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Process Optimization of Lentiviral Vector Production Using Design of Experiments

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Abstract

Optimizing process parameters for lentiviral vector production is crucial for achieving high-quality and high-yield products for gene therapy applications. Lentiviral vectors are pivotal tools in delivering genetic material into target cells. Their efficiency and safety are directly influenced by production conditions.

This application note delves into the significance of fine-tuning process parameters for transient lentivirus production, including plasmids, transfection reagents, culture medium, and culture conditions. By systematically optimizing these parameters, vector titers can be enhanced, and process consistency as well as reproducibility can be ensured.

For further information, visit
https://shop.sartorius.com/de/p/fectovir-lv/FectoVIR_LV
https://shop.sartorius.com/de/p/hek-vip-nb/HEK_ViP_NB

Introduction

Lentiviral vectors (LVs) are one of the most popular viral vectors for *ex vivo* cell and gene therapy. Their ability to transduce dividing as well as non-dividing cells and to stably integrate their transgene into the host cell genome has made LVs valuable tools for cell and gene therapy, including CAR-T cell therapy (Munis, 2020; Bulcha, 2021).

Efficient production processes are critical to meet the growing demand for LV therapies. However, due to the inherent sensitivity of the LV lipid membrane to process parameters such as temperature and production medium osmolarity, the development of efficient production processes is challenging (Bandeira, et al., 2012). Therefore, it is advisable to conduct detailed process examination to identify and optimize relevant parameters. A design of experiments (DoE) strategy is a valuable tool to identify such parameters in a cost- and time-efficient way. Hence, the DoE software MODDE® (Sartorius) was employed in this study to identify and optimize critical parameters of a transient LV production process. As a first step, the new FectoVIR®-LV (Sartorius) transfection reagent was tested and compared to the conventional polymer-based reagent PEIpro® (Sartorius). Aiming to determine the optimal DNA quantity and DNA:transfection reagent ratio, a DoE was performed at shake flask scale. The results were transferred to a controlled LV production process performed in the Ambr® 15 (Sartorius).

Subsequently, further process parameters comprising dissolved oxygen (DO), stirrer speed, the impact of a pH- or temperature shift, media, and plasmids were evaluated through DoE and separate factor at a time approaches. MODDE® (Sartorius) was employed in this study to identify and optimize critical parameters of a transient LV production process.

Materials and Methods

Routine Cultivation

A commercially available suspension HEK293 cell line was thawed and cultivated in an internally developed HEK medium, the Sartorius HEK media 4Cell® HEK ViP NB, HEK ViP NX, HEK TF, and two commercially available HEK media. Cells were subcultured to a VCD of 0.3 – 0.4E6 cells/mL every three to four days. Cells were cultivated in plain bottom shake flasks with a vented cap at a culture volume of 30 mL, 37 °C, 5% CO_2, 120 rpm and 50 mm orbit. Cells were adapted to culture media for three to five passages prior to LV production.

Lentivirus Production in Shake Flaks Scale

One day before transfection, cells were seeded to a density of 1.9 × 10E6 cells per mL in 22 mL culture medium. Transfection was performed with plasmid concentration indicated as µg per 1 × 10E6 cells at a final cell density of 3E6 cells/mL. A four-plasmid system (pALD Aldevron) or the pPLUS® LV packaging plasmids (Sartorius) were employed. As transfection reagents, PEIpro® (Sartorius) or FectoVIR®-LV (Sartorius) were used at indicated ratios with the DNA. Pre-complexation of DNA and transfection reagent was performed in the indicated culture medium using 5%, i.e., 1.5 mL of the final culture volume for FectoVIR®-LV, and 10% for PEIpro®. Mixing the components at an overhead shaker for 20 seconds at 15 rpm was followed by incubation of 30 or 20 min at room temperature for FectoVIR®-LV or PEIpro®, respectively. Subsequently, the transfection mixture was added to the culture, followed by addition of 6 mL fresh culture medium. For temperature shift experiments, temperature was reduced to 32 °C directly after addition of the transfection mixture, at 24 h or 48 h post-transfection. LVs were harvested 72 h post-transfection and filtered with 0.45 µm PES filters (Sartorius) before titer analytics.

