower than for the iron free AMBIC 1.293A media test (21.5%), with the exception of condition 30–14 (FeEDTA base-EDTA spike) that had a transfection efficiency of 14.9% (Figure 4). We then probed if further decreasing free iron concentration would improve transfection efficiency without detrimental effects on cell health in a second screen. AMBIC 1.293A formulations tested in the second screen had 30 μM FeEDTA with 14–108 μM EDTA spikes to probe the effect of further iron chelation, and 15 μM FeEDTA with 0–14 μM EDTA spikes to determine whether cell health could be maintained with a lower base iron concentration. Growth profiles of all the 15 and 30 μM FeEDTA cultures appeared to depend on EDTA concentration (Supporting Information S1: Figure S8), as the final Passage 3 VCD and viability both decreased as the EDTA concentration increased (Figure 4). Transfection efficiency during Passage 3 also increased with EDTA concentration for all conditions, and all formulations supported transfection efficiencies at or above the 14.9% maximum seen in the first study (Figure 4). Taken together, the results from the two studies show that the base FeEDTA concentration and amount of EDTA supplementation must be balanced to maximize VCD and viability while maintaining acceptable transfection efficiency. Cultures with 15 μM FeEDTA showed a decrease in cell health with any EDTA chelation, but maintained good transfection efficiency. At 30 μM FeEDTA, chelation with 14–56 μM EDTA maintained a balance between cell health and transfection efficiency, and at 60 μM FeEDTA, cell health could be maintained but transfection efficiency was poor even upon the addition of EDTA.

rAAV production was attempted in media formulations 15–0, 30–14, and 30–56 (Fe-FeEDTA) because the associated screening cultures showed an acceptable balance between terminal VCD, terminal viability, and transfection efficiency (Figure 4). Cultures in FeEDTA/EDTA spiked AMBIC 1.293A showed consistent VCD and viability profiles relative to each other and the BalanCD medium control during rAAV production (Supporting Information S1: Figure S9). Additionally, the rank orders for VG titer and transfection efficiency of the cultures, BalanCD, 30–14, 30–56, then 15–0, were the same (Figure 5). Since the 30–14 medium supported 1.5- and 3.6-fold higher rAAV production than the 30–56 and 15–0 mediums, respectively, 30–14 was deemed the final AMBIC 1.293 formulation. Even though the VG titer and transfection efficiency in BalanCD were 2.6- and 1.2-fold higher than those in AMBIC 1.293,

