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Optimized production of HIV-1 virus-like particles by transient transfection in CAP-T cells

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Abstract HIV-1 virus-like particles (VLPs) have great potential as new-generation vaccines. The novel CAP-T cell line is used for the first time to produce Gag-GFP HIV-1 VLPs by means of polyethylenimine (PEI)-mediated transient transfection. CAP-T cells are adapted to grow to high cell densities in serum-free medium, and are able to express complex recombinant proteins with human post-translational modifications. Furthermore, this cell line is easily transfected with PEI, which offers the flexibility to rapidly generate and screen a number of candidates in preclinical studies. Transient transfection optimization of CAP-T cells has been performed systematically in this work. It is determined that for optimal production, cells need to be growing at mid-exponential phase, Protein Expression Medium (PEM) medium has to be added post-transfection, and cells can be transfected by independent addition of DNA and PEI with no prior complexation. A Box-Behnken experimental design is used to optimize cell density at time of transfection, DNA/cell and PEI/cell ratios. The optimal conditions determined are transfection at a density of 3.3E + 06 cells/mL with 0.5 pg of DNA/cell and 3 pg of PEI/cell. Using the optimized protocol, 6×10^{10} VLP/mL are obtained, demonstrating that CAP-T is a highly efficient cell line for the production of HIV-1 VLPs and potentially other complex viral-based biotherapeutics.

Keywords Transient gene expression · HIV-1 virus-like particles · CAP-T · Design of experiments

Introduction

The majority of vaccines commercially available today are based on inactivated or live-attenuated viruses. Although they are immunologically potent, these classical vaccines often present side effects on vaccinated individuals and they represent a risk at manufacturing level before being inactivated or attenuated. Recombinant vaccines are a safer alternative; however, they show less immunogenicity and higher doses may be required (Kushnir et al. 2012). Virus-like particles (VLPs) represent a novel approach in the generation of recombinant vaccines as they combine some advantages of classical and new-generation vaccines. VLPs are formed by the spontaneous assembly of the main virus structural protein(s), but they do not contain the viral genetic information and they are non-infectious. Therefore, no safety concerns are associated to their manufacture, manipulation, and administration. On the other hand, they have shown to be immunologically more potent than recombinant subunit vaccines, as they present the antigens in a repetitive form and in a very similar conformation than in the wild virus. For these reasons, VLPs represent high potential as novel vaccine candidates (Roldao et al. 2010; Buonaguro et al. 2013).

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One of the viruses for which VLPs have been studied as potential vaccines is HIV. The main structural protein of the virus (Gag polyprotein) is able to self-assemble upon expression in a heterologous system, without the need of any other viral component, giving rise to VLPs that resemble the structure of immature HIV virions. VLP assembly takes place in the vicinity of the plasma membrane, and the particles are released from cells by a budding process taking part of the membrane as a lipid envelope (Buonaguro et al. 2013). Gag-based VLPs can expose on their surface membrane proteins from the host cells, HIV surface antigens (when co-expressed with Gag) (Hammonds et al. 2007; Tagliamonte et al. 2011), or proteins from other viruses (Garnier et al. 1995; Haynes et al. 2009). Therefore, they can be exploited as platforms for displaying a variety of antigens.

HIV-1 VLPs have been generated using a variety of expression systems (Pillay et al. 2009; Sakuragi et al. 2002; Scotti et al. 2009; Tagliamonte et al. 2010); however, mammalian cells are one of the most suitable platforms for Gag VLP production because they are able to perform post-translational modifications (PTMs) very similar to those of the wild-type virus (Cervera et al. 2013). Protein production in mammalian cells can be achieved by generation of stable cell lines (SGE) or by transient gene expression (TGE). While the former has been up to now the preferred approach in industry (high titers and lot-to-lot consistency are its main advantages), TGE has been relegated to early development stages of drugs and vaccines, or as the system to obtain research-grade material. However, in the last years, extensive efforts have been made toward the improvement of production titers and scale-up of TGE strategies (Bandaranayake and Almo 2014; Geisse 2009). As a result, titers of 2 g/L of protein using a TGE approach at 6 L scale (Daramola et al. 2014) and a TGE-based protein production at more than 100 L scale (Girard et al. 2002; Tuvesson et al. 2008) have been reported. Moreover, TGE offers the flexibility to easily change the protein to be expressed and milligram to gram quantities of protein can be obtained within 2–4 weeks (Backliwal et al. 2008a).

TGE has been extensively used in HEK293 and CHO cell lines. However, new emerging cell lines suitable for transient protein expression have been developed (Geisse 2009). CAP (Cevec Pharmaceuticals) is a novel cell line derived from human amniocytes and transformed by the E1 and pIX functions of Ad5 (Schiedner et al. 2000). CAP-T cells are based on CAP, but they express the large T antigen of simian virus 40 (SV40LT) which supports episomal replication of plasmids containing SV40 ori (Wolfel et al. 2011). This advantageous feature allows working both at early development stages (using TGE on CAP-T cells) and production levels (using SGE on CAP cells) without the need of changing the cell line (Geisse 2009). Moreover, CAP-T cells are adapted to grow in suspension and in serum-free media to high cell densities, which make them suitable for large-scale TGE, and are highly transfectable. There are limited reports on this cell line because of its novelty; however, it has been previously described to support expression of difficult-to-express proteins (Fischer et al. 2012) and virus production (Genzel et al. 2013).

The aim of this work is to establish an optimized TGE approach to produce HIV-1 derived Gag-GFP VLPs using the CAP-T cell platform. The plasmid DNA used in this work codes for a HIV-1 Gag polyprotein fused to GFP that allows easy quantification of the fluorescently labeled VLPs and transfection efficiency. Polyethylenimine (PEI) Max is used as a transfection reagent for CAP-T cells. Besides its many advantages (easy to use, efficient with suspension cells, compatible with some serum-free media, and cost-effective) (Baldi et al. 2007), it has been previously reported as the most efficient reagent for this cell line (Fischer et al. 2012). Production of correctly assembled VLPs is initially demonstrated. Then, the variables affecting TGE are discriminated between discrete and continuous factors. The former are addressed one by one in a stepwise optimization, and the latter are optimized by a Design of Experiments (DoE) approach. High production levels are reported, demonstrating the suitability of the CAP-T system for this specific production of HIV-1 VLPs. To the best of our knowledge, this is the first reported case of VLP expression in CAP-T cells by means of TGE.

