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A Novel Scalable Production Platform for Gene Therapy Vectors Based on Human Suspension Cell Lines

By Kerstin Hein, Simon Fradin, Helmut Kewes, Martina Graßl, and Nicole Faust

Abstract

A rapid increase in the number of gene therapy trials and products has led to a comparable increase in the need for industrial production of viral gene therapy vectors such as lentiviral, adeno-associated, and adenoviral vectors. Current production systems are limited with respect to scalability and robustness. With our CAP® and CAP-T™ cell lines, we have developed a novel system for high-density suspension culture, efficient and reproducible transfection, and highly efficient production of viral vectors. By upstream process optimization, we have obtained a robust and high-density fed-batch culture system which can be scaled in any current bioreactor format. A design-of-experiments approach has been employed to optimize transient production of lentiviral vectors with significantly higher titers than can be obtained with adherent HEK293T cells.

Introduction

Over the last several years, the number of clinical trials in the field of gene therapy has grown immensely. With better understanding of the molecular causes of diseases such as muscular dystrophy[1] and cystic fibrosis[2], it has become possible to address these diseases through intervention by genetic means. One of the crucial aspects of gene therapy is the efficient delivery of the genetic material to the patient´s cells either *in vivo* or *ex vivo*. Many approaches rely on the use of viral vectors. Classically γ-retroviral or adenoviral (AV) vectors have been employed but are now increasingly being replaced by lentiviral (LV) and adeno-associated virus (AAV) vectors. While initially targeting rare diseases, gene therapy is also tackling high-incidence diseases like Alzheimer´s and Parkinson's.[3] This is leading toward the need for scalable production technologies for viral gene therapy vectors. Most current production platforms rely on the use of adherently grown HEK293 or HeLa cells which severely hamper scale-up. Suspension-adapted HEK293 cell lines are difficult to handle and do not grow to very high densities, making large-scale processes difficult to control. In order to provide a solution to these production issues, we have developed our human CAP® suspension cell line along with the CAP-T™ cell line to provide an efficient, scalable platform for the production of AAV and LV vectors.

Process Development with the CAP Cell Line

CAP cells were originally derived from primary human amniotic fluid cells obtained by routine amniocentesis. They were immortalized by the transfection of plasmid-carrying AV E1/pIX functions of human adenovirus serotype 5 (Ad5).[4] After immortalization and clonal derivation, the resulting cell line was adapted to suspension culture and grown in serum-free media. To obtain a robust, high-density suspension culture system supporting scalable production of viral vectors, we performed upstream process optimization with the CAP cell line.

Materials and Methods

Batch Process and Metabolite Analysis

Cells from a CAP cell-derived clone were seeded at a density of 3×10^5 cells/mL. They were cultured for 10 days in four parallel reactors in a DASbox® mini bioreactor system (Eppendorf) in 180 mL of CAP-CDM medium (CEVEC, Merck Millipore) supplemented with 6 mM glutamine. Process parameters were set to 37°C, DO 40%, pH 7.1. Every 24 hours, samples were analyzed for glucose, lactate, glutamine, and glutamate with the YSI 7100 MBS biochemistry analyzer.

Fed-Batch Processes

CAP fed-batch processes were performed four different ways: (1) 1 L stirred tank bioreactor (cellferm-pro, Eppendorf) with 3-blade pitched impeller at 300 rpm, 37°C, DO 40%, pH 7.1; (2) 700 mL Erlenmeyer flasks at 185 rpm with an orbit of 5 cm, 37°C, 5% CO_2 in a humidified incubator; (3) 15 mL stirred tank ambr15® microscale bioreactors (Sartorius) with pitched impellers at 1300 rpm, 37°C, DO 40%, pH 7.1; and (4) a 10 L BIOSTAT® RM 20 CultiBag® (Sartorius) wave-motion system at 42 rpm (rocker angle 5.4°), 37°C, DO 40%, pH 7.1.

