Simplifying Progress

Plenty of Lenti - A multi-angle approach towards method optimization of lentivirus production

Luisa Vetter[1,2], Kathrin Teschner[1], Niklas Krämer[1,2], Alyssa Vetter[1], Sandra Klausing[1]

[1]Sartorius Xell GmbH, Alte Verler Strasse 1, 33689 Bielefeld, Germany
[2]Cell Culture Technology, Faculty of Technology, Universitaetsstrasse 25, 33615 Bielefeld, Germany
Presenting author: Alyssa Vetter, alyssa.vetter@sartorius.com
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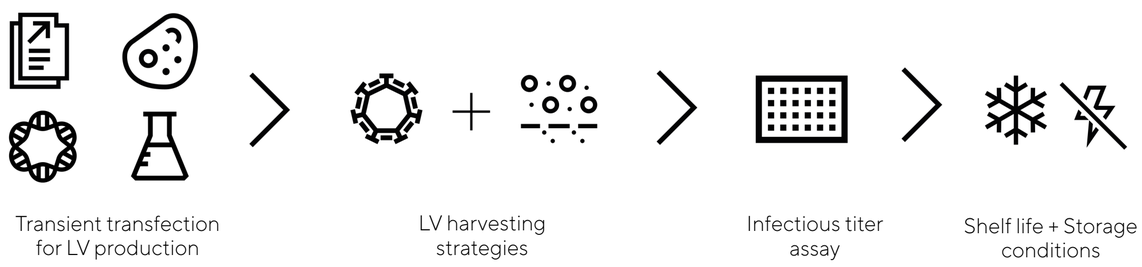
Introduction

Lentiviral vectors (LVs) are increasingly used for cell therapy applications as they can transduce a variety of cell types as well as dividing and non-dividing cells. The latter is a game changer for treatment options, as LVs can also infect hematopoietic and T-Cells, cell types that have been proven difficult to transduce for other viral vectors. However, challenges remain. One that has yet to be overcome is the current demand of LV quantities and required quality for clinical use. Therefore, more efficient production processes for high quality LVs are essential. Here, we show how the upstream section of VSV-G enveloped LV production processes in HEK293 suspension cells can be tweaked to help overcome these limitations.

Workflow

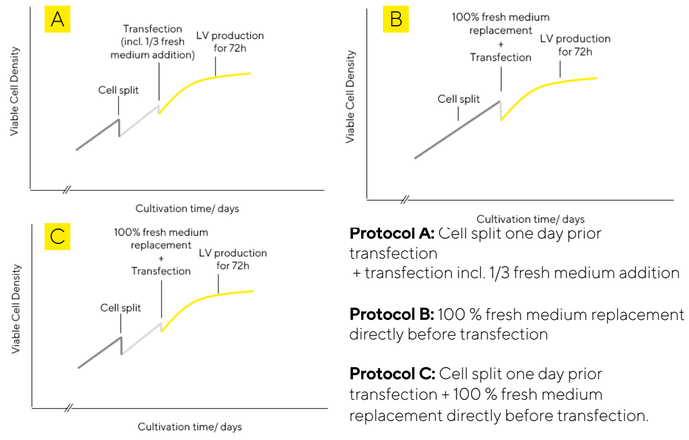


Results

Comparison of transfection protocols for transient LV production

LV production in HEK293 cells was studied by applying different transfection protocols, analyzing the effect of fresh medium and cell split before transfection. Protocol B and C, which include 100 % fresh medium addition directly before transfection show a positive effect on titer (genomic and infectious) compared to protocol A, where fresh medium addition was only 1/3 of final culture volume at transfection. Additionally, the titer can be further increased when a cell split one day prior transfection is reimplemented (Protocol C).

(A) Schematic overview of transfection protocols



Protocol A: Cell split one day prior transfection
+ transfection incl. 1/3 fresh medium addition

Protocol B: 100 % fresh medium replacement directly before transfection

Protocol C: Cell split one day prior transfection + 100 % fresh medium replacement directly before transfection.