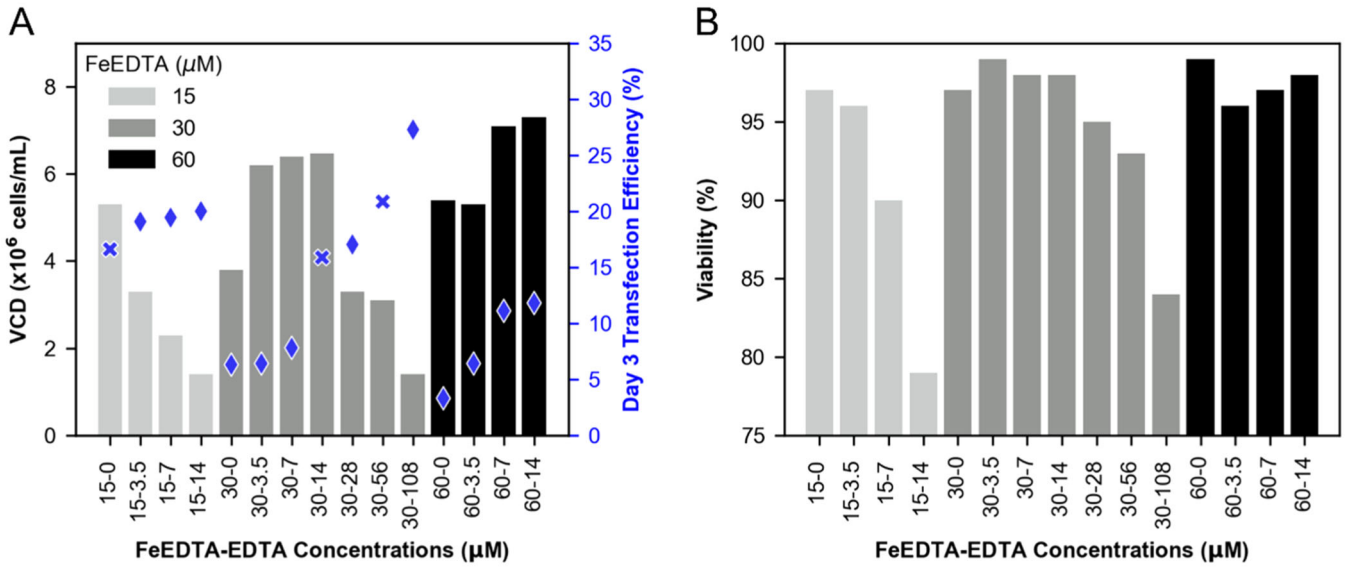

FIGURE 4 | (A) VCD (left axis) plotted with transfection efficiency (blue, right y-axis) and (B) viability of HEK293s singlet cultures expressing an eGFP plasmid three passages after growth in AMBIC 1.293A with varying base concentrations of FeEDTA in μM and added EDTA in μM (categorical x-axis, FeEDTA-EDTA). The shading of the bars denotes added FeEDTA concentrations in μM (30, light grey; 60, dark grey; 60, black). Top performing formulations are denoted with an "X" and all others are denoted with a diamond as the blue transfection efficiency marker in (A). Cells were transfected at the start of the third passage in the respective AMBIC 1.293A formulations.

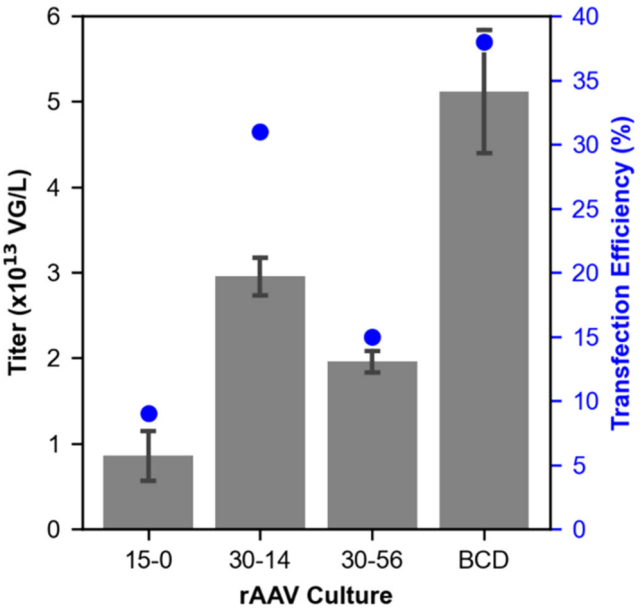

FIGURE 5 | rAAV2-eGFP titers (left y-axis) and transfection efficiencies (blue, right y-axis) for cultures in top performing AMBIC 1.293A formulations (15–0, 30–14, 30–56) versus BalanCD HEK293 medium (BCD). Error bars show the standard deviation of technical triplicates of singlet cultures.

respectively, we deemed this result to be an acceptable baseline for production of rAAV using the AMBIC media.