Cells were inoculated at either 5×10^5 or 1×10^6 cells/mL in CAP-CDM medium with 6 mM stable glutamine. Feeding started at day 3. Cell density and viability were determined via automated cell counting (Cedex XS, Roche; and Vi-CELL® XR, Beckman Coulter) plus trypan blue staining.

Results and Discussion

As a baseline for fed-batch development, we performed the metabolite analysis during a batch process (**Figure 1**). Full consumption of glucose and glutamine was observed after days 5–6. This was accompanied by a lactate accumulation peak of 2.5 g/L after six days of batch culture, which became the major carbon source. The glutamate accumulation was limited to a 2.5-fold increase, with the concentration reaching 2.5 mM after 10 days of batch culture.

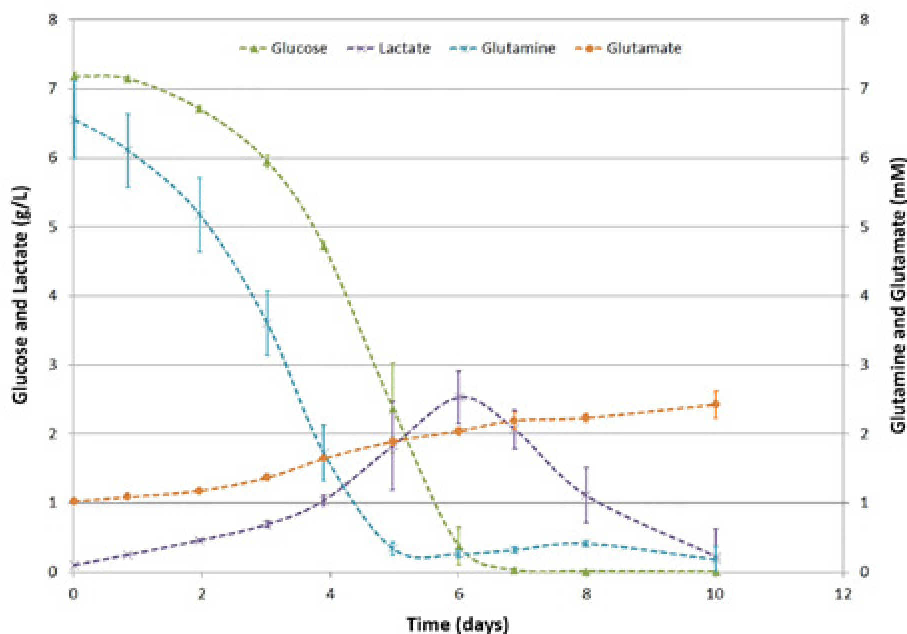


FIGURE 1. Metabolite analysis of CAP cells in stirred tank bioreactor batch culture.

Based on metabolite analysis results and an additional amino acid analysis (data not shown), we designed a fed-batch process that included glucose and glutamine supplementation starting at day 3 post-seeding. This fed-batch process can support cell growth and viability for up to 10 days post-seeding (**Figure 2**).