Materials and methods

Cell line and culture conditions

CAP-T cells were kindly provided by Cevec Pharmaceuticals (Cologne, Germany). Cells were cultured in suspension in Protein Expression Medium (PEM, Invitrogen, Grand Island, NY, USA) supplemented with 4 mM of GlutaMAX (Invitrogen, Paisley, UK). Cells were routinely maintained at exponential growth phase with viabilities over 95 % in 125 mL disposable polycarbonate Erlenmeyer flasks (Corning, New York, NY, USA). Erlenmeyer flasks were placed in an orbital shaker (orbit diameter 16 mm, Stuart, Stone, UK) at 130 rpm in a humidified incubator at 37 °C and 5 % CO_2 in air. Cell counts, viability, and vitality assay were performed using the NucleoCounter®NC-3000 automatic cell counter (Chemometec, Allerod, Denmark) according to manufacturer's instructions.

Plasmid DNA

The pGag-EGFP plasmid used for transfection codes for a Rev-independent HIV-1 Gag protein fused in frame to the enhanced GFP. The plasmid from the NIH AIDS Reagent Program (Cat #11468) (Hermida-Matsumoto and Resh 2000) was constructed by cloning the Gag sequence from

pCMV55M1-10 (Schwartz et al. 1992) into the pEGFP-N1 plasmid (Clontech, Palo Alto, CA, USA). Plasmid DNA amplification and purification was carried out as previously reported (Gutierrez-Granados et al. 2013).

Standard transient transfection

VLPs were generated by transient transfection of CAP-T cells with pGag-EGFP plasmid DNA using 40 KDa linear polyethylenimine (PEI Max, PolySciences, Warrington, PA, USA) as transfection reagent. The standard transfection protocol consisted of the steps explained here. However, as part of the optimization study, changes are introduced in the final protocol as detailed in the "Results" section. Prior to transfection, cells exponentially growing in PEM were subjected to a medium exchange to FreeStyle™ 293 (Invitrogen, Grand Island, NY, USA) supplemented with 0.1 % Pluronic (Invitrogen, Paisley, UK) by centrifugation at 200×*g* during 5 min. In this step, cells were concentrated to 5×10^6 cells/mL in 4 mL of medium. DNA at 1 pg/cell and PEI at 3 pg/cell (DNA:PEI mass ratio of 1:3) were diluted separately in FreeStyle™ 293 and vortexed for 10 s then the DNA solution was added to the PEI solution and the mixture was vortexed three times for 3 s. After 15 min of incubation at room temperature, 2 mL of complexes were added to the concentrated culture. Culture volume was completed with PEM (24 mL) 5 h post-transfection (hpt). The percentage of GFP expressing cells was assessed by flow cytometry using a fluorescence-activated cell sorter (FACS) (BDFACS Canto, BD BioSciences, San Jose, CA, USA).

Confocal microscopy

The visualization of VLP producer cells was performed using a FluoView®FV1000 confocal microscope (Olympus, Tokyo, Japan). Transfected cells were dyed with Hoechst and CellMask™ and subsequently observed under the microscope as previously reported (Gutierrez-Granados et al. 2013).

Quantitation of Gag-GFP VLPs by spectrofluorometry

The concentration of Gag-GFP VLPs was assessed by spectrofluorometry using an in-house developed and validated quantification technique (Gutierrez-Granados et al. 2013). The equation used to convert RFU values to Gag-GFP concentration values is the following:

$$\text{Gag-GFP}\ (\text{ng/mL}) = (3.245 \times \text{RFU-1.6833}) \times 36 \qquad (1)$$

where Gag-GFP is the estimated concentration of polyprotein and RFU is the measured GFP fluorescence intensity in the samples. To further convert Gag-GFP concentration to VLP concentration, it was assumed that one VLP contains 2500 Gag-GFP monomers (Chen et al. 2009) with a molecular weight of 84 kDa per monomer. Percentage of assembled Gag-GFP molecules was assessed using Vivaspin500 centrifugal filter units (300 kDa MWCO; Sartorius Stedim Biotech, Goettingen, Germany) to separate Gag-GFP monomers from VLPs and then quantifying both species by p24 ELISA as described in Gutierrez-Granados et al. (2013). The percentage of assembled Gag-GFP was over 80 %, so monomeric Gag-GFP molecules were not taken into account for further quantification.

VLP purification

VLPs from CAP-T transfected cell culture supernatants were further purified using a common retrovirus purification protocol consisting of virus particle pelleting by ultracentrifugation through a sucrose cushion (Transfiguracion et al. 2003). Briefly, a volume of 35.5 mL of clarified supernatant harvested 96 hpt was layered on top of a 30 % sucrose cushion (3 mL) and centrifuged at 26,000 rpm (115,254×*g*) for 3 h at 4 °C using a SW32 rotor in a Beckman Optima L100XP centrifuge. Pellets were resuspended in pre-chilled PBS and incubated overnight at 4 °C. Size exclusion chromatography (SEC) was performed using PD-10 Desalting Columns (GE Healthcare, Little Chalfont, UK) according to manufacturer's instructions. Elution was carried out with PBS.

VLP characterization by nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was employed for VLP characterization in a NanoSight®LM20 device (NanoSight Ltd., Amesbury, UK) at the Service of Preparation and Characterization of Soft Materials (Institut de Ciència de Materials de Barcelona, ICMAB, CSIC, Bellaterra, Spain). Sample analyses were performed as previously reported (Gutierrez-Granados et al. 2013).

VLP characterization by transmission electron microscopy

VLP samples were prepared by air-dried negative staining method at the Servei de Microscòpia (UAB, Bellaterra, Spain) as previously reported (Gutierrez-Granados et al. 2013) before the examination in a Jeol JEM-1400 transmission electron microscope (Jeol, Tokyo, Japan) equipped with a Gatan ES1000W Erlangshen CCD Camera (Model 785).

VLP characterization by tunable resistive pulse sensing

Purified VLP samples were measured using an Izon qNano (Izon Science Europe Ltd., Magdalen Centre, The Oxford Science Park, Oxford, UK). Polystyrene calibration particles with a concentration of 1.2×10^{13} particles/mL and mode size

of 110 nm were purchased from Thermo Fischer Laboratories (Waltham, MA, USA). All samples were dispersed in PBS for analysis. Particle concentration and size were calculated using Izon Control Suite Software V3.2 on a minimum of 500 particle events.

