LENTIVIRAL VECTOR PURIFICATION USING NANOFIBER ION EXCHANGE CHROMATOGRAPHY

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ABSTRACT

Lentiviral vectors (LVs) are used in cell and gene therapies due to their ability to transduce both dividing and non-dividing cells while carrying a relatively large genetic payload and providing long-term gene expression via gene integration. Current cultivation methods produce titres of $10^5$ to $10^7$ TU/mL thus it is necessary to concentrate LVs as well as to remove process and product related impurities. In this work we used a packaging cell line WinPac-RD-HV for LV production to simplify upstream processing. A direct capture method based on ion-exchange chromatography and cellulose nanofibers for LV concentration and purification was developed. This novel scalable stationary phase provides a high surface area that is accessible to LV and therefore, has potential for high capacity operation compared to traditional bead-based supports. We were able to concentrate LVs 100-fold while achieving a two-log removal of host cell protein and maintaining up to a 90% yield of functional vector.
INTRODUCTION

The encouraging results coming from ongoing gene therapy clinical trials and recently approved therapies such as Kymriah\textsuperscript{1} means there is a strong interest in processes for the scalable and cost-effective production and purification of viral vectors which are considered to be a major roadblock to the commercialisation of gene therapies\textsuperscript{2}. Current pricing for the commercially available gene therapies starts at half a million dollars per treatment, thus the number of patients with access to these therapies is small. In addition there are significant potential applications of gene therapy in various chronic illnesses and in oncotherapy.

Lentiviral vectors (LVs) which, unlike other retroviral vectors, can transduce non-dividing cells, thus providing a wider range of potential applications, are important tools in cell and gene therapy. Currently, cell lines used for LV production provide titres of $10^5$ to $10^7$ TU/mL\textsuperscript{3} whereas $10^{11}$-$10^{12}$ TU per patient\textsuperscript{4,5} are being used for clinical applications. Therefore, it is necessary to extensively concentrate LV preparations as well as to remove process (e.g. serum proteins) and product related impurities (e.g. non-infective vector) which can cause unwanted immune responses in patients\textsuperscript{6}. Small-scale purification and concentration can be achieved by ultracentrifugation but there are several disadvantages to this approach: the method is time consuming, there are limited scale-up possibilities, some impurities can be co-purified that elicit an immune response, and the success of the process is strongly dependent on well trained operator’s skills. Alternative methods that can provide scalable production include tangential flow filtration (TFF) and chromatography. Currently, chromatography is dominated by porous bead stationary phases, which were designed for purification of therapeutic proteins such as mAbs. This is not adequate for LV purification since binding sites located within particle pores are typically not accessible to the considerably larger viral vectors; therefore alternative stationary phases are necessary\textsuperscript{7}.

Cellulose nanofibers are a new scalable purification platform. They are fabricated by
electrospinning a non-woven fibre structure with diameters in the sub-micron range\textsuperscript{8–10}. The resulting adsorbent has an open structure with a large surface area accessible to viral vectors, and allows operation at high flow rates due to mass transfer based on convection rather than diffusion thus substantially shortening processing time. The application of nanofibers with different ligand densities in Adenovirus type 5 vector purification has recently been reported\textsuperscript{11}.

This work investigates whether nanofiber based ion-exchange chromatography can provide a scalable LV purification process. Typically, LV is produced via transient plasmid (pDNA) transfection of adherent HEK293 cells in multiple T175 flasks or cell factories where 48-72h post transfection LV-containing media (LCM) is harvested and processed. In order to circumvent problems associated with transient plasmid transfection and the consequent removal of the plasmid DNA as well as its sourcing problems, we used a continuous vector producer cell line WinPac-RD-HV\textsuperscript{12} and the Corning®HYPERFlask® system (total cell attachment surface area of 1720 cm\textsuperscript{2}, equivalent number of ten T175 flasks) to produce LVs used in our studies.

The WinPac-RD-HV cell line produces a LV vector with an RD-pro envelope protein derived from cat endogenous retrovirus RD114 and GFP reporter gene thus avoiding problems associated with VSV-G cytotoxicity and allowing monitoring of LV infectivity via flow cytometry using the GFP reporter. Since LV vectors are structurally complex, in addition to the infectivity assay, we also measured LV recovery with several additional methods targeting different aspects of LV particle. The RNA genome was quantified via RT-qPCR using primers specific for the GFP gene\textsuperscript{12}, lentivirus-associated p24 capsid protein via ELISA, and reverse transcriptase enzyme via SYBR Green I-based product-enhanced reverse transcriptase (SG-PERT) assay\textsuperscript{13,14}. 
RESULTS

LV production

We performed three upstream process runs designated as harvest A, B, and C (Fig. 1a) to study the impact of different seeding cell densities on LV titre. Because LV is released from the cells into the media, we were able to collect multiple batches of lentivirus containing media (LCM) from a single HYPERFlask. In harvest A, 4.6 x 10⁴ cells/cm² WinPac-RD cells were seeded under antibiotic selection and 3 days post seeding (dps) first media exchange was performed using antibiotic-free complete media. LCM from 4 dps showed no infectivity while LCM from 5 and 6 dps showed very similar low infectivity levels of 3.9 x 10⁴ TU/mL and 8.9 x 10⁴ +/- 3.2 x 10⁴ TU/mL, respectively. The highest titre of 3.1 x 10⁵ +/- 0.6 x 10⁵ TU/mL was obtained on the 7dps and this LCM batch was stored in a 1L bottle at -80°C.