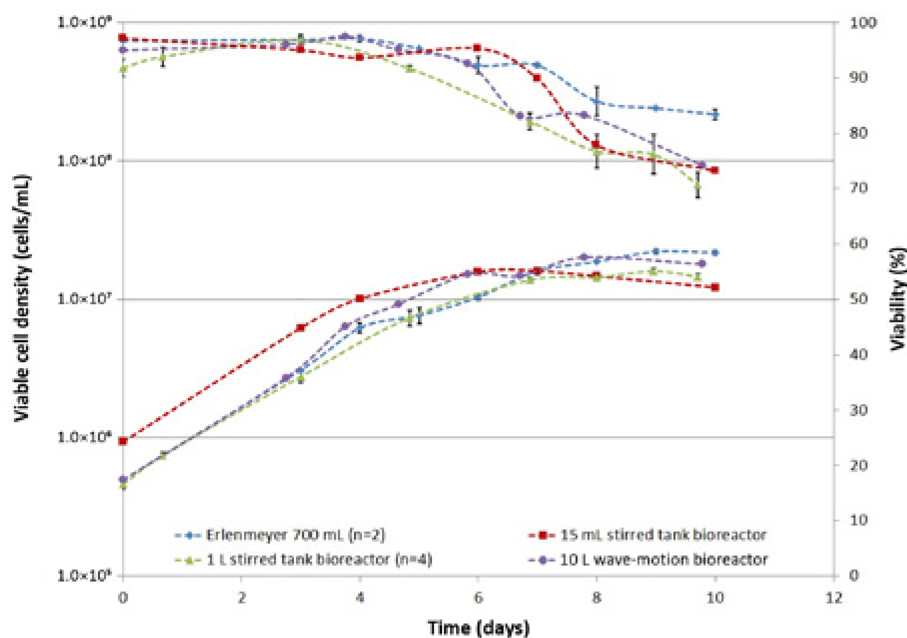


FIGURE 2. Growth performance of a CAP cell clone in various culture systems.

To assess the transferability of the process to different culture systems, CAP fed-batch processes were performed using four different culture systems (**Figure 2).** The growth curves in all the systems tested are highly comparable. The cells reached viable cell densities of approximately 2×10^7 cells/mL by days 7–8, which could be maintained up to day 10. The overall variability of viable cell density was around 15% amongst the different culture conditions. A similar variability was observed for cell viability. While some of the variability might be attributed to differences in the four culture systems, the variability of the cell counts due to the counting method also has to be considered. The relative standard deviation of cell counts using an automated cell counter fluctuates from 1%–15% depending on the device and type of cells counted (particularly for high cell densities).[5,6] Taking this into consideration, we detected no significant differences in viable cell numbers among the different culture systems. High cell viability appeared to be maintained for the longest period with the Erlenmeyer flask, which was the only system in which pH was not controlled. This indicates the potential for further optimizing pH regulation. Overall, the data show good scalability of the process to higher volumes. On the other hand, the good reproducibility in 15 mL microscale stirred tank bioreactors makes the upstream process easily accessible to further process optimization by a design-of-experiments (DoE) approach.

Process Development for Transient LV Production with the CAP-T Cell Line

To obtain a cell line for efficient transient protein expression and vector production, the CAP cells have been further modified through stable integration of the SV40 large T antigen. CAP-T cells can be transfected very efficiently by a variety of biochemical transfection reagents resulting in high-titer protein production.[7] This feature renders the CAP-T cell line an efficient system for the transient production of viral vectors. Initial experiments (data not shown) have shown that transient co-transfection of CAP-T cells with a four-plasmid system leads to efficient production of LV vectors. We chose to further optimize the system with a DoE approach.