Optimization of transient transfection using design of experiments

Transient transfection was optimized in order to maximize VLP production (DoE response). The three variables chosen for optimization were cell density at the time of transfection, DNA/cell ratio, and PEI/cell ratio. A Box-Behnken design was selected to define the optimal value for each variable. The three variables were screened at three levels: a low level coded as −1, a medium level coded as 0, and a high level coded as +1 as indicated in Table 1. Box-Behnken experimental results were fitted to a second-order polynomial equation described below (Eq. 2) by non-linear regression analysis:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (2)$$

where Y is the response in RFU, β_0 is the offset term, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient, and X_i and X_j are the independent variables. This equation was used to predict the optimum values of the independent variables using the Solver feature of Microsoft Excel 2010 and R software (RStudio, Inc., Boston, MA, USA).

Statistical analysis

Statistical analysis of the models was performed using SigmaPlot 11.0 (Systat Software Inc.) and R Studio software (RStudio, Inc., Boston, MA, USA). The quality of the fit of the model equation is expressed by the coefficient R^2 obtained by regression analysis. Additionally, a lack of fit test was performed to compare the experimental error to the prediction error. The overall significance of the model was determined by analysis of variance (ANOVA) F test, whereas the significance of each coefficient was determined by the corresponding t test.

Results

Initial characterization of cell growth and transfection

As starting point of the study, some aspects related to the characterization of cell growth and transfection were assessed. CAP-T cell line is adapted to grow in suspension in PEM, and FreeStyle™ 293 has been previously reported as the transfection medium for these cells (Fischer et al. 2012). Particularly, the need to use two different media, one for cell growth and one for cell transfection, was first investigated with the aim to avoid medium exchange, which may add complexity to the process. First, transfection in PEM medium was performed and no GFP expressing cells were obtained (data not shown). In fact, PEM has been previously reported as incompatible with PEI transfection (Fischer et al. 2012). Second, cell growth in FreeStyle™ 293 was tested and compared with growth in PEM. CAP-T cells grew up to 7×10^6 cells/mL with a duplication time of 25 h in PEM medium. However, they only grew up to 3.6×10^6 cells/mL in FreeStyle™ 293 with a duplication time of 49 h, with lower viabilities (Fig. 1a). Transfection in FreeStyle™ 293 was required, while cell growth in PEM allowed obtaining higher cell densities with higher viabilities. Thus, medium exchange could not be avoided due to the lack of a culture medium that simultaneously supports high cell densities and transfection of CAP-T cells.

Gag-GFP VLP production upon transfection with the standard protocol in CAP-T cells was studied. The percentage of GFP-positive cells was 30 % at 24 hpt and almost 60 % at 48 hpt. Then, the percentage of GFP-positive cells decreased (Fig. 1b), although the concentration of GFP+ cells was maintained over time up to 120 hpt (data not shown). Cells were able to grow up to 6×10^6 cells/mL after transfection, while the untransfected culture grew up to 7.9×10^6 cells/mL with higher viabilities (data not shown).

Green fluorescent cells were visualized by confocal fluorescence microscopy 48 hpt. Green fluorescence was observed in the cytoplasm of transfected cells (Fig. 1c), but Gag-GFP molecules were particularly enriched in the vicinity of the plasma membrane. By staining the lipid membrane with CellMask™ (red), co-localization of green and red fluorescence was visualized as yellow regions on the membrane, where the virus particle budding occurs. Then, the kinetics of Gag-GFP expression upon transient transfection of CAP-T cells was evaluated by monitoring green fluorescence intensity in harvested supernatants at different times post-transfection (Fig. 1b). Gag-GFP fluorescence in cell culture supernatants increased continuously after transfection and stabilized at 120 hpt. Maximum fluorescence was 120 RFU, which corresponds to 4×10^{10} VLPs/mL according to Eq. (1). Cell viability was maintained over 80 % until 120 hpt.

The Gag-GFP VLPs produced in CAP-T cells upon transfection were further studied in order to demonstrate that Gag-GFP was correctly assembled into VLPs with the expected characteristics. Representative micrographs from VLP purified material were obtained by transmission electron microscopy (TEM) (Fig. 1d). Roughly spherical virus particles surrounded by a lipid envelope can be distinguished with a particle diameter around 138 ± 25 nm. The same sample was analyzed by NTA and tunable resistive pulse sensing (TRPS). NTA results showed a main

Table 1 Box-Behnken design, results, and ANOVA analyses

	−1		0		1
Cell density (E + 06 cells/mL)	2		5		8
DNA/cell ratio (pg/cell)	0.5		1.25		2
PEI/cell ratio (pg/cell)	3		5		8
Experimental run	Cell density	DNA/cell ratio	PEI/cell ratio	Max fluorescence in SN (RFU[a])	Viability (%)
1	0	1	1	80.96	64.70
2	1	0	−1	117.20	55.30
3	0	1	−1	102.17	85.60
4	0	0	0	130.15	53.10
5	1	0	1	81.67	77.00
6	1	1	0	78.01	49.50
7	0	−1	1	134.40	88.30
8	−1	1	0	103.07	64.00
9	1	−1	0	162.63	68.30
10	0	0	0	138.17	67.60
11	−1	0	−1	116.42	84.20
12	−1	−1	0	171.33	80.80
13	0	−1	−1	174.26	83.40
14	−1	0	1	110.56	79.90
15	0	0	0	131.93	67.60
Model	*F* test, *p* value[b]		Lack of fit test, *p* value[c]	R^2	
A (fluorescence-based)	0.0003		0.279		0.988
B (viability-based)	0.0948		0.598		0.860
Parameters (model A)	Coefficient		*t*		*p* value
Constant	129.856		40.265		<0.0001
[Cells]	−7.734		−3.916		0.0112
[Cells][b]	−8.789		−3.023		0.0293
[DNA]	−34.802		−17.622		<0.0001
[DNA][b]	7.694		2.647		0.0456
[PEI]	−12.806		−6.484		0.0013
[PEI][b]	−14.603		−5.023		0.004
[Cells] × [DNA]	−4.091		−1.465		0.2029
[Cells] × [PEI]	−7.418		−2.656		0.0451
[DNA] × [PEI]	4663		1670		0.1558

[a] RFU: relative fluorescence units

[b] *p* values under 0.05 are considered statistically significant with 95 % confidence, and *p* values under 0.1 are considered statistically significant with 90 % confidence

[c] *p* values associated to lack of fit test above 0.05 mean that the hypothesis arguing that the model is suitable cannot be rejected

peak size of (average statistical mode) 169.5 ± 60 nm (Fig. 1e), and 46 % of the particles in the supernatants were determined to be between 100 and 200 nm. Accordingly, when particle size analysis was performed by TRPS technique, a mean size of 154 nm was determined.