Ambr® 15 Cultures

For LV production at Ambr® 15 scale, culture medium in microbioreactors was equilibrated to culture conditions (37 °C, 30% or 50% DO, pH 7.1) for one day. The microbioreactors were then inoculated with the indicated cells at a viable cell density (VCD) of 2.8 × 10E5 cells/mL in 14.7 mL final volume per vessel. Cultures were stirred at 545 or 600 rpm (upstirring). After three days, cells were passaged to a VCD of 2.0 × 10E6 cells/mL in 11 mL final volume. The next day, transfection was performed using the same parameters described for shake flask experiments. FectoVIR®-LV was used as a transfection reagent and pre-complexation was performed in 5% of the final culture volume. Based on the smaller final volume, 0.715 mL transfection mixture and 2.5 mL fresh medium were added. Transfection was performed in sets of six microbioreactors. VCD and viability were measured daily using an automated cell counter. As indicated, a pH shift to 6.8 was conducted after transfection, whereas a pH shift to 6.6 was executed 18 hours post-transfection. LV-containing supernatants were collected at 72 h post-transfection, filtered with 0.45 µm PES syringe filter, and stored at -20 °C until further analysis.

Infectious Titer Assay

A flow cytometry-based assay was used to determine the infectious titer of the produced LVs. In short, adherent HEK293T cells were seeded in 12-well plates with 8 µg/mL hexadimethrine bromide-containing culture medium and transduced with the LV particles. A media exchange was performed 24 h post-transduction to wash out hexadimethrine bromide-containing medium, and 72 h post-transduction, cells were washed twice with PBS, trypsinized, and measured in the iQue®3 flow cytometer.

DoE Approach

A D-optimal design was employed for all DoE approaches to maximize information while minimizing estimate uncertainty by reducing the covariance matrix. This design is especially advantageous when the number of experiments is limited. For the first DoE approach, the following parameters were used: transfection reagent (FectoVIR®-LV or PEIpro®), DNA amount per 1 million cells (minimum 0.5 µg, maximum 1 µg), DNA: transfection reagent ratio (minimum 1:1, maximum 1:2). The second DoE approach comprised the following parameters: DNA amount per 1 million cells (minimum 0.2 µg, maximum 0.5 µg) and DNA-TF reagent ratio (minimum 1:1.5, maximum 1:3). For the third DoE approach, the following parameters were used: stirrer speed (545 or 600 rpm), DO (30% or 50%), culture medium (4Cell® HEK ViP NB or internally developed variant), pH shift (none, shift to 6.6, or shift to 6.8).

Results

Use of FectoVIR®-LV Increased Lentivirus Titers by More Than 3-Fold Compared to PEIpro®

FectoVIR®-LV is a next-generation transfection reagent, free of animal components, and developed for the transient transfection of LV-producing HEK293 cell systems. The performance of FectoVIR®-LV was evaluated in comparison to the gold standard transfection reagent PEIpro® using a competitor four-plasmid system. For cultures transfected with FectoVIR®-LV, cell growth slowed down from 24 h post transfection (Figure 1). In comparison, when PEIpro® was used, reduced cell growth was observed from 48 h post transfection (Figure 1). In both cases, use of a 1:2 ratio of DNA to transfection reagent had the largest impact on cell growth (Figure 1), but did not impact transducing titer (Figure 2).Compared to PEIpro®, transducing titer was increased by 4-fold when FectoVIR®-LV was used for transfection (Figure 2). This was also confirmed by statistical analysis with MODDE®, which identified the transfection reagent as the major variable influencing LV transducing titer (Figure 3). Within the ranges tested, neither high DNA:transfection reagent ratios nor the DNA concentration per one million cells/mL had an impact on transducing titer (Figure 3). In conclusion, by using FectoVIR®-LV instead of PEIpro®, LV yields can be increased 4-fold.