Materials and Methods

Cell Culture

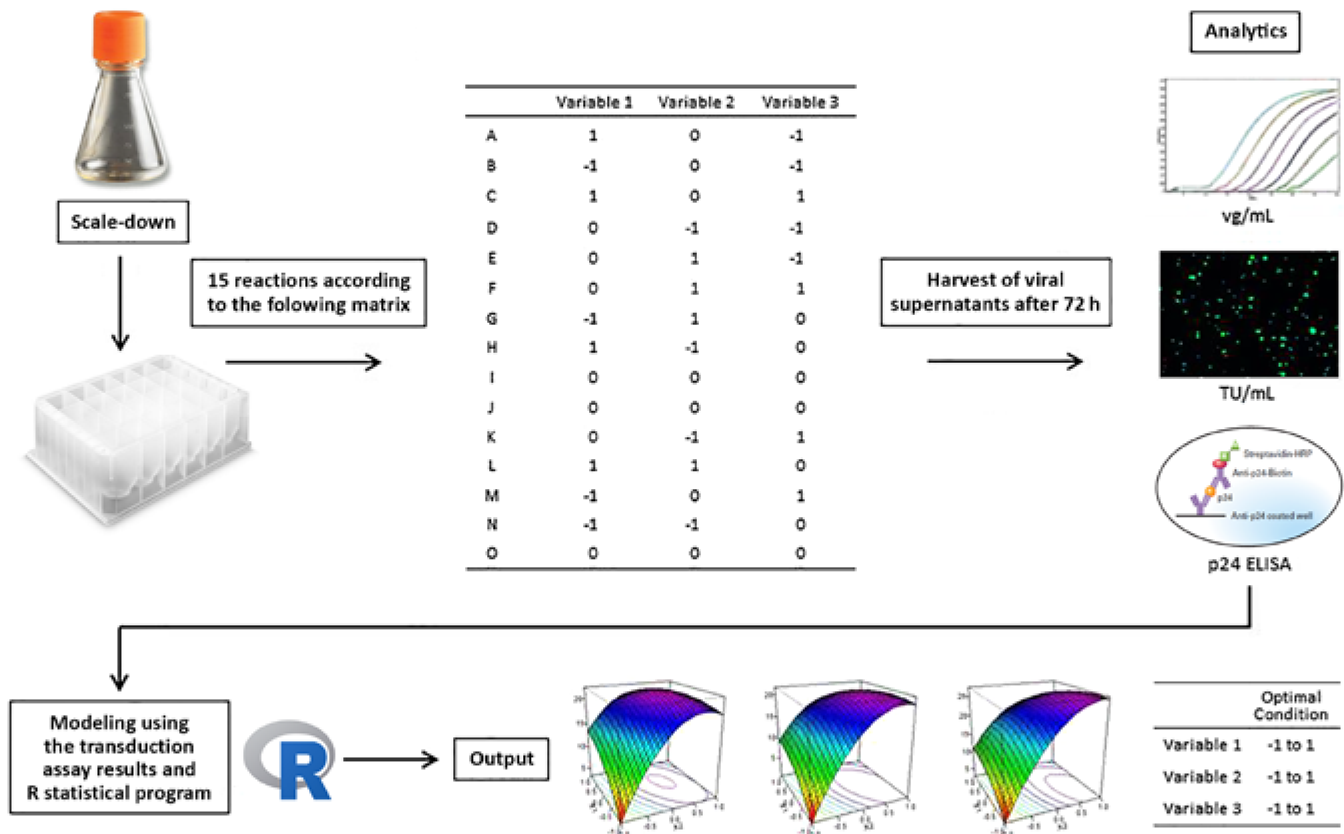
CAP-T™ cells were grown in suspension using a Multitron 2 shaking incubator (Infors) at 5% CO_2, 37°C, 185 rpm with a diameter of 5 cm. Gibco® PEM (Thermo Fisher Scientific) growth medium supplemented with 4 mM stable glutamine (BioChrom) and 5 µg/mL blasticidin was used. Cells were passaged every 3–4 days and adjusted to a cell density of 1×10^6 cells/mL. Cell number and viability were measured with the NucleoCounter® NC-3000™ (ChemoMetec) image cytometer according to the manufacturer's instructions.

Transient Transfection

Transient transfection of CAP-T cells was performed according to standard protocol. In brief, CAP-T cells in the exponential growth phase were spun down (150×*g*, 5 min), washed with PBS, and resuspended in FreeStyle™ 293 medium (Thermo Fisher Scientific). DNA and polyethylenimine (PEI-MAX) (Polysciences) were each diluted in FreeStyle 293, then mixed and incubated for 15 min before being added to the cell suspension. After 5 hours of incubation at 185 rpm, PEM supplemented with 4 mM glutamine was added to the transfection mixture.

Transfection Scale-Down

We scaled down the volume of transient transfection from 15 mL in shake flasks to 2 mL in 24 deep well plates. In order to define the optimal values of a variety of parameters such as cell density, PEI and DNA amounts, and concentrations of additives such as sodium butyrate, a Box-Behnken design was used. Factors anticipated to influence LV production were each tested at three different values: a low level coded as –1, a medium level coded as 0, and a high level coded as +1 as indicated in the matrix within **Figure 3**. The resulting total of 15 transfection reactions was then carried out in a 24 deep-well plate format.