Stepwise optimization of the transient transfection protocol for CAP-T cells

Several features of the transfection process were further studied in order to improve the standard production protocol

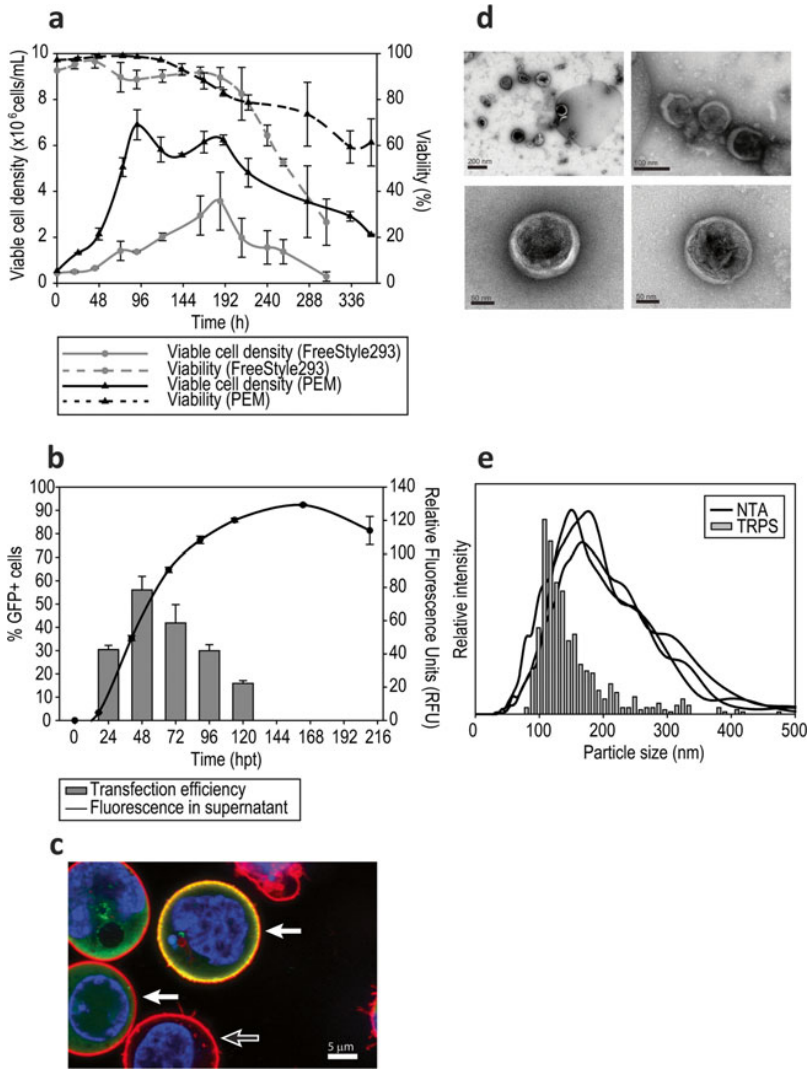

Fig. 1 **a** Cell growth in 125-mL disposable Erlenmeyer flasks of CAP-T cells in batch. **b** Percentage of GFP+ cells and fluorescence signal in cell culture supernatants at different times post-transfection of CAP-T cells transfected with the standard protocol. **c** Confocal fluorescence microscopy images of CAP-T producer cells 48 hpt. Cells were subjected to double staining: cell nucleus was stained with Hoechst (*blue*) and membrane was stained with CellMask™ (*red*). The *green fluorescence* indicates the presence of Gag-GFP molecules. *Solid arrows* indicate transfected cells, and the *empty arrow* indicates a non-transfected cell. **d** Electron microscopy image of negatively stained Gag-GFP VLPs. **e** NTA and TRPS analysis of purified Gag-GFP VLP samples. All experiments were performed in triplicate. *Error bars* represent standard deviation in all cases

described before. These include cell state at the time of transfection, DNA-PEI complexation, and PEM addition after transfection. The mentioned factors are considered as discrete variables affecting transient transfection that need to be addressed individually (Thompson et al. 2012).

Study of cell state at the time of transfection

A growth curve in PEM medium was performed in order to study if cell state has an influence on the productivity of CAP-T cells, and cell density, viability and vitality were analyzed every 24 h (Fig. 2a). Cells grew up to 8.7×10^6 cells/mL with viabilities over 80 % and with a duplication time of 25 h. The vitality assay indicates the percentage of cells with high concentrations of thiol-containing molecules. These molecules are easily oxidized; thus, they are able to protect cells from the reactive oxygen species. That is the reason why thiol-containing molecules have an essential role on maintaining the redox state of cells (Circu and Aw 2010). The percentage of healthy cells was maintained high (over 70 %) during the exponential growth phase. Viability and vitality of cells decreased after reaching the maximum cell concentration.

Additionally, cells obtained at various times of the growth curve in PEM (indicated with gray arrows in Fig. 2a) were transfected using the standard protocol (as described in "Materials and methods" section). The percentage of GFP positive cells (24 hpt) and fluorescence in supernatant (120 hpt) of each transfected cells were analyzed (Fig. 2b). Transfection efficiency was higher at days 2, 3, and 4 after cell seeding, which coincides with the mid-exponential phase than when cells were transfected at 1, 5, and 7 days after seeding. VLP production was significantly (p value <0.001) increased when transfected cells were derived from mid-exponential phase (days 2–4), obtaining almost threefold fluorescence than when transfected cells were derived from days 1 and 7 after seeding. Thus, the best performance coincided with mid-