Figure 1: *Reduced cell density and viability when using higher DNA:Transfection reagent ratios. A commercially available HEK293 cell line was cultivated in 4Cell® HEK ViP NB at shake flask scale, and transfected either with FectoVIR®-LV or PEIpro® using indicated DNA amounts, DNA:transfection reagent ratios, and a competitor LV packaging plasmid system.*

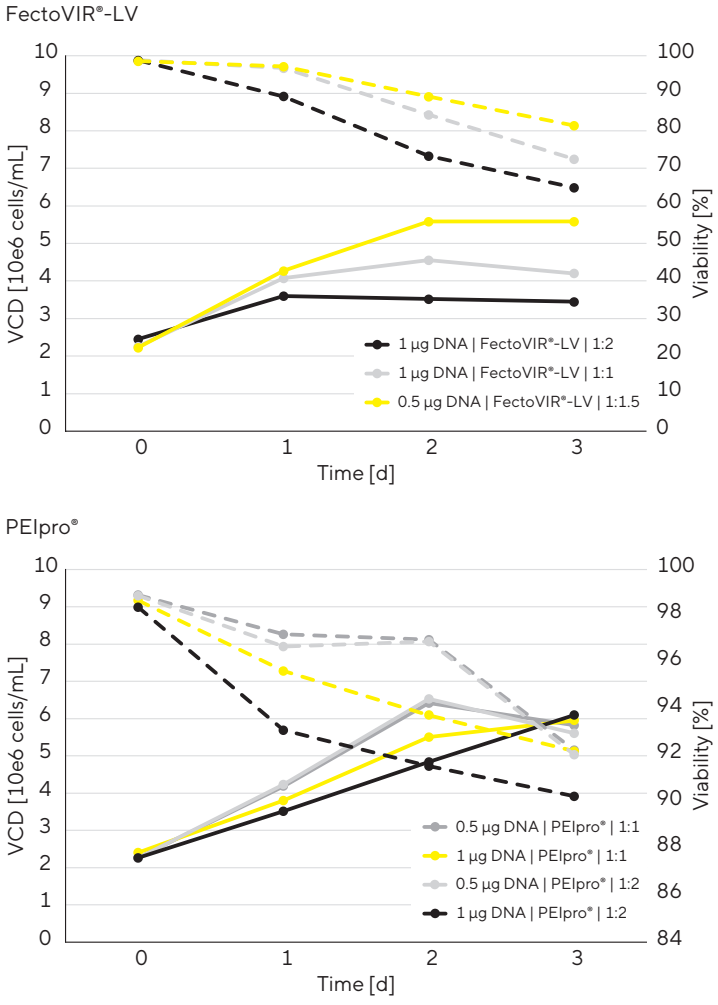


Figure 2: *FectoVIR®-LV- increased transducing titer by a factor of four compared to PEIpro®-transfected cells. A commercially available HEK293 cell line was cultivated in 4Cell®HEK ViP NB in shake flask scale, and transfected either with FectoVIR®-LV or PEIpro® using indicated DNA amounts and DNA:transfection reagent ratios and competitor LV packaging plasmids. Harvest was performed 72 h after transfection and transducing titer was analyzed by flow cytometry. Depicted is the mean of duplicates ± standard deviation of one experiment.*