	Variable 1	Variable 2	Variable 3
A	1	0	-1
B	-1	0	-1
C	1	0	1
D	0	-1	-1
E	0	1	-1
F	0	1	1
G	-1	1	0
H	1	-1	0
I	0	0	0
J	0	0	0
K	0	-1	1
L	1	1	0
M	-1	0	1
N	-1	-1	0
O	0	0	0

	Optimal Condition
Variable 1	-1 to 1
Variable 2	-1 to 1
Variable 3	-1 to 1

FIGURE 3. Optimization workflow for lentiviral production using the CAP-T cell line.

Harvest of Viral Supernatants

Twenty-four hours before harvest, 25 U/mL Novagen® Benzonase® (Merck Millipore) and 2 mM $MgCl_2$ were added to the cells. Twenty-four hours later, viral supernatants were harvested. Then 2 mL of cell suspension were transferred to a reaction tube and spun down at 1000×*g* for 5 min. Viral RNA was purified with the High Pure Viral Nucleic Acid Kit (Roche). The supernatant was clarified by 0.45 µm filtration and used directly for analysis or stored at −80°C.

Viral Titer Determinations

After 72 h, viral supernatants were harvested and analyzed for viral particles by: (1) ELISA for p24; (2) RT-qPCR for viral genomes (vg); and (3) transduction assay on HEK293T cells for transducing units (TUs).

1. ELISA. p24 levels were determined by ELISA (Clontech, Takara Bio). Viral supernatants and a p24 standard were diluted in PEM growth medium and quantified by binding of anti-p24-biotin conjugate following the manufacturer's instructions.

2. RT-qPCR. Viral RNA was purified from viral supernatant with the High Pure Viral Nucleic Acid Kit (Roche) according to the manufacturer's instructions. Purified RNA was then quantified in RT-qPCR with an Agilent Mx3005P system. Primer-probe was directed against the WPRE element, as a part of the viral vector.

3. Transduction Assay. Viral supernatants were diluted in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific) plus 10% fetal calf serum and 2 mM glutamine, and incubated with a defined number of HEK293T cells. Two days (48 h) later, HEK293T cells were trypsinized and analyzed with the image cytometer for % of transduced green fluorescent protein (GFP)-expressing cells. TUs were then calculated according to the following formula:

$$\mathrm{TU/mL} = [(P*N*DF)/(100*V)]*1000$$

with P = % GFP⁺ cells, N = cell number at time of transduction, V = volume of dilution, and DF = dilution factor.

Modeling

Since TUs are the most significant parameter for functional LV particles, modeling was done using the results from the transduction assay. An overview of this workflow is shown in **Figure 3**. The R Project for Statistical Computing software was used for statistical analysis. Significance of the model and coefficients was verified by the analysis of variance (ANOVA) *F*-test.

Results and Discussion

In a first step to increase LV production with the CAP-GT technology, cell densities and DNA/PEI amounts/ratios were optimized for the best LV production conditions. Three different coding levels for each variable were chosen according to **Table 1** and covering a range of 2–6×10⁶ cells/mL and a DNA to PEI ratio from 1:1 to 1:6.

Transfection was performed according to the Box-Behnken design as shown in **Table 2**. For LV production, a system of the third generation was used consisting of four plasmids.[8] The ViraSafe™ (Cell Biolabs) vector system comprises: (1) the plasmid pCpgV encoding the *gag* and *pol* gene functions of HIV-1; (2) pCMV-VSV-G for the expression of the VSV envelope glycoprotein G (from the vesicular stomatitis virus); and (3) pRSV-Rev for the HIV-1 Rev protein. The transfer vector pLenti-GFP (Cell Biolabs) encodes for GFP which also allowed monitoring transfection efficiency and transduction units by detecting GFP fluorescence.

Transfection efficiency detected by GFP expression was a first read-out for functionality of the chosen DNA/PEI ratio (last column of **Table 2**). Cell number, viability, and GFP expression were measured 72 h after transfection using the NucleoCounter® NC-3000™ (ChemoMetec) according to the manufacturer's instructions.