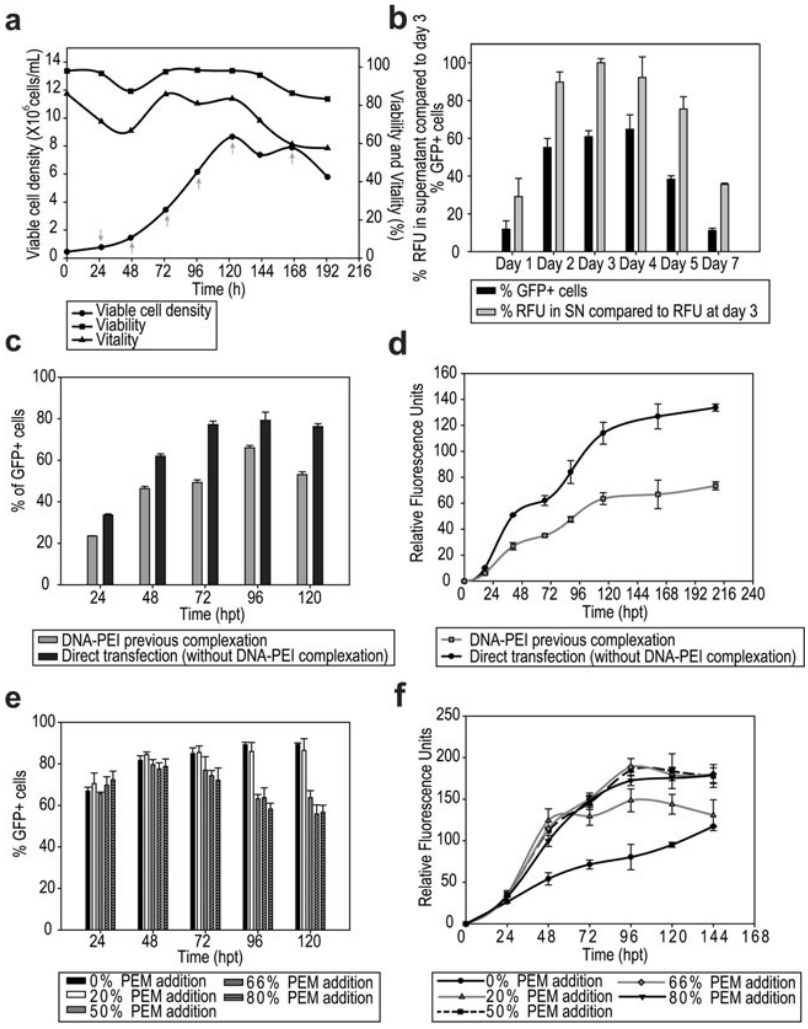

Fig. 2 Stepwise optimization of the standard transfection protocol. **a** Cell growth in 1 L disposable Erlenmeyer flasks of CAP-T cells in PEM. *Arrows* indicate the times at which transfections were performed. **b** Percentage of GFP+ cells 24 hpt of the cells transfected on different times of the curve. Fluorescence in cell culture supernatants 120 hpt expressed as percentage respect fluorescence obtained at day 3. **c**, **d** Percentage of GFP+ cells and fluorescence in cell culture supernatants after transfection when CAP-T cells are transfected with and without prior DNA-PEI complex formation. **e**, **f** Percentage of GFP+ cells and fluorescence in cell culture supernatants after transfection when different percentages of PEM were added 5 hpt. All experiments (except **a**) were performed in triplicate. *Error bars* represent standard deviation in all cases

exponential phase, when cells are healthier and highly dividing. All subsequent transfections were performed 2 days after seeding cells at 0.5×10^6 cells/mL in an attempt to maximize production and gain reproducibility of the process.

Study of direct addition of DNA and PEI avoiding pre-complexation step

Transfection with prior DNA-PEI complexation was studied in comparison with the direct transfection approach, consisting in adding DNA and PEI solutions separately to the culture. This approach has been successfully used in HEK293 and CHO cell lines (Backliwal et al. 2008b; Schlaeger and Christensen 1999) and also in CAP-T cells (Fischer et al. 2012), yielding good transfection efficiencies and product levels. The standard protocol was compared to a transfection performed by adding 1 mL of DNA solution and 1 mL of PEI solution, both at the same final concentration than in the standard protocol but in this case without previous complexation. The percentage of GFP-positive cells and the fluorescence in cell culture supernatants were monitored every 24 hpt. Both transfection efficiency and fluorescence in supernatants (Fig. 2c, 2d) were significantly higher (p value <0.001) when DNA and PEI were added separately in contrast to DNA-PEI complex formation prior to transfection. Since complexation of DNA and PEI did not contribute to an improvement in VLP production, all subsequent transfections were performed using the direct transfection method.

Study of PEM addition after transfection

Finally, the addition of PEM medium after transfection was studied in order to determine the effect of different percentages of PEM in VLP production medium. To this end, five transfections were performed using the standard protocol, this time without DNA-PEI complexation. After 5 hpt, the culture was completed with 80 % of the final volume with different percentages of PEM (0, 20, 50, 66, and 80 %) and FreeStyle™

293 (80, 60, 30, 14, and 0 %). Transfection efficiency and fluorescence in supernatants were analyzed every 24 hpt. The percentage of GFP-positive cells was higher in the cultures that contained 0 and 20 % of PEM (Fig. 2e), probably due to the absence of cell growth after transfection (data not shown). Conversely, when 50 % or more PEM was added after transfection, the percentage of GFP-positive cells decreased over time (from 48 to 120 hpt), probably due to the fact that cells were able to grow after transfection up to 2×10^6 cells/mL (data not shown). Regarding VLP production, Fig. 2f shows that addition of PEM after transfection allowed increasing VLP titers in comparison to the transfection where no PEM was added. A minimum of 50 % of PEM was required to maximize VLP titers.

Summarizing, in order to achieve better performance of CAP-T cells producing Gag-GFP VLPs by transient transfection, cells should be transfected in the mid-exponential phase, DNA and PEI should be added separately, avoiding complex formation before transfection and the final transfected cells should be cultured in a medium containing at least 50 % of PEM.

Transient transfection optimization by design of experiments

Besides the discrete variables studied in the previous section, there are some parameters influencing transient transfection that should be considered as continuous. These are DNA to cell ratio, PEI to cell ratio, and cell concentration at the time of transfection. Continuous variables were studied by means of a design of experiments (DoE). A response surface methodology (RSM), specifically a Box-Behnken design, was selected as it is an efficient methodology in terms of the number of experimental runs to be performed related to the statistically relevant information that can be obtained (Montgomery 1997).

It should be highlighted that the design space must be carefully considered when using DoE approaches since the results will depend on it. Regarding cell density and DNA to cell ratio, the working ranges were set based on the literature (Backliwal et al. 2008a; Cervera et al. 2013; Fischer et al. 2012; Thompson et al. 2012). In the case of PEI to cell ratio, an empirical identification of the working range was performed. Since PEI has a toxicity effect on cells, the upper limit was set at 8 pg/cell based on a toxicity assay (data not shown). 3 pg/cell was set as the minimum PEI necessary to obtain transfected cells. Working ranges for each variable are presented in Table 1.