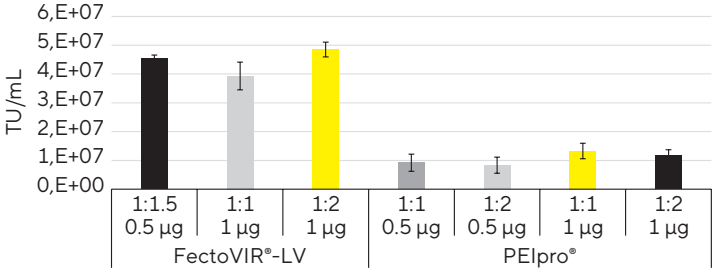
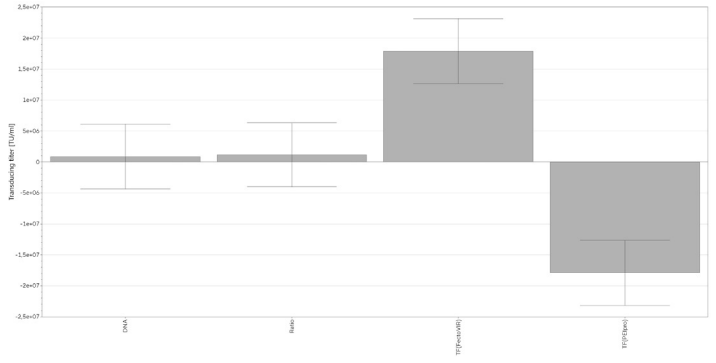


Figure 3: *Analysis with MODDE® identified FectoVIR®-LV as the major impact factor on transducing titer. Results from the transducing titer assay were analyzed in MODDE® with help of a coefficient plot. FectoVIR®-LV correlated strongly with an increase in transducing titer ("TF(FectoVIR)"), while PEIpro® showed a negative impact on TU ("TF(PEIpro)"). Neither the DNA concentration per one million cells ("DNA") nor the ratio of DNA to transfection reagent ("Ratio") had an impact on transducing titer.*



Optimization of DNA Concentration for Transfection

Since the previously analyzed range of 0.5 to 1 µg DNA per one million cells did not impact transducing titer, lower DNA amounts, i.e., 0.2 to 0.5 µg, were tested in a second DoE approach with FectoVIR®-LV to define the optimal amount for LV production. Given that the total amount of transfection reagent also decreases when reducing DNA quantity, higher ratios were included to assesses whether the total amount of transfection reagent influenced product yields. Compared to using 0.5 µg DNA per one million cells which yielded in the highest overall titer, use of 0.2 µg led to a four-fold decrease and the lowest transducing titer (Figure 4). Importantly, lower titer caused by reduced DNA amounts could not be restored by increased DNA to transfection reagent ratios (Figure 4). This was in line with statistical analysis in MODDE® identifying the DNA concentration as the major factor influencing LV transducing titer (Figure 5). MODDE® analysis further indicated a significant effect of the DNA to transfection reagent ratio, while a combinatorial effect of DNA amount and DNA to transfection reagent ratio was not significant (Figure 5). From a biological perspective, differences between transducing titers were neglectable for the DNA to transfection reagent ratios tested. In conclusion, 0.5 µg DNA per one million cells was identified as the optimal DNA concentration for the tested transient LV production system. It was combined with a DNA to transfection reagent ratio of 1:2 for further process optimization in a pH-controlled system.

Figure 4: *DNA concentrations less than 0.5 µg per one million cells are detrimental for the transducing titer and cannot be restored by higher DNA: transfection reagent ratios. A commercially available HEK293 cell line was cultivated in 4Cell® HEK ViP NB at shake flask scale and transfected with FectoVIR®-LV using indicated DNA amounts, DNA:transfection reagent ratios and a competitor LV packaging plasmids. Harvest was performed 72h after transfection and transducing titer was analysed by flow cytometry. Since the samples were part of a screening experiment, the data shown here are from individual measurements and no statistical analysis was performed.*