TABLE 1. Coding levels for Box-Behnken design.

Coding Levels	Number of cells/mL	DNA pg/cell	PEI pg/cell
–1	2×10⁶	1.0	2.0
0	4×10⁶	1.5	4.0
1	6×10⁶	2.0	6.0

TABLE 2. Matrix design of Box-Behnken experiment to determine optimal cell density, DNA/PEI ratios, and resulting transfection efficiency.

Reaction	Number of cells/mL	DNA pg/cell	PEI pg/cell	GFP-positive (%)
A	1	0	–1	48.10
B	–1	0	–1	49.00
C	1	0	1	53.60
D	0	–1	–1	71.47
E	0	1	–1	3.08
F	0	1	1	56.26
G	–1	1	0	70.93
H	1	–1	0	56.03
I	**0**	**0**	**0**	**73.14**
J	**0**	**0**	**0**	**69.04**
K	0	–1	1	32.11
L	1	1	0	72.87
M	–1	0	1	60.73
N	–1	–1	0	64.06
O	**0**	**0**	**0**	**67.86**

Supernatants were harvested after 72 h by centrifugation and clarified by filtration (0.45 µm). To identify the optimal conditions for viral production, the viral titers in reaction samples A–O were measured by ELISA, RT-qPCR, and transduction assay in HEK293T cells (as shown in **Figure 4, A–C**). Samples I, J, and O are identical triplicates and serve as an internal control to display comparable values within all three assays. The highest p24 levels were around 30 ng/mL while the genomic viral titers reached about 2×10^9 vg/mL. Interestingly, p24 values are not very predictive of infectious particles, as seen when comparing **Figure 4A** to **Figure 4C**. Vector copy numbers are a better prognosis for the quality of particles, but also here, differences in the transducing units (TU/mL) are found (**Figure 4B** and **Figure 4C**). For sample E, viral titers were below detection limit.