In harvest B we increased the amount of cells seeded in a single HYPERFlask to 7.5 x 10⁴ cells/cm². Infective LV was detected earlier in harvest B (3 dps) than in harvest A (5dps) (Fig. 1a) and it continued to increase until 10 dps. LCM was not collected on 8 and 9 dps thus the 10 dps batch contained LV produced over 72h. There is insufficient data on LV half-life and stability at 37°C in the literature, especially LV vectors carrying other envelope proteins rather than VSV-G¹⁵–¹⁸, therefore this is an element which needs to be addressed in the near future in order to develop efficient harvesting strategies. We observed a significant drop in infectivity at 11 dps in harvest B which can be explained by cell detachment due to overgrowth observed while collecting 10 dps batch as well as containing LV produced in 24h. Overall, higher total amounts of infective LV were obtained in harvest B (1.5 x 10⁹ TU) than harvest A (2.4 x 10⁸ TU) which can be explained by higher density of seeded cells and larger volumes of collected LCM.

In harvest C we further increased the amount of seeded cells (1.4 x 10⁵ cells/cm²). Surprisingly, we obtained a relatively high infectivity immediately on the 2dps which can be
attributed to higher initial cell density and higher production over 48h (Fig. 1a). The infectivity dropped by 61% on 3 dps and stayed at approximately the same level during 4 and 5 dps. On 6dps the infectivity increased by 51% and that level was maintained until 9 dps when it started to decrease. Overall, harvest C provided the highest amount of infective material ($2.0 \times 10^9$ TU) compared to two previous harvests. By comparing the three production runs the importance of upstream process development (UPD) in order to obtain high titre and good quality LV material prior to embarking on downstream process development (DSP) is illustrated. Sanber et al\textsuperscript{12}, who developed the Win-Pac-RDpro cell line, showed that high LV titres could be maintained for 4 days with titres in the range of $10^6$ TU/mL when cells were seeded at a similar density to our harvest C. We obtained titres around $10^5$ TU/mL which can be explained by the differences in infectivity assay execution where we did not perform the spinoculation step. Spinoculation has been shown to increase LV titres two to three times\textsuperscript{12}. Manceur et al\textsuperscript{19} recently reported titres of $10^7$ TU/mL from a suspension based inducible cell line obtained after media composition optimisation. This clearly shows that UPD is an area of extensive development since it has a significant impact on LV infective titres as well as DSP due to optimised media composition and quality of LV particles.

**LV clarification**

Harvest A 7 dps batch was defrosted overnight at 4°C. An infectivity assay performed after clarification (low speed centrifugation and filtration with Millex HP PES Express 0.45 µm) revealed 80% loss of infectivity due to this freeze thaw protocol (Fig. 1b). To determine the origin of this loss we studied the infectivity at each step. Bandeira et al\textsuperscript{20} reported 83% losses in infectivity following a freeze thaw cycle performed slowly on ice, therefore our initial defrosting approach was a plausible culprit for this infectivity loss. In further production runs we decided to store our LCM in smaller volumes (100 to 200 mL) and defrost them quickly.
in water bath at 37°C. Batches from harvest B (5-7,10dps) were rapidly thawed in a water bath at 37°C and their infectivity compared to that prior to storage at -80°C (Fig. 1c). The average recovery was 116 +/- 23%, we conclude therefore that this approach is significantly better.

In order to investigate if low speed centrifugation had an impact on LV infectivity, we tested all batches from harvest C and several batches from harvest B (5-7 dps, 10dps). Infectivity recovery after this step was 95 +/- 20% (Fig. 1c) therefore we can conclude that low speed centrifugation has no negative impact on LV infectivity. Bandeira et al\textsuperscript{20} have reported 70% recovery after low speed centrifugation performed at 4°C under similar conditions (time and speed) while we performed ours at room temperature. Infectivity of several batches from harvest B (4-7dps, 10dps) and all batches from harvest C were also tested after filtration with Millex HP PES Express 0.45 µm to accommodate LV particle size of 100 nm. With no further optimisation our recovery compared to starting infectivity (before low-speed centrifugation) was 108 +/- 17% thus showing no loss of infectivity. This is comparable to 91% recovery obtained with scalable Sartopore 2 depth filters\textsuperscript{20}.