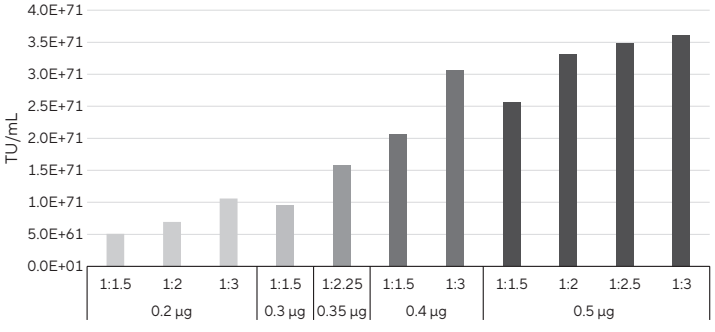
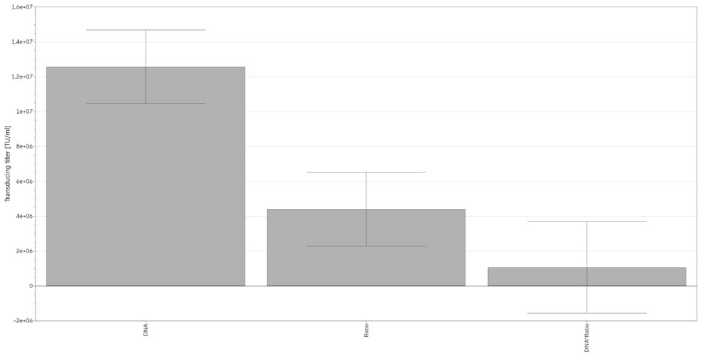


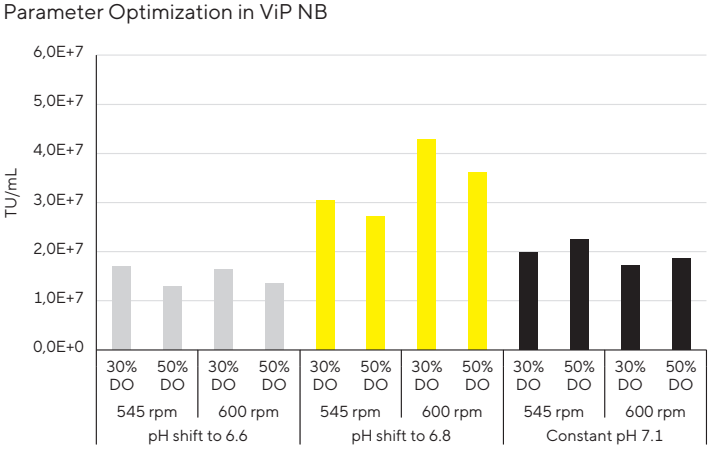
Figure 5: *Analysis with MODDE® identified the DNA concentration as the major impact factor on transducing titer. Results from the transducing titer assay were analysed in MODDE® with help of a coefficient plot. The DNA concentration correlated strongly with an increase in transducing titer. Also, a positive correlation of DNA to transfection reagent was observed, while the combinatorial effect of DNA amount and DNA to transfection reagent ratio was not significant.*



LV Titer Increase by pH Shift After Transfection

For cell and gene therapy applications, controlled large-scale processes are employed for LV production. To investigate parameters relevant for such a process, a DoE approach was performed using the Ambr® 15, an automated microbioreactor system replicating large scale bioreactors. In addition to DO and stirrer speed, the impact of a pH shift after transfection as well as of the culture medium was assessed. Transducing titer- and MODDE® analysis revealed no significant impact of DO, stirrer speed, and culture medium (Figure 6; Figure 7). However, pH was critical. A pH shift from 7.1 to 6.8 right after transfection increased LV titer ~2.5-fold (Figure 6). In contrast, a pH shift from 7.1 to 6.6 at 18 h post transfection was detrimental for product yields (Figure 6). Further experiments will address whether the effect was caused by the pH difference or the timing of the pH shift. Taken together, the pH after transfection was identified as the most critical parameter for LV production in a controlled system.