3.4 | Transfer of AMBIC Cells and Media for Process Development

Cells banked in AMBIC 1.293 were transferred from Site A to Site B for process development. Before executing DOE studies to optimize VG titers, a benchmarking experiment was performed to establish baseline levels of rAAV production in BalanCD medium versus AMBIC 1.293 at Site B (Supporting Information S1: Table S8). Set points for incubation time, complexation volume, DNA mass/cell, PEI:DNA ratio, and VCD at transfection were set based on the results of previous studies (Chen et al. 2024; Coplan et al. 2024; Fu et al. 2023a; Grieger et al. 2016; Gu et al. 2018; Guan et al. 2022; Zhao et al. 2020). A 1:1:1 molar plasmid ratio was used to set a baseline. VG titer was 4.2-fold higher and the terminal VCD was 2.2-fold higher in BalanCD versus AMBIC 1.293, which revealed that there was a gap in volumetric and specific productivities between the baseline in-house and commercial production systems.

3.5 | Screening DOE

A resolution IV fractional factorial sDOE was performed to determine which transfection conditions would need to be optimized in subsequent experiments (Supporting Information S1: Table S9) because such a design allowed for analysis of high impact factors (main effects) without confounding them with two-factor interactions (Mandenius and Brundin 2008; Vera Candioti et al. 2014). Low, medium, and high values for seven transfection parameters were set based on literature (Chen et al. 2024; Coplan et al. 2024; Fu et al. 2023a; Grieger et al. 2016; Gu et al. 2018; Guan et al. 2022; Zhao et al. 2020), and VG titer, transfection efficiency, and cell viability were measured as responses. Three parameters, PEI:DNA ratio, transfection VCD, and pHelper/pGOI, were identified as significant factors impacting VG titer ($p < 0.05$, Supporting Information S1: Table S10). PEI:DNA ratio had the largest normalized effect on VG titer, and the presence of this negative effect is consistent with observations from other studies (Coplan et al. 2024; Fu et al. 2023a). Cellular responses to PEI toxicity observed in other studies are attributed to activation of protein kinase C (Chung et al. 2023), and this protein has been shown to persist in purified rAAV preparations (Leibiger et al. 2024a). We saw that 6/7 highest titer, 8/10 highest transfection efficiency, and 8/9 highest average viability sDOE conditions were run with a PEI:DNA ratio of 1 (Supporting Information S1: Table S9). Cell density had the next largest effect on VG titer, and 6/8 highest titer conditions had a starting VCD of 4×10^6 cells/mL (Supporting Information S1: Table S9). A PLS model was generated from the sDOE data and predictive capability was tested by running the optimal setpoint condition in a shake flask study. The measured VG titer was 6.14×10^{12} VG/mL, which was 1.4-fold higher than predicted (Supporting Information S1: Table S10). This difference is within measurement error ranges seen in qPCR values for VG titer (Martinez-Fernandez de la Camara et al. 2021; Werling et al. 2015), but use of the model-derived optimal transfection VCD of 5.8×10^6 cells/mL, which was outside of the tested design space, may have contributed to this discrepancy as well.

3.6 | Optimization DOE

The results of the sDOE and a survey of literature were used to inform parameters that would be fixed versus further explored in the central composite face (CCF) optimization DOE (opDOE). Despite contributing the most significant main effect in the sDOE, we set PEI:DNA ratio to 1 for the opDOE because of its negative impact on VG titer, transfection efficiency, and cell viability in the sDOE (Supporting Information S1: Table S9). Total DNA mass per cell, incubation time, and complexation volume were fixed to 1 pg/cell, 7 min, and 3 mL (10%), respectively, to match the optimal settings identified by the sDOE model (Table 1). Transfection VCD, which was a significant variable in the sDOE, was studied at a higher range in the opDOE (2×10^6–6×10^6 cells/mL) to determine whether the high optimal VCD derived from the SDOE model (5.8×10^6 cells/mL) would support higher VG titers versus lower VCDs. Although pHelper:pGOI ratio was significant ($p = 0.0299$) in the sDOE while pHelper:pRepCap was not ($p = 0.176$), we chose to explore both parameters in the opDOE because of their relation to gene expression. Previous studies have shown that it is commonly advantageous to have the pGOI as the limiting reagent, but consensus is mixed on whether pRepCap, pHelper, or both plasmids should be supplied in excess versus pGOI (Coplan et al. 2024; Gu et al. 2018; Liu et al. 2024; Park et al. 2024b; Zhao et al. 2020).