A three-factor three-level Box-Behnken design was constructed using the selected concentration ranges for each variable as the design space boundaries. A 15-experiment matrix was defined (Table 1) in which the central point was triplicated to assess the pure experimental error. Transfections were performed with a 2 day-old culture previously seeded at 0.5×10^6 cells/mL, without pre-complexation and adding in all cases 50 % of PEM after transfection, according to the conditions defined as optimal in the previous section.

The response variables considered in this study were the maximum fluorescence in cell culture supernatants after transfection and the viability at the time of maximum production (Table 1). The data were fitted to a second-order model by non-linear regression analysis obtaining a model for each one of the response variables. The statistical significance of the non-linear regressions was confirmed by ANOVA analysis (Table 1).

The data from model A were used to construct the response surface plots where the interactions between the factors can be depicted (Fig. 3a–i). Noticeably, cell density does not have a strong influence on fluorescence outcome as can be seen in Fig. 3a–c, where cell density increases from 2 to 8×10^6 cells/mL and the maximum fluorescence does not change. Otherwise, DNA and PEI have a stronger influence on VLP production with smaller associated p values than the p value for cell density (Table 1). The optima for DNA and PEI are located in the lower boundaries of the design space, as can be seen in Fig. 3d–f, g–i, respectively, the increase in DNA/cell ratio or PEI/cell ratio was translated into a decrease of fluorescence in supernatant. A statistically significant interaction between cell concentration and PEI/cell ratio was identified (p value 0.0451, see Table 1). As cells are more concentrated at the time of transfection, the amount of PEI/cell required to achieve maximum fluorescence decreases (Fig. 3a–c).

The effect of cell density, DNA and PEI on viability of the cultures was explored by analyzing a third model (C) whose response variable was $Y = \text{Fluorescence} \cdot (\text{Viability}/100)^n$ (3), where n is between 0 and 1. When $n = 0$, the viability has no importance in the model; thus, the weight of viability in this particular model increases with n. Figure 3j shows the value of each factor studied as a function of the different n values tested. As can be observed, DNA concentration is not influencing the viability of the cultures, as it remains constant regardless of the weight of the viability in the model. Otherwise, PEI and cell density have an influence on viability: the more important viability is in the model (n increases), the lower PEI and cell concentration need to be to achieve the optimum.

Model A was used to predict an optimal combination of the three factors to maximize fluorescence signal in supernatants, but a restriction was applied to this optimization: the viability predicted by model B with the conditions found should be at least 88 % which is the maximum viability attained experimentally. With these conditions, a predicted maximum fluorescence in supernatant of 170.31 ± 10.95 RFU was obtained, with a viability of 88 ± 15.34 % predicted by model B. The optimization of model A without restricting the viability predicted a fluorescence of 177.64 RFU (only a 4 % increase) while the predicted viability was dropped to 74 %. Thus, the optimal concentration of each variable was 0.5 pg of DNA/cell, 3 pg of PEI/cell (PEI:DNA mass ratio of 6:1) and 3.3×10^6 cells/mL at the time of transfection.