Figure 6: *A pH shift to pH 6.8 directly after transfection increased transducing titer by a factor of two compared to constant pH of 7. 1. A commercially available HEK293 cell line was cultivated in 4Cell® HEK ViP NB (left) and an internal development medium (right) for 3 passages at s hake flask scale. Following inoculation, cells were cultivated for one more passage in the Ambr® 15 at 30 % and 50 % DO at 545 rpm and 600 rpm upstirring, pH 7.1, and 37°C. Transfection was performed using FectoVIR®-LV with 0.5 µg DNA per one million cells, a DNA:transfection reagent ratio of 1:2, and competitor LV packaging plasmids. By CO_2-gassing, a pH shift was performed to pH 6.8 directly after transfection or to pH 6.6 at 18 h post-transfection. Harvest was performed 72 h after transfection and transducing titer was analyzed by flow cytometry. Depicted is the mean of duplicates ± standard deviation of one experiment.*



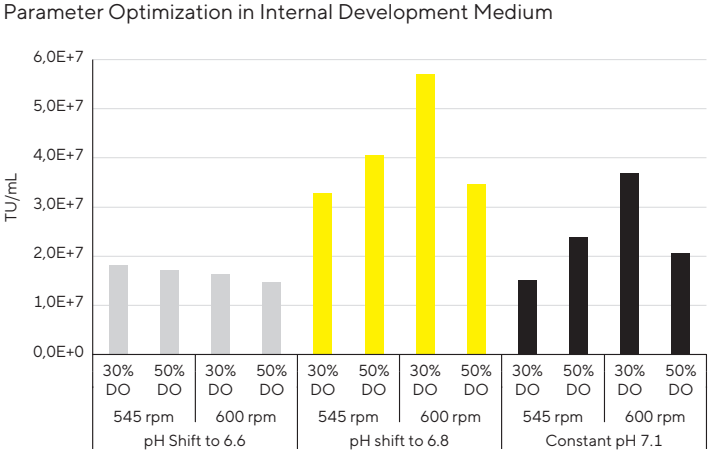
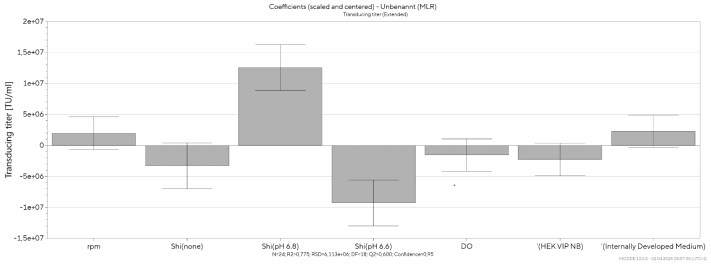


Figure 7: *Analysis with MODDE® identified the pH shift as the major impact factor for LV transducing titer. Results from the transducing titer assay were analysed in MODDE® with help of a coefficient plot. A pH shift from 7.1 to 6.8 immediately after transfection positively correlated with an increase in transducing titer. In contrast, a detrimental impact was observed for a pH shift to 6.6 at 18 h post transfection.*



Temperature Shift Has a Minor Impact on LV Titer

In addition to pH shifts, a temperature shift is a well-established tool for enhancing production outcomes in recombinant antibody production processes. To test whether a temperature shift was also beneficial for lentivirus production, cultures were shifted from 37 °C to 32 °C right after-, at 24 h, or 48 h post transfection in a shake flask experiment. In terms of cell growth, a temperature shift right after transfection resulted in slightly lower VCDs compared to all other conditions tested (Figure 8). However, no major impact on LV titer was observed (Figure 9). Notably, LV titer was slightly improved when performing the temperature shift at 24 h post transfection. Whether such minor beneficial effect can also be observed in a fully controlled production process, remains to be investigated (Figure 9).

Figure 8: *Temperature shift had no major impact on cell growth. A commercially available HEK293 cell line was cultivated in 4Cell® HEK ViP NB at shake flask scale, and transfected either with FectoVIR®-LV, 0.5 µgDNA per one million cells, a DNA:transfection reagent ratio of 2, and a competitor LV packaging plasmid system. A temperature shift from 37 to 32 °C was performed immediately after-, 24 h, or 48 h post transfection.*