(B) Infectious and genomic titer

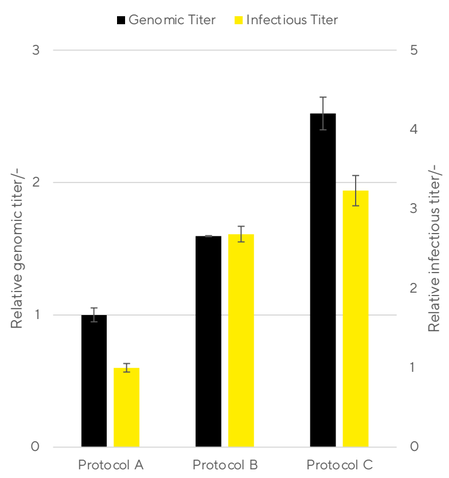


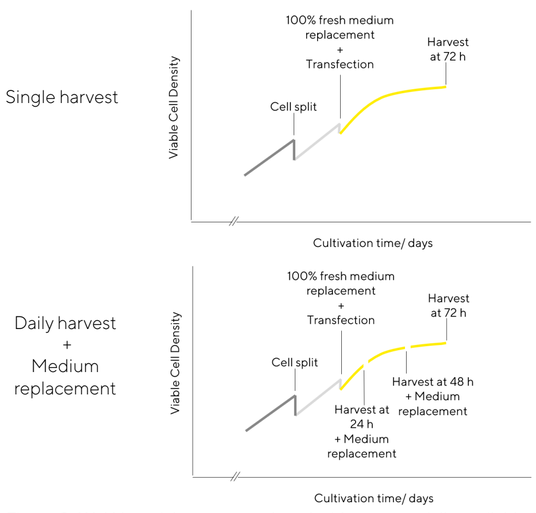
Figure 1: (A) A commercially available HEK293 cell line was cultured in HEK ViP NB medium (Sartorius Xell GmbH) and transfected with a 4-plasmid system (Aldevron) in 125 mL plain shakers using PEI-MAX as transfection reagent. Cells in protocols A and C were split one day prior transfection. For Protocol A, fresh medium addition was 1/3 of final culture volume at transfection, whereas for Protocol B and C, a complete medium replacement directly prior transfection was performed (100% fresh medium). LV particles were harvested 72 h post transfection and 0.45 µm filtered. (B) Genomic titer was determined via ddPCR using Viral RNA Extraction Kit (Invitrogen) and 1-Step ddPCR-Kit (Biorad), while infectious titer was measured via adherent infectious titer assay using flow cytometry. Shown are mean titer values relative to titers generated by Protocol A ± MAD of n=2.

Comparison of different harvesting strategies for LV production

A stability study for LVs was performed at 37 °C, in which the fragility of the vector during the production process was investigated. A decrease in infectious viruses below detection limit after 24 h was observed (data not shown). Based on these results, we compared different harvesting strategies where one single harvest step 72 h post transfection (pTF) versus multiple harvest steps including daily medium replacement were performed in order to factor in virus fragility at 37 °C.

The different harvesting strategies revealed that the main virus production timeframe is 24 h to 48 h pTF, whereas virus production beyond this point is comparatively low. Despite the vector fragility, obtained data demonstrate no advantage in transducing units or total virus quantities when using multiple harvest steps, wherefore a single harvest is more expedient.