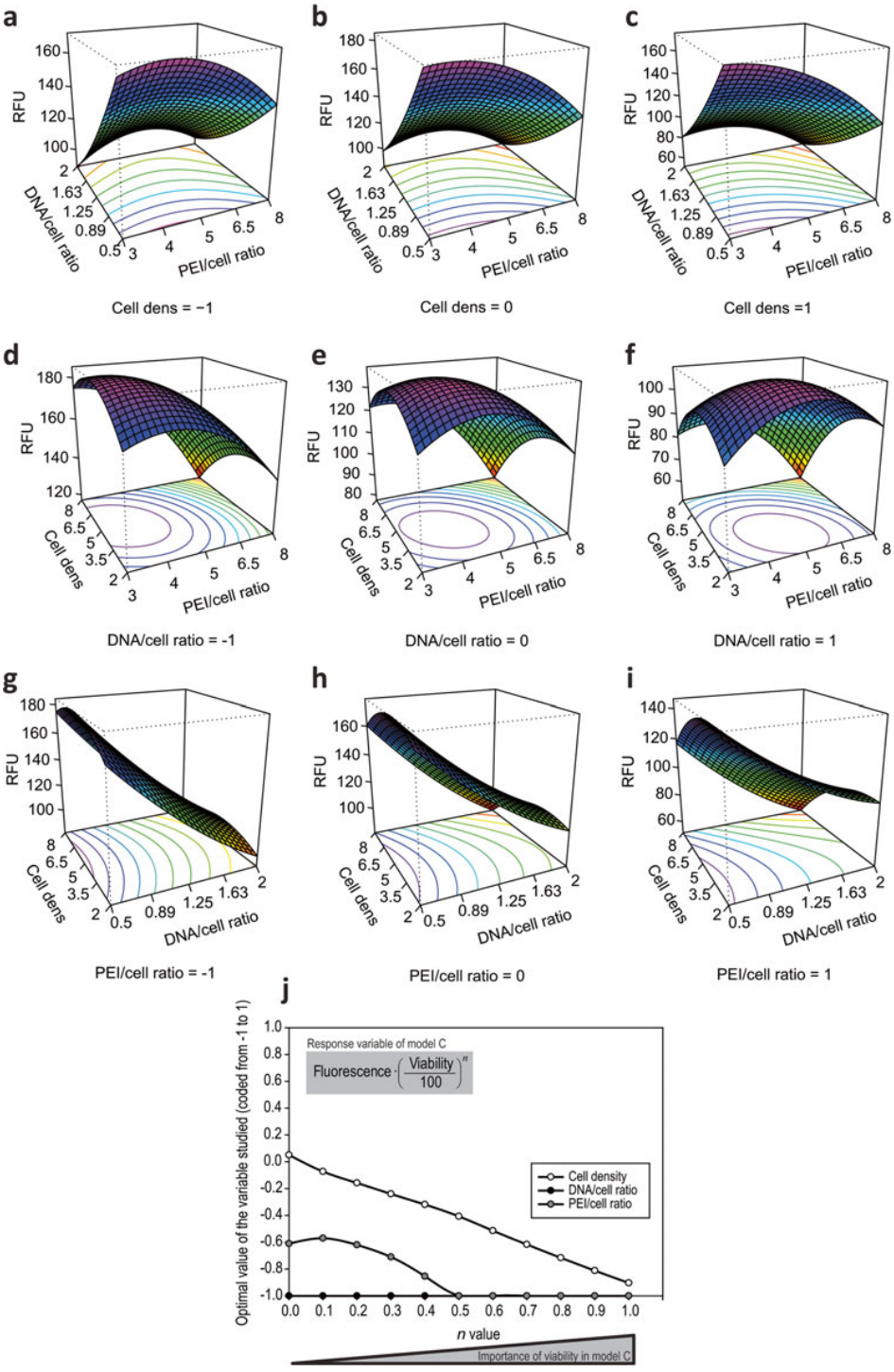

Fig. 3 Response surface graphs based on Box-Behnken experimental results. Maximum fluorescence in cell culture supernatants as a function of **a**, **b**, **c** cell density vs. DNA/cell ratio; **d**, **e**, **f** cell density vs. PEI/cell ratio; and **g**, **h**, **i** DNA/cell ratio vs. PEI/cell ratio. The graphs were constructed by depicting two variables at a time and maintaining the third one at a fixed level. **j** Analysis of the influence of cell density, DNA, and PEI on cell culture viability. The optimal values of each of the factors studied are represented as a function of the *n* value. The value of *n* is an indication of the importance of cell culture viability in the model; thus, the influence of each factor in viability can be depicted

Validation of the model

A confirmation experiment was carried out with the aim of validating the model. To this end, the optimal cell density, DNA and PEI to cell ratios predicted by the model were tested experimentally. With these transfection conditions, the experimental output was a maximum fluorescence in supernatant of 172.83 ± 19.61 RFU (Fig. 4a), which was within the range of the predicted response (170.31 ± 10.95 RFU), confirming model adequacy (Fig. 4b). Cell culture viability at the time of maximum fluorescence (96 hpt) was 90.43 ± 0.86 % (Fig. 4a), in good agreement with the predicted 88 % viability.

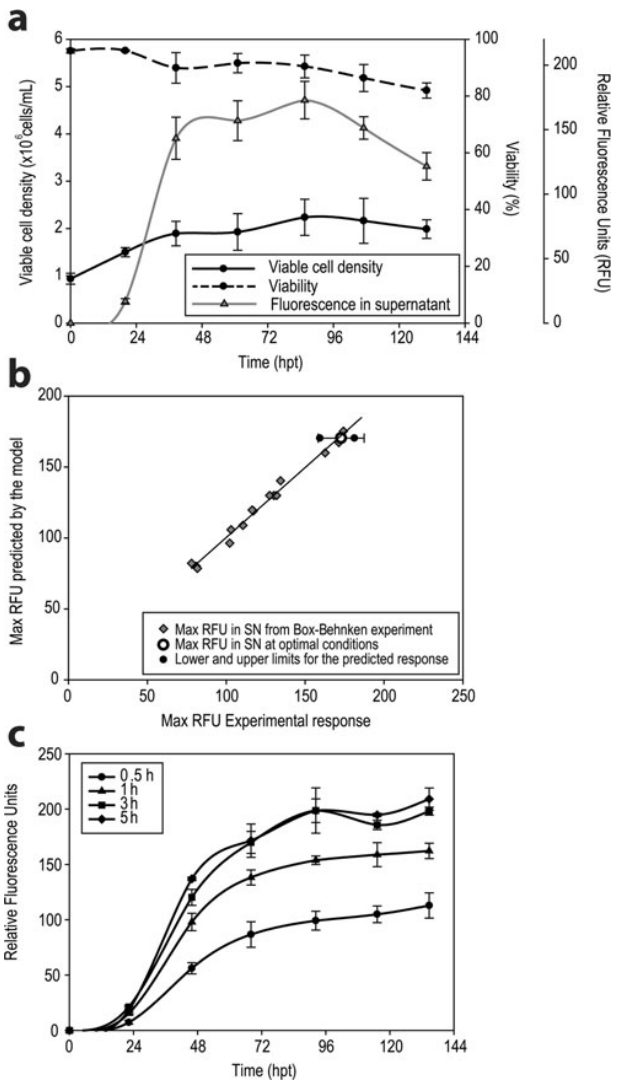

Fig. 4 Model validation. **a** Cell growth, viability, and fluorescence in supernatant after transfection with the optimal conditions. **b** Correlation between the experimental and Box-Behnken model predicted fluorescence values in RFU. The *white dot* represents the experimental vs. predicted value when the optimal conditions for transfection are used, and *solid dots* represent the upper and lower bounds for the predicted value, calculated aspredicted value $\pm 1.96 \times \sqrt{\mathrm{MS}}$, with a confidence interval of 95 %. **c** Fluorescence in cell culture supernatants over time after transfection with the optimal conditions, but with different times of PEM addition after transfection. All experiments were performed in triplicate. *Error bars* represent standard deviation in all cases (*MS* mean squared error)

Finally, to further refine the protocol, different times of PEM addition were tested. These included 0.5, 1, 3, and 5 hpt. Figure 4c shows that a minimum time of 3 h after transfection is necessary before adding PEM to the culture in order to obtain maximum fluorescence signal in supernatants. When PEM addition was performed earlier, transfection process was altered likely because of the interference that PEM medium has on PEI-mediated transfection, and consequently, fluorescence intensities were lower.

Discussion

The systematic optimization of a transient transfection protocol allowed to develop a simple, robust, and high yielding method for the production of HIV-1-based VLPs in CAP-T cells. A concentration of 5.8×10^{10} VLPs/mL was achieved, which represents a 44 % of improvement compared to the initial conditions. CAP-T cells were proven to be a suitable platform for Gag-based VLP production. Generated VLPs presented the expected size (~120–150 nm) and morphology consistent with immature HIV-1 particles and similar to VLPs produced in Sf9 or HEK293 (Cervera et al. 2013; Valley-Omar et al. 2011). CAP-T cells have been used for influenza virus production before (Genzel et al. 2013); nonetheless, to the best of our knowledge, this is the first evidence of HIV-1-based VLP production in this cell line.

Despite the recent novelty of CAP-T cells, it has already been demonstrated that they represent a powerful system for expression of heterologous proteins (Fischer et al. 2012). A comparison of Gag-based VLP production levels between CAP-T and other reported systems is summarized in Table 2. These results support that CAP-T cells are a suitable and promising alternative to classical systems because they are able to produce higher titers of complex VLPs in the correct conformation. Noticeably, the high expression levels of VLPs upon transfection would allow obtaining, in 1 L of culture, enough material to immunize 500 mice (Table 2).