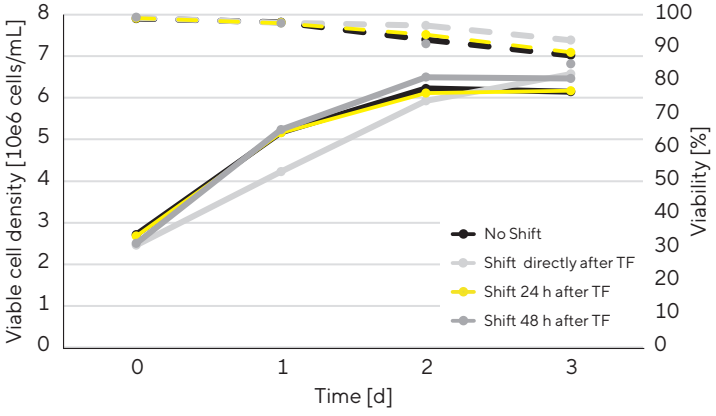
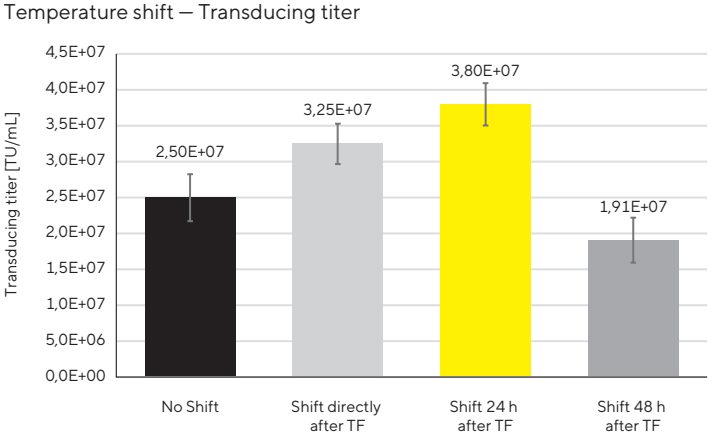


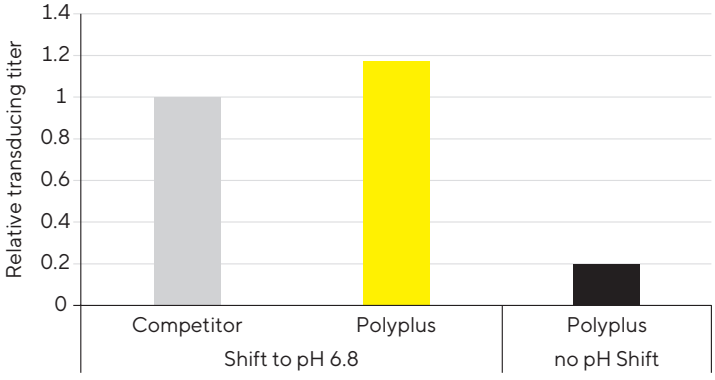
Figure 9: *Infectious titer was slightly improved upon temperature shift at 24 h post transfection. A commercially available HEK293 cell line was cultivated in 4Cell® HEK ViP NB at shake flask scale, and transfected either with FectoVIR®-LV, 0.5 µg DNA per one million cells, a DNA:transfection reagent ratio of 2, and a competitor LV packaging plasmid system. A temperature shift from 37 to 32 °C was performed immediately after-, 24 h, or 48 h post transfection. Harvest was performed at 72 h after transfection and transducing titer was analyzed by flow cytometry. Depicted is the mean of duplicates ± standard deviation of one experiment.*



The Optimized LV Production Process Is Transferable to Other Plasmid Systems and Culture Media