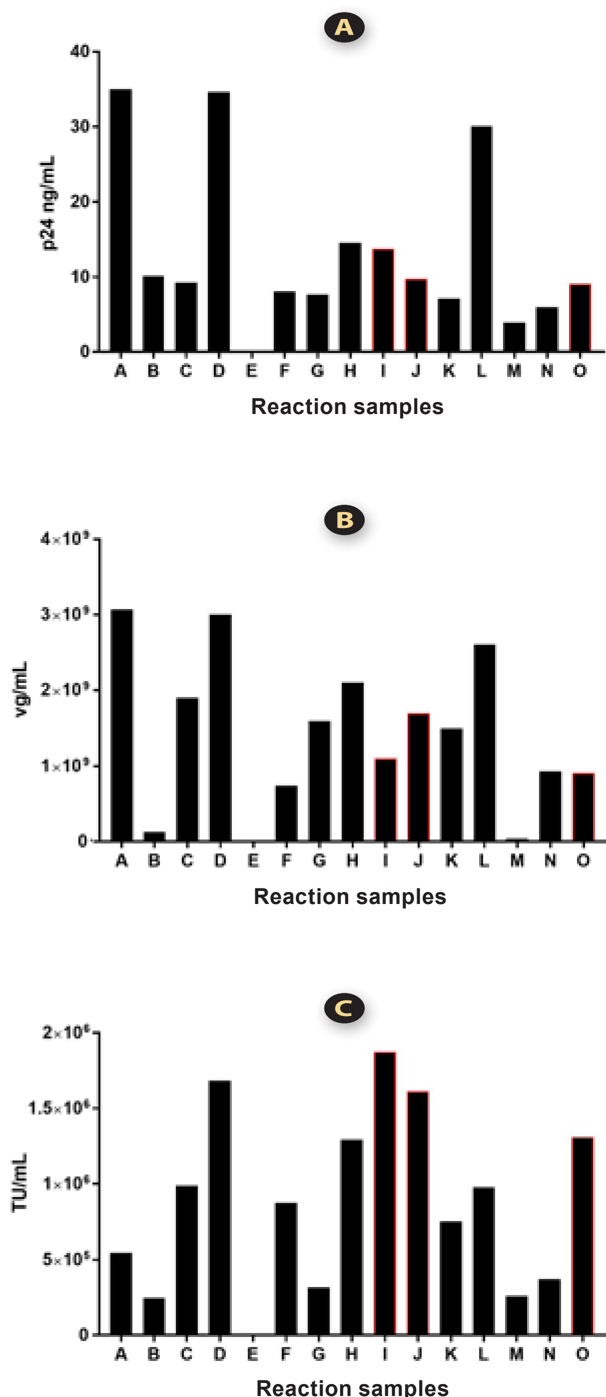


FIGURE 4. Viral titers of the Box-Behnken samples determined by three different methods: (A) ELISA for p24 levels; (B) RT-qPCR for vg/mL; and (C) transduction assay in HEK293T cells for TU/mL.

The most important quality of the LV vectors produced is their ability to efficiently transduce the target cells. For this reason, TU/mL were used as a readout parameter for modeling and were adjusted to a second-order polynomial regression analysis that identified the result as significant, with a regression coefficient (R^2) of 0.915. Further confirmation of the statistical significance of the model is deduced from the p-value of 0.031 (<0.05). The statistical significance of each single regression coefficient was analyzed by Student's t-test (**Table 3**). For visualization of results, response surface graphs were generated using the R statistical software. Keeping one variable constant, the relation between the two other variables and their influence on the response is depicted in coded values in each graph (**Figure 5**). Low and intermediate levels of DNA result in the highest TU/mL, as indicated by the values on the y-axis of the three different graphs. From the first to the last graph, higher amounts of DNA require higher amounts of PEI, as

TABLE 3. Regression coefficients for Box-Behnken experimental design and optimal conditions for the three variables: cell density, DNA, and PEI amount.

	Coefficient	*t*-test	*p*-value
Constant	1599066	9.6934	0.00019
Cells	325910	3.2262	0.0233
DNA	−241525	−2.3909	0.0623
PEI	50075	0.4957	0.6411
Cell²	−588868	−3.9602	0.0107
DNA²	−270578	−1.8197	0.1285
PEI²	−499738	−3.3608	0.0201
Cells × DNA	−64900	−0.4543	0.6687
Cells × PEI	106500	0.7455	0.4895
DNA × PEI	452050	3.1642	0.0250

	Degrees of Freedom	Sum of Squares	Mean Squares	*F*-value	*p*-value
Model	9	4.4×10^{12}	4.9×10^{11}	5.9915	0.031
Error	5	4.1×10^{11}	8.16×10^{10}	—	—

Variable	Optimum Coding Level	Optimal Value
Cell density	0.2935	4.6×10^6 cells/mL
DNA pg/cell	−0.6647	1.2
PEI pg/cell	−0.2192	3.6

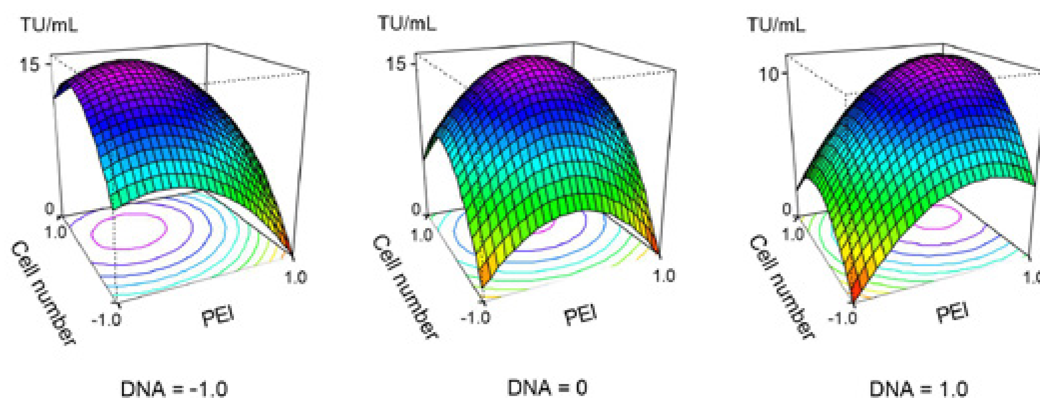


FIGURE 5. Response surface graphs based on Box-Behnken experimental TU/mL results (relative values). For each graph, DNA amount/cell is constant while the relation between cell density and amount of PEI under the design condition is depicted in coded values.