The opDOE PLS models for transfection efficiency (Y_1) and VG titer (Y_2) generated from the collected data (Supporting Information S1: Table S11) had excellent fit ($R^2 > 0.95$), good

TABLE 1 | Optimization DOE transfection parameters, model-derived verification settings, and associated validation run results.

Input parameter	Low/medium/high or fixed setting	Verification setting
PEI:DNA ratio	1:1 (Fixed)	1:1
Cell density ($\times 10^6$ cells/mL) [X_1]	2/4/6	4.35
pHelper:pGOI [X_2]	0.2:1/2.6:1/5:1	1:2
pHelper:pRepCap [X_3]	0.2:1/2.6:1/5:1	1:2
DNA mass (pg/cell)	1.0 (Fixed)	1.0
Cocktail volume (mL)	3 (Fixed; 10% culture volume)	3
Incubation time (min)	7 (Fixed)	7
Output measurement[a]	**Simulated value**	**Measured value**
Transfection efficiency (%) [Y_1]	53.25	58.95 ± 1.48
Titer (VG/L) [Y_2]	1.86×10^{13}	$1.43 \times 10^{13} \pm 3.90 \times 10^{12}$

[a]Measurement values are reported as the average ± the standard deviation of biological duplicates.

predictability ($Q^2 > 0.75$), and excellent centerpoint reproducibility (Supporting Information S1: Figure S10). First order terms for transfection VCD (X_1) and pHelper/pGOI (X_2) were significant ($p < 0.05$) in the opDOE model for VG titer (Supporting Information S1: Table S12) as was observed for the sDOE (Supporting Information S1: Table S10) and a previous study on protein production in HEK293 (Bollin et al. 2011). In addition, the second order term for transfection VCD (X_1X_1) was significant in the opDOE models for transfection VCD and transfection efficiency (Supporting Information S1: Table S12). The opDOE model optimal setpoint had a 25% lower VCD of 4.35×10^6 cells/mL, 86% lower pHelper:pRepCap of 0.5, and 4% higher pHelper:pGOI of 0.5 (Table 1) versus that of the sDOE. These settings for VCD and pHelper:pRepCap are similar to those reported from other DOE studies, but the pHelper/pGOI setpoint is 3.8- to 6.5-fold lower (Coplan et al. 2024; Zhao et al. 2020). Despite the difference in our pHelper/pGOI setting versus literature, running the optimal setpoint condition in shake flasks yielded 1.43×10^{13} VG/L with a 59.0% transfection efficiency, which were 23% lower and 11% higher than the predicted values, respectively (Table 1).

Analysis of the contour plots for transfection efficiency and VG titer provides further insight into how both responses trended in response to the input transfection VCD and plasmid ratios. The maximum VG titer and transfection efficiency predictions occurred at mid-range VCDs between 3.5×10^6–4.5×10^6 cells/mL (Figure 6), and this trend is also noticeable when only visualizing data points for the measured opDOE conditions (Supporting Information S1: Figure S11). Measured VG titer was highest for conditions with transfection VCD set to 4×10^6 cells/mL, and these conditions had the highest transfection efficiencies and lowest terminal viabilities (Supporting Information S1: Figure S11). A mid-range transfection VCD may be optimal due to the needs to balance the use of sufficient cells to accumulate appreciable quantities of vector and problems resulting from the well-described "cell density effect" that occurs at high VCDs due to nutrient and plasmid transfer limitations observed in batch cultures (Lavado-García et al. 2022; Mendes et al. 2022; Moço et al. 2023). Nutrient limitations can be alleviated by culturing cells in perfusion, but molecular causes of transfection efficiency and gene expression limitations may need to be solved using cell line engineering (Lavado-García et al. 2020). Modulation of calcium signaling pathways may help increase rAAV production, as VG titers were higher in cultures with upregulated ITPRIP (Inositol 1,4,5-Trisphosphate Receptor Interacting Protein), a protein that may control nuclear calcium level (Barnes et al. 2021). Recent studies have also shown that enhancing endoplasmic reticulum gene expression (Fu et al. 2024), knocking out pro-apoptotic genes (Park et al. 2025; Strasser et al. 2021), and inhibiting interferon pathways can increase rAAV yields (Wang et al. 2023; Wang et al. 2025).