Even though CAP-T cells represent a strong platform, there are many variables influencing TGE yields that can be optimized. These variables are the medium chosen for transfection, cell density at the time of transfection and the cell state, DNA concentration, transfection reagent, its mass ratio to DNA, and the DNA-PEI complexation conditions. After study of the latter, the final transfection protocol is as follows: cells are seeded in PEM at 0.5×10^6 cells/mL, after 2 days, thus in mid-exponential phase, they are subjected to a medium exchange to FreeStyle™ 293 prior to transfection. In this step, cells are concentrated to 3.3×10^6 cells/mL in 30 % of the final volume. DNA at 0.5 pg/cell and PEI at 3 pg/cell (DNA:PEI mass ratio of 1:6) are diluted in FreeStyle™ 293 and vortexed for 10 s then both solutions are added separately to the concentrated culture. Culture volume is completed with PEM 3 hpt.

Although PEM is not compatible with PEI transfection, it better supports cell growth and protein production compared to FreeStyle™ 293. Addition of PEM after transfection allows improvement of VLP production, which is in good agreement with many reported TGE protocols that also include a dilution step with fresh medium after transfection to improve cell

Table 2 Comparison of production levels of HIV-1 Gag-based VLPs in different systems

VLP	Cell line	Production approach	Gag/L (μg)	Fold-improvement of CAP-T compared to alternative system	Number of mice that could be immunized with 1 L of culture[b]	Reference
Gag-GFP	CAP-T	TGE	20130[a]	1	503	This work
Gag-GFP	HEK293SF-3F6	TGE	4170[a]	5	104	(Cervera et al. 2015)
Gag-GFP	HEK293SF-3F6	TGE	950[a]	21	24	(Cervera et al. 2013)
Gag	High Five	SGE	655	31	16	(Tagliamonte et al. 2010)
GagRT and GagTN	T.ni ProTM cells and Sf9	Baculovirus infection	504	40	13	(Pillay et al. 2009)
Gag	*Saccaromyces cerevisiae* spheroplasts	Yeast transformation	196	102	5	(Sakuragi et al. 2002)

[a] Converted from relative fluorescence units according to (Gutierrez-Granados et al. 2013)

[b] Assuming a mouse is immunized with two doses of 20 μg of VLPs according to (Tagliamonte et al. 2010) and not considering purification losses

growth and increase mRNA levels and consequently, protein concentration (Backliwal et al. 2008b; Fischer et al. 2012; Tuvesson et al. 2008). On the other hand, the availability of new chemically defined and serum-free media that can support both high cell densities and transient transfection is crucial to overcome the potential limitations associated with medium replacement steps, especially in bioreactors (Geisse 2009; Tuvesson et al. 2008). In this regard, media development projects are ongoing by the authors to address this issue. The lack of such culture media makes that many authors end up using medium replacement strategies in bioreactor, even at scales of 100 L because of the associated benefits (addition of nutrients to improve cell performance and removal of metabolism by-products that may interfere with cell transfection) (Girard et al. 2002; Haldankar et al. 2006). Alternatively, large-scale TGE involving medium exchange could be performed by perfusion mode (Ansorge et al. 2009) or by continuous centrifugation (Tuvesson et al. 2008).

Optimization of the discrete variables affecting TGE have led to reduction of steps and increased simplicity of the protocol, which make it very suitable for routinely producing recombinant proteins. Moreover, efforts towards variability minimization are also relevant in TGE processes. Namely, the health of the cells at the time of transfection may be a source of variability if it is not appropriately considered (Carpentier et al. 2007; Grosjean et al. 2002; Liu et al. 2008); thus, transfection of cells 2 days after seeding in PEM enables to avoid this specific source of variability. Furthermore, direct transfection strategy enables to circumvent variability arising from the complex formation, thus making the protocol more reproducible and the number of steps is minimized, which simplifies the process. Valuably, the direct transfection approach is advantageous for process scale-up and automation. Since DNA-PEI complexation depends on many variables such as the medium composition, mixing, or incubation time, accurate control of all variables involved in this step is not operationally trivial when working with large-scale bioreactors and also it hinders process automation (Raymond et al. 2011).

Optimization of cell density, DNA, and PEI to cell ratios was carried out by a DoE approach which is a powerful tool to rapidly identify the optimal combination of the main process variables and has been used in other works to improve the yields of transiently expressed proteins (Backliwal et al. 2008a; Pillay et al. 2009; Thompson et al. 2012). The main advantages of this approach are that statistically relevant results are obtained with fewer experiments compared to the "one factor at a time" strategy and that interactions between variables can be depicted. The optimal PEI to DNA ratio found for CAP-T cells is in good agreement with previously reported results (Fischer et al. 2012). Noticeably, the DNA concentration was kept relatively low, what can be regarded as an advantage, since DNA is one of the major-cost factors of TGE, and producing high quantities of high-quality DNA for transfection is a bottleneck in TGE scale-up (Pham et al. 2006).

In the specific case of Gag-based VLP production, cell culture viability becomes very relevant. Considering that dead cells would theoretically release their intracellular content into the supernatant, contaminating the VLP preparations with intracellular proteins as well as free Gag-GFP monomers, a higher viability at the time of harvest is preferred. That is why optimal conditions are determined by restricting a minimum cell culture viability value, even with the associated decrease in fluorescence compared to the optimization without restricting the viability value. Not surprisingly, PEI is the variable with the highest effect on viability, so it needs to be minimized in order to avoid toxicity effect and gain viability of the culture. On top of the relevance of PEI/cell ratio by itself, its statistically significant interaction with cell density in the second-order model suggests that volumetric concentration of PEI is also important since the more concentrated cells

are at the time of transfection, the lower volume is used to transfect and PEI is more concentrated, thus more toxic to the cells.

In conclusion, although there is room for further improvement of TGE in this cell line by applying a number of strategies previously reported (Backliwal et al. 2008a; Cervera et al. 2015; Vink et al. 2014), high expression of HIV-1-based VLPs was achieved in this work upon variables optimization. Future efforts toward scale-up of this process should allow the rapid and efficient production of enough material for pre-clinical studies.

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Compliance with ethical standards This article does not contain any studies with animals or human participants performed by any of the authors.

Conflict of interest Jens Wölfel is an employee of CEVEC Pharmaceuticals, the company that has developed the CAP-T cell line and has contributed with scientific advice to this work.

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