(A) Schematic overview of harvesting strategies



(B) Total infectious LV particles

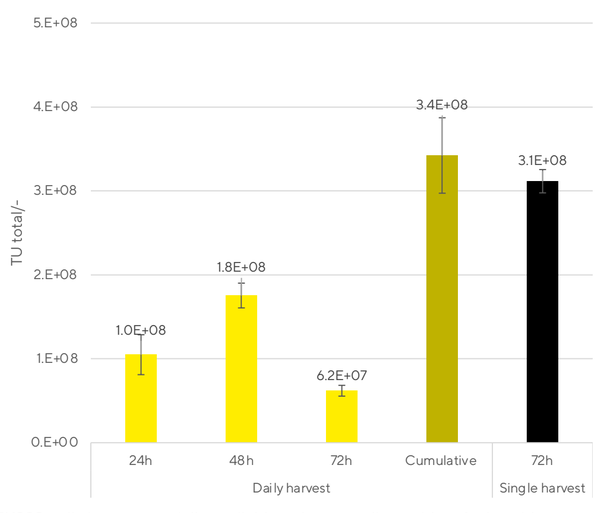


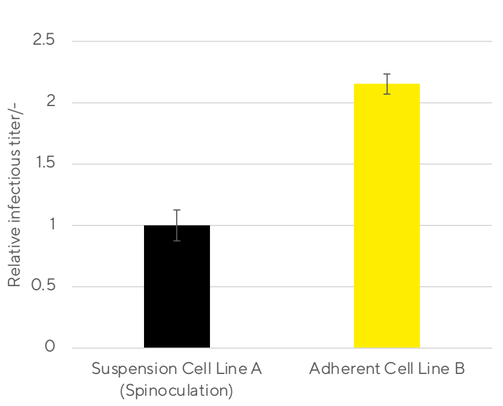
Figure 2: (A) LV particles were produced with commercially available HEK293 cells in commercially available culture medium with a 4-plasmid system (Aldevron) in 125 mL plain shakers using PEI-MAX as transfection reagent and transfection protocol C. LV particles were either harvested in a single harvest step 72 h pTF or harvested every 24 h including a complete medium replacement. (B) LV particles were stored at -80 °C until titer determination using adherent infectious titer assay with flow cytometry. Shown are mean values ± MAD from n=2. Titers are normalized to the production volume.

Infectious titer assays in adherent- and suspension-based systems and LV stability after freeze-thaw cycles

In order to simplify the infectious titer assay, two protocols were compared using either suspension- or adherent-based systems. Performing the assay in suspension cells has the advantage that FCS supplementation can be omitted. But results show that cells can be more effectively infected by the LV particles in adherent-based systems. Those protocols have the upside of being more convenient as the indispensable washing steps can be performed faster.

LVs are known to be fragile vectors e.g regarding temperature, pH or shear forces [1]. Therefore, an experiment was performed to assess their stability with respect to repeated freeze-thaw cycles using infectivity assay in adherent cells. Results show no decrease in LV infectivity after four cycles of freeze-thaw with consideration of methodological deviation, demonstrating that their stability is not at risk. Also, a filtration step of the fresh harvest using 0.45 µm PES filters does not compromise the titer outcome.

(A) Comparison of protocols for infectious titer assay



(B) Effect of multiple freeze-thaw cycles on LV infectivity

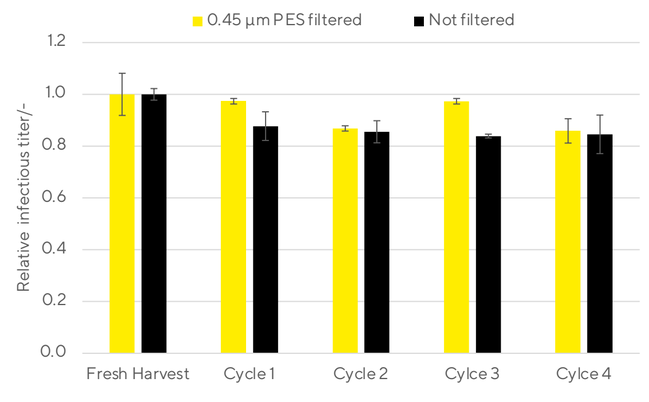


Figure 3: (A) For suspension cell-based assay a commercially available HEK293 cell line A was transduced with LV particles based on standard spinoculation protocol [2] with 8 µg/mL hexadimethrine bromide and flow cytometry. For adherent cell-based assay a commercially available HEK293 cell line B was transduced with the same LV particle sample based on standard protocol [3] for adherent cells with 8 µg/mL hexadimethrine bromide and flow cytometry. (B) LV particles were produced with commercially available HEK293 cells in commercially available culture medium with a 4-plasmid system (Aldevron) in 125 mL plain shakers using PEI-MAX as transfection reagent. LV particles were harvested 72 h pTF and either 0.45 µm filtered using PES filters or not filtered. Aliquots were frozen at -80 °C for 60 min and thawed at 4 °C for the corresponding number of cycles. Infectious titer was determined using adherent cell-based infectious titer assay.