Optimal model ranges for pHelper/pGOI were response dependent, as VG titer predictions peak when pHelper/pGOI < 1 while those for transfection efficiency are maximized when pHelper/pGOI is 1–3. Previously established VG titer models using DOE methodologies had optimal conditions with pHelper/pGOI > 1 (Coplan et al. 2024; Zhao et al. 2020), which matches our results for optimizing transfection efficiency, but not VG titer. Interestingly, multidimensional ddPCR VG analysis in our opDOE that monitored three genome targets (promoter, transgene, and polyA tail) provides insight into how cultures with different pHelper/pGOI ratios could have similar single-target qPCR VG titers but may contain different distributions of full and partially full capsid populations (Supporting Information S1: Figure S12). The opDOE centerpoint and verification cultures produced rAAV at comparable single-target qPCR titers of $1.19 \times 10^{13} \pm 2.32 \times 10^{12}$ VG/mL and $1.43 \times 10^{13} \pm 3.90 \times 10^{12}$ VG/mL, but the 3D ddPCR analysis suggests that the different pHelper:pGOI ratios between these conditions affected the percentage of triple positive droplets predicted to contain full-length genomes. The centerpoint cultures had a transfected pHelper:pGOI > 1 and an estimated 28% of packaged genomes being full-length, while the verification cultures had a transfected pHelper:pGOI < 1 and a predicted 7% of full-length genomes. These results suggest that pHelper:pGOI may affect full genome packaging efficiency between conditions that have similar VG titers, which could confound efforts to optimize models for single target VG titers. Subsequent DOEs focused solely on varying plasmid ratios as inputs and studying full and partial capsid content as responses could better resolve trends, especially since the normalized effects of plasmid ratios on VG titer in our study and others are small compared to other variables tested (Coplan et al. 2024; Wang et al. 2024). Cell line engineering