seen by the shift of the optimal response indicated by the small circle on the basis of the graph and the pink shade on top of the surface graph. The optimal cell density for all three DNA amounts is found at similar values between 0 and 0.5.

Solving the model equation gave the optimal amounts for each variable. The best cell density was 4.6×10^6 cells/mL. A DNA amount of 1.2 pg/cell and PEI amount of 3.6 pg/cell resulted in the best LV titers. This means a DNA to PEI ratio of 1:3 is the most beneficial for transient transfection and viral production.

The statistics showed that, as expected, cell density itself, and DNA/PEI in relation to each other, are the most important variables in this experiment, significantly influencing the outcome of LV production (highlighted in green, **Table 3**).

It is known that additives such as sodium butyrate[9] and valproic acid[10] can significantly influence LV or virus-like particle (VLP) production. Further optimization for these variables following the Box-Behnken design was performed and resulted in our current optimized protocol for transient LV production with CAP-T cells. Before optimization, the genomic LV titers obtained by transient production with CAP-T cells were in a comparable range to what we could obtain with adherently grown HEK293T cells (**Figure 6**). After the DoE-based optimization, these titers could be improved 20-fold **(Figure 6)** and a comparable increase was found in TU/mL.

Similar efforts to optimize the production of AAV vectors are currently under way.

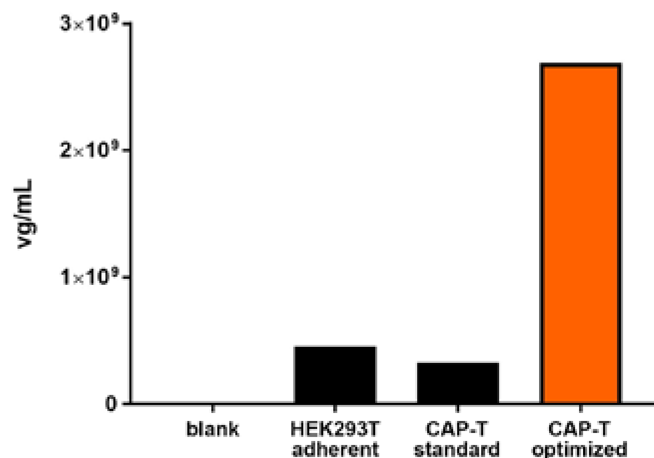


FIGURE 6. Comparison of viral production using the optimized protocol in CAP-T cells, with adherent HEK293T standard, and CAP-T standard protocol. Viral supernatants, generated by transient transfection of HEK293T or CAP-T cells with the same four-plasmid system, were harvested, and after Benzonase digest, viral RNA was purified. Copy numbers were determined in RT-qPCR using a linearized transgene plasmid as a standard.

Conclusions

The human CAP suspension cell line represents a cellular production system which can be easily scaled up in serum-free culture systems. We have developed an upstream process which allows high-density cell growth in fed-batch cultures for up to 10 days. The CAP cell line has been further modified to express SV40 large T antigen (CAP-T cells), and both cell lines present the basis for our CAP-GT production platform for viral vectors. Highly efficient and reproducible transfection protocols make the CAP-GT platform an ideal system for the scalable production of gene therapy vectors. Transient production of LV vectors has been optimized, and we are currently working on the optimization of transient production of AAV viral vectors. In addition, packaging and producer cell lines are being developed, obviating the need for high-volume transient transfections.

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