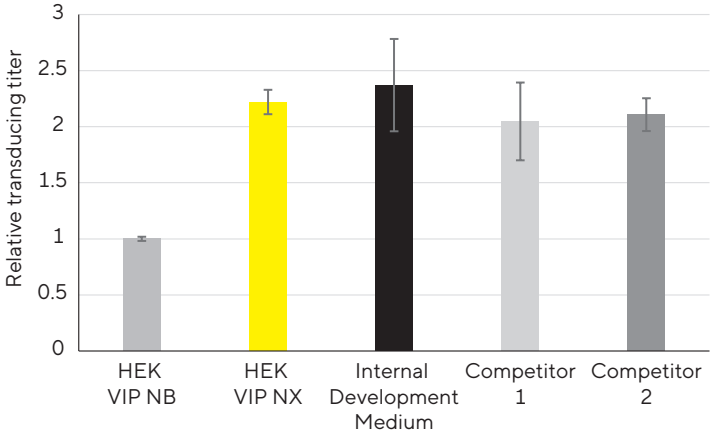
After having established an optimized LV upstream process, the impact of plasmid system and cell culture medium on LV titer was investigated using the Ambr® 15. At first, the optimized process was employed to evaluate the impact of changing from competitor to pPLUS® LV packaging plasmids. Transducing titer was comparable for both plasmid systems used (Figure 10). Importantly, also for the pPLUS® LV packaging system, a pH shift to pH 6.8 directly after transfection was highly beneficial (Figure 10). Hence, the optimized protocol is applicable for different plasmid systems.

Figure 10: *Transducing titer was comparable when exchanging the LV packaging plasmids. Commercially available HEK293 cells, were cultivated in 4Cell® HEK ViP NB in an Ambr® 15. Transient transfection was performed as specified in the methods. The pH shift directly after transfection continued to prove positive even if plasmids are exchanged. Transducing titer was measured by flow cytometry. Depicted is the mean of biological duplicates ± standard deviation of one experiment.*



In a second step, the transferability of the established LV production process to other cell culture media was investigated. In the Ambr® 15, LV production with 4Cell® HEK ViP NB, which was used in all previous experiments, was compared to 4Cell® HEK ViP NX, an internal medium formulation, and to two competitor media. Depending on the culture medium, an increase in the transducing titer of about two-fold was observed in comparison to 4Cell® HEK ViP NB (Figure 11). This shows that optimizations already carried out can also be transferred to other media. It is therefore also worthwhile to screen cell culture media again in a process that has already been optimized.

Figure 11: *More than two-fold transducing titer increase by exchanging the cell culture medium. Commercially available HEK293 cells, were cultivated in 4Cell® HEK ViP NB, HEK ViP NX, Internal Development Medium, as well as in Competitor medium 1 and 2 in an Ambr® 15. Transient transfection was performed as specified in the methods. Transducing titer was measured by flow cytometry. Depicted is the mean of biological duplicates ± standard deviation of one experiment.*



Conclusion

During the process development of LVs for gene-modified cell therapies, we recommend using a DoE approach to optimize process parameters and maximize titers for manufacturing scale. In this application note, we demonstrated the superior performance of FectoVIR®-LV as a transfection reagent for lentiviral production in HEK 293 cell systems compared to the gold standard transfection reagent PEIpro®.

Key findings include:

- **Enhanced Titer:** FectoVIR®-LV increases lentiviral titer up to 3.6-fold compared to PEIpro®.
- **Optimized DNA Use:** Optimal DNA amount is 0.5 µg per 1 million cells, balancing cost and efficiency without compromising titer.
- **DNA: Transfection Reagent Ratio:** While higher DNA: transfection reagent ratios statistically affect titer, biologically, differences are negligible, allowing flexibility in reagent use.
- **Process Economics:** Reduced DNA amount and transfection reagent usage with FectoVIR®-LV leads to cost savings while improving titers.
- **Controlled Process Parameters:** In controlled environments, a pH shift from 7.1 to 6.8 immediately after transfection significantly enhances titer, while other factors like DO and stirrer speed show no significant impact.
- **Choice of Culture Medium:** A more than two-fold increase in transducing titer is possible when exchanging the culture medium, even though process parameters were optimized on another culture medium.

Overall, FectoVIR®-LV used with Sartorius Xell media on HEK293 cell systems facilitates efficient and cost-effective lentiviral production, with pH adjustment being a critical parameter for optimizing yields in controlled processes. We noted that the temperature shift could be also beneficial to increase titers. Further studies are recommended to explore more parameters that could affect productivity and cost.

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