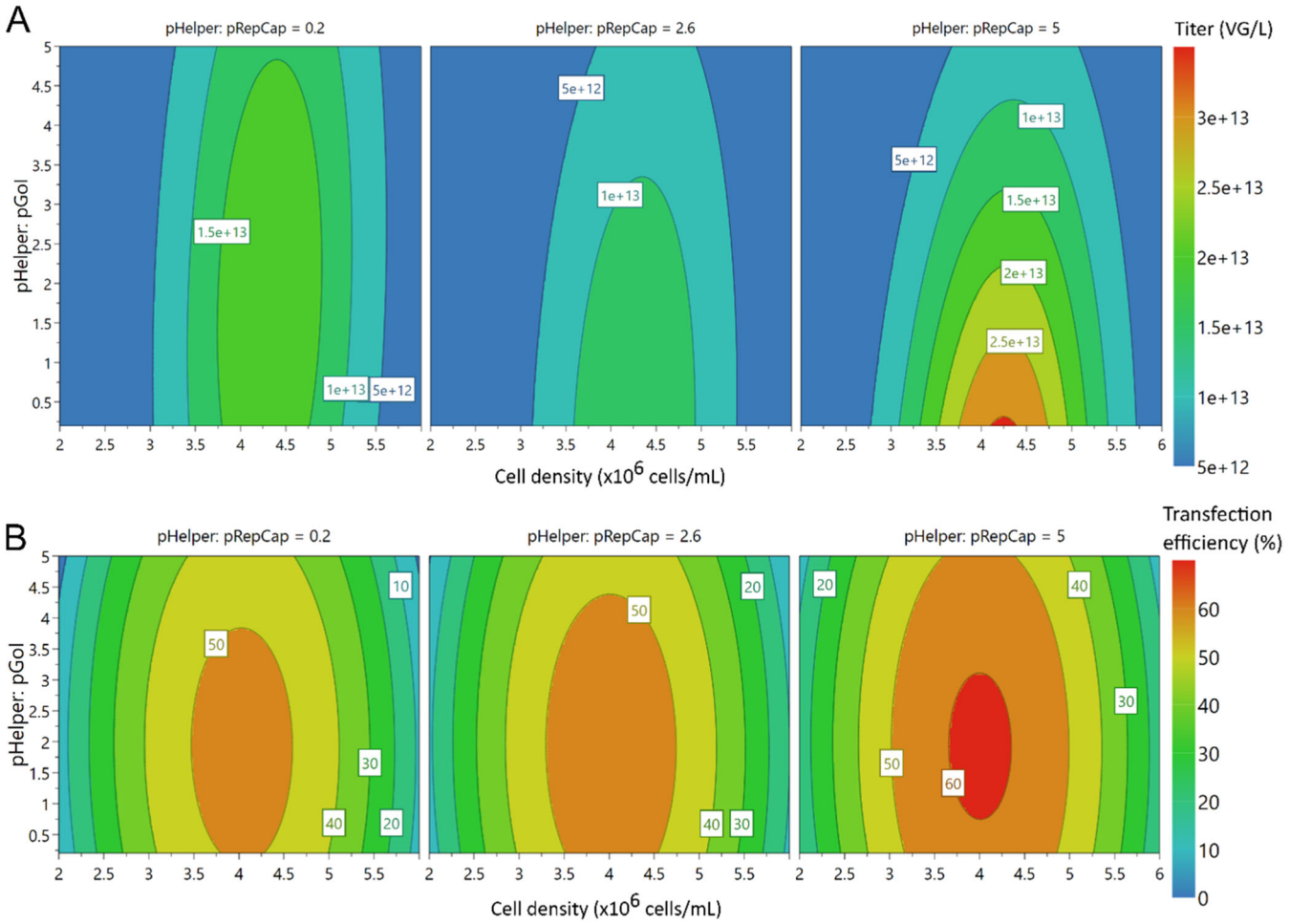

FIGURE 6 | 4D contour plots depicting how (A) VG titer and (B) average transfection efficiency trend with changes in cell density at transfection (x-axis), the ratio of pHelper:pGOI (y-axis) and the ratio of pHelper:pRepCap (held constant as denoted above each subplot).

strategies to modulate cell cycle and DNA transcription pathways may also help increase full capsid populations. Recent omics studies have shown that components of the nuclear pore complex and transcription factors are upregulated to allow for viral gene expression during rAAV production, but DNA templated transcription pathways are downregulated. (Gurazada et al. 2025; Patra et al. 2024; Tworig et al. 2024). Upregulating genes that directly or indirectly modulate single stranded DNA viral genome replication can increase the full capsid population, as shown in a study that saw a higher percentage of full capsids after upregulating SKA2 (Barnes et al. 2021). Despite the potential for future optimization, DOE conditions with high VG titer produced using the AMBIC platform had VG titers that were statistically similar to those measured from high titer DOE conditions our team has tested using commercial media at Site B ($p = 0.25$, *t*-test, Supporting Information S1: Figure S13) (Fu et al. 2023a).

3.7 | Production of rAAV2-eGFP for Analytical Characterization

The in house process was transferred to Site C for vector production at 1 L flask scale and for analytical characterization to evaluate the comparability of vector derived from the in-house versus commercial (Expi293) systems. When the AMBIC process was run at Site C, pre-purification VG titer was approximately twofold higher (2.46×10^{13} VG/L) than that at Site B (1.22×10^{13} VG/L). This result demonstrates good alignment between process performance across sites, but differences between sites may have arisen due to use of different equipment for cell culture or VG titer.