LV shelf life under different storage conditions

Shelf life and functional stability play a great role when it comes to LV production. The traditionally used temperature for long term storage is -80 °C [4], but how is functionality affected when using -20 °C instead ? And how stable are LVs when stored at +4 °C, as this condition is often relevant for purification processes (e.g. ultracentrifugation) and short term storage [5,6]?

Here, LV shelf life was investigated under three different storage conditions. Results show that LV particles are not only stable at -80 °C but also -20 °C for up to 6 month, indicating -20 °C as possible long term storage alternative. Stability at +4 °C is given for up to 1 week, facilitating purification procedures. After 3 month at +4 °C a 10-fold decrease in functional titer is noted and a 100-fold decrease after 6 month.

LV infectious titer after storage at -80 °C, -20 °C, +4 °C

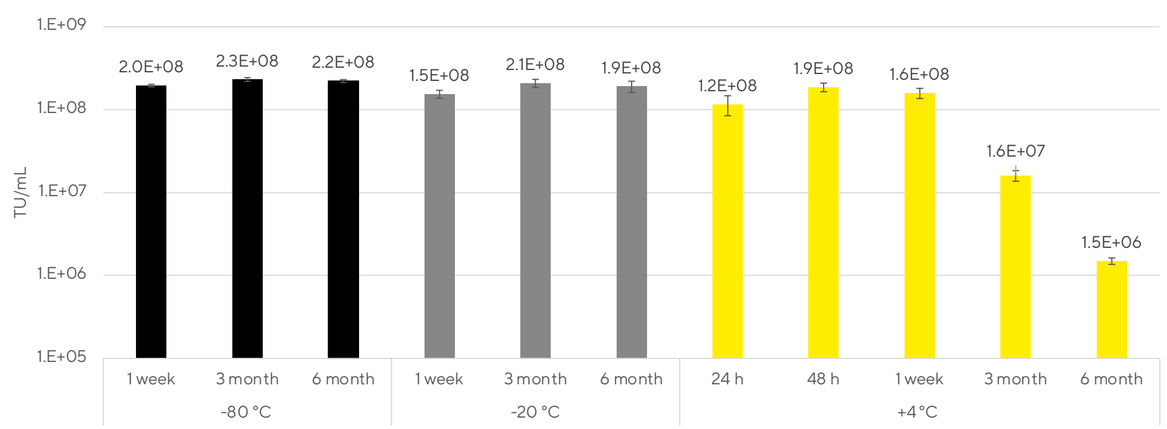


Figure 4: A commercially available HEK293 cell line was transfected with a 4-plasmid system (Aldevron) in 125 mL plain shakers using LV MAX transfection kit (Thermo Fisher). LV particles were harvested 72 h pTF, 0.45 µm filtered and stored at either -80 °C, -20 °C or +4 °C for the corresponding time. Infectious titer was determined by adherent infectious titer assay using flow cytometry. Shown are mean values ± STD of n=3.

Summary and Conclusion

- Addition of fresh medium during transfection process as well as splitting cells one day prior to transfection have a positive effect on LV yield, indicating that N-1 perfusion for fresh medium supply could be beneficial for upscaling.
- Despite vector fragility at 37 °C, one single harvest step at the production end is more expedient than multiple harvest steps during the production process.
- When storing LVs, not only -80 °C, but also -20 °C is worth considering with regards to LV infectivity. Also +4 °C can be used for short term storage purposes. Furthermore, LVs are even stable after multiple freeze thaw cycles.
- LV infectious titer is dependent on assay protocols and set up, with adherent protocols beeing more suitable.
- This work shows the importance of optimizing every step in order to improve LV production and therefore making LV based cell therapies more accessible in the future.

References

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