3.8 | Capsid Identity by Intact Protein LC-MS

Intact viral capsid protein (VP) analysis of purified vector performed by LC-MS achieved good separation of the three viral capsid proteins as evidenced by peak resolution in the fluorescence chromatogram and total ion current (Supporting Information S1: Figure S14). Peaks were then assigned to the AAV2 VP1, VP2, and VP3 proteins based on the deconvoluted mass spectra. We observed that the detected molecular weights of the VP proteins matched closely with their theoretical values for the in-house and commercial samples (Table 2). N-terminal removal of methionine and acetylation of alanine in VP1 and VP3 were detected in both samples, which are modifications that have been observed previously (Table 2) (Jin et al. 2017; Smith et al. 2024). The collected data shows that the in-house material is comparable to the commercial material in terms of viral protein molecular mass and demonstrates the viability of

TABLE 2 | Theoretical and detected rAAV2-EGFP capsid protein (VP) molecular weights.

Parameter	VP1	VP2	VP3
Amino acid sequence	2(Ac)-735	139-735	204(Ac)-735
Theoretical MW (Da)	81,856	66,488	59,974
Literature (Waters) detected MW (Da)[a]	81,854	66,486	59,974
Commercial platform detected MW (Da)	81,855	66,488	59,974
AMBIC platform detected MW (Da)	81,856	66,489	59,975

[a]Theoretical VP masses reported in (Lam et al. 2022).

TABLE 3 | Transducing and vector genome titers for rAAV2-eGFP used for analytical characterization.

Source (location manufactured)	VG:TU ratio[a]	Transducing titer (TU/L)[a]	Purified genome titer (VG/L)[b]	Crude genome titer (VG/L)[b]
Commercial platform (internal)	2404 ± 168	$9.36 \times 10^{11} \pm 6.53 \times 10^{10}$	2.25×10^{15}	3.98×10^{13}
AMBIC platform (internal)	1194 ± 80	$1.91 \times 10^{12} \pm 1.28 \times 10^{11}$	2.28×10^{15}	2.46×10^{13}
Addgene standard (external)	1670 ± 69	$1.22 \times 10^{13} \pm 5.06 \times 10^{11}$	2.04×10^{16}	N/A

[a]VG:TU ratio and TU titers are reported as the calculated value ± the standard error of the mean (SEM) for biological triplicates for all points in the linear region of the standard curve ($n = 12$ for Commercial, AMBIC; $n = 9$ for Addgene). SEM was calculated based on the standard error of the estimated slope for each trend line.
[b]VG titers are reported as the average of three technical replicates.

LC-MS for rapid and repeatable serotype verification agnostic of the upstream production process.

3.9 | TU Titer by In Vitro Transduction and Flow Cytometry

Regression analysis revealed that the relationship between the eGFP+ cell fraction (out of 1) and the MOI was linear when MOI was < 700 VG/cell with $R^2 > 0.97$ for all three vector samples tested (Supporting Information S1: Figure S2C). Median fluorescence of the eGFP+ cells varied by $< 60\%$ (Supporting Information S1: Figure S2A). and eGFP+ cell percentages ranged from 2% to 45% in the linear region, depending on the vector preparation (Supporting Information S1: Figure S2C). VG/TU ratios calculated from the slopes of the eGFP+ cell fraction versus MOI regression lines were 1194 VG/TU for in-house, 2404 VG/TU for commercial, and 1670 VG/TU for Addgene vector preps, indicating that the TU titers are ~3-log lower than the VG titers (Table 3). Multi-log differences in VG and TU titer have also been observed in other studies that were over 4-log for rAAV2-GFP (Zeltner et al. 2010) and over 5-log for rAAV8-eGFP (François et al. 2018) when helper-free transduction assays were used. Our helper-free VG/TU ratios could be lower (meaning particles are deemed more infectious) than the previous rAAV2 study (Zeltner et al. 2010) due to differences in the vector production systems, in vitro transduction systems, and flow cytometry methods. Of note, our in vitro transduction workflow was in suspension format, which may have facilitated more efficient transport of viruses to cell surface receptors. Although not utilized here, addition of helper virus during transduction can also lower the measured VG/TU ratio, as VG/TU ratios on the order of 1–2 log have been recorded for rAAV2 vectors in most (Chahal et al. 2014; François et al. 2018; Mayginnes et al. 2006; Zeltner et al. 2010), but not all (Grimm et al. 1999), studies that utilize helper adenovirus to increase assay signals.

Although we did not induce AAV genome replication in our assay, eGFP signals were clearly above background, and we saw measurable differences in functional titer between vector preps. As a lower VG/TU value indicates that an rAAV preparation is more infectious, our analysis indicates that the in-house vector prep is more infectious than the commercial vector and the external Addgene reference material. The differences in VG/TU ratio between vectors tested here may have arisen due to differences in the upstream host cells and media, transfection reagent, or the downstream purification process (Eisenhut et al. 2024). Interestingly, comparisons of our vectors (in-house and commercial) produced internally and purified by affinity chromatography versus the externally produced vector (Addgene) purified by iodixanol gradient ultracentrifugation (IGUC) suggest that purification method may affect assay performance. The top of the linear range for the Addgene vector is 1.7- and 2.8-fold higher than for the in-house and commercial vectors (Supporting Information S1: Figure S2C), respectively, but fluorescence for the Addgene vector saturates at lower MOIs than for the other tested vectors (Supporting Information S1: Figure S2B). Although there is not definitive consensus around whether partial and empty capsids do (Gao et al. 2014; Lee et al. 2023; Troxell et al. 2023) or do not (Pan et al. 2023) affect transduction efficiency, and therefore the point at which assay response may saturate, it is possible that the more homogeneous nature of the Addgene vector prep due to full capsid enrichment via IGUC enabled a more linear response over a larger MOI range but caused faster eGFP signal saturation. Taken together, these observations suggest that care must be taken when preparing vector for transduction testing so that differences in downstream processing do not cause unintended variability in VG/TU ratio measurements.

4 | Conclusion

This study demonstrated the value of developing an rAAV manufacturing workflow spanning media development, process

optimization, and analytical testing. We showed that reducing calcium and balancing iron levels relative to those in our original culture medium for antibody production enabled development of AMBIC 1.293, a formulation that can support rAAV production. A DOE approach was then used to increase VG titers of the AMBIC HEK293 upstream process to match titers achieved using commercial platforms in our labs. Purified vector generated from the in-house process using AMBIC 1.293 culture medium had comparable viral capsid protein moelcular masses by intact LC-MS and a lower VG/TU ratio than vector produced from a commercial platform. Since cell density at transfection was identified as a process parameter with a significant impact on VG titer, the established optimization and analysis workflows could be used for development of media and feeds that support cell growth to higher densities and address issues with cell density effects during batch viral vector production. Additional work to further characterize the relationship between pHelper:pGOI and capsids predicted to contain full length genomes by ddPCR may also aid in setting process parameters that improve product quality. The engineering of novel HEK293 hosts with modulated pathways for calcium signaling and single stranded DNA genome replication may also help alleviate issues with transfection limitations at high cell density and loading of capsids with partial rAAV genomes, respectively. The media and methods developed here can also serve as a starting point for defining additional production and product quality parameters for processes with rAAV cargo designed for therapeutic use.

Author Contributions

Q.F., E.A.G., N.N., and D.J.M. conceived the manuscript. N.N., Q.F., Y.W., Y.S.L., E.A.G., and T.M.L performed experiments. E.A.G., Q.F., N.N., and T.M.L. analyzed data. D.J.M., S.Y., M.B., and K.H.L. provided input on data analyses. E.A.G., Q.F., and N.N. wrote the manuscript. T.M.L. and D.J.M. edited the manuscript. D.J.M., S.Y., M.B., and K.H.L. acquired funding and supervised the work. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.