**Tangential Flow Filtration**

TFF has previously been shown to concentrate infective LVs\textsuperscript{21,22} therefore we initially explored this option. We tested four hollow fibres with different pore sizes (100, 300, 500, and 750kDa MWCO) in our preliminary diafiltration experiments with 20 mM Tris pH 7.4. Hollow fibres with 300 and 500 kDa MWCO performed best in removing the majority of Foetal Calf Serum (FCS) proteins and retaining LV (Supplement Fig. S1). We opted to pursue the 500 kDa MWCO option. We performed five concentration experiments with different LV batches where we removed 89+/-7% of the total protein (Fig. 2, DC proteins assay). When we used a more specific ELISA assay to track HEK293 Host Cell Proteins (HCP) we found 19+/-2% is still in the retentate but since the LV membrane and core contain
HCPs\textsuperscript{23,24} a certain amount is expected. Unfortunately, infectivity assay showed that \textasciitilde80\% of LV was lost and similar results were obtained by both RT-qPCR and p24 ELISA. Although TFF has been previously successfully used to concentrate LV\textsuperscript{21,22}, the studies in question utilized LV pseudotyped with VSV-G envelope protein, transient transfection to produce LV, and FCS in the diafiltration mix to preserve vector stability while our source material was LV pseudotyped with RDpro envelope protein from a stable producing cell line. The influence of the source material (producer cell line and pseudotyped envelope) on LV stability while undergoing TFF concentration is an unexplored area. Nevertheless, one purification platform does not necessarily provide a satisfactory solution for all LV constructs\textsuperscript{25} and alternative approaches need to be implemented, thus we decided to pursue `ion-exchange chromatography and cellulose nanofibers for LV concentration.

**Shear impact on LV – control experiment**

In order to eliminate the possibility of LV loss due to entrapment in the chromatography rig (AKTA Pure) or nanofibers themselves, as well as to take into account the impact of shear forces on LV stability, we performed an experiment in which we passed LV containing media through the equipment and non-derivatized regenerated cellulose (RC) nanofiber adsorbent. Runs were done in triplicate and the whole system was washed with the loading buffer (flow rate 100 CV/min, 10 mL/min). Due to the complexity of this viral vector we employed multiple analytics to fully understand any potential losses thus LV recovery was monitored by four assays: infectivity, RT-qPCR, SG-PERT, and p24 ELISA (Fig.3). High recoveries were obtained with all four assays with infectivity recovery of 96\%/\-6\% and RT-qPCR recovery of 110\%/\-6\%. SG-PERT and p24 ELISA gave very similar recoveries of 88\%/\-16\% and 89\%/\-9\%, respectively. In conclusion, all four assays show that there is no significant loss of LV in the FPLC system and no entrapment of viable LV in the nanofibers themselves as
well as a no apparent impact of shear forces generated in the system on the functionality of
the vector.

**IEX separation of LV**

LCM from harvest B (6 dps) was four times diluted with loading buffer and loaded onto the
regenerated cellulose nanofibers derivatised with a quaternary amine (RCQ). Following a
wash step, bound material was eluted using a linear gradient over 120 column volumes (CVs)
to a final concentration of 1M NaCl. A representative chromatogram and elution profile is
shown (Fig.4a, b). Silver stained SDS-PAGE gel analysis of collected fractions (Fig.4c)
revealed that the majority of protein content was found in the flow through while smaller
amounts were also present in early elution fractions (E3, E4) as seen by the \( A_{254}/A_{280} \) ratio in
the representative chromatogram (Fig.4b). Western blot analysis using a p24 polyclonal
antibody (Fig.4d) show that the majority of p24 is in elution fractions E5 and E6
(conductivity range of 38.12-63.67 mS/cm and NaCl concentration of 0.6-0.9M). Although
there are small traces of p24 in E3-E4 and E7-E8, when comparing the p24 band in the load
to E5-E6 there is a clear concentration of p24 in the two elution fractions. In E5-E6 we also
detected the p55 gag, a polyprotein which during LV maturation releases p24 and several
other LV structural proteins. Surprisingly, we also found that this antibody recognises a non-
specific protein (>62kDa) from FCS (Supplementary Fig. S2) which was present in the load
but did not bind to the RCQ. We also re-examined our TFF diafiltration samples and found
that the non-specific cross reacting protein is present in both the permeate and retentate thus
demonstrating improved performance of RCQ compared to TFF in the reduction of this
process related impurity.

The infectivity assay based on GFP expression provides the most direct information on the
quality of the purified LV material. To be infectious, LV particles need to have the following:
1) structurally preserved envelope and envelope protein in order to enter target cells, 2)
functional genome and gene of interest (e.g. GFP), 3) reverse transcriptase (RT) enzyme and integrase in order to express the gene of interest, and 4) a functional nucleocapsid monitored via p24-lentivirus associated p24 ELISA assay. Overall infectivity recovery was 27+/−3% with 24+/−4% in E5-E6 (Fig.4e). This recovery was similar to that obtained with TFF therefore additional quantitative analytics were used to provide further insight. RT-qPCR total recovery was 71+/−5% with 2+/−0.1% in the flow through and 54+/−3% in E5-E6. Compared to TFF this is a significantly higher recovery.

SG-PERT quantifies reverse transcriptase encapsidated within the LV particle and has been shown to correlate with the infectivity13,14. The advantage of this assay is that it has a much broader dynamic range, higher sensitivity, and it can be completed in less than a day. SG-PERT total recovery compared to the load was 138+/20% with 5+/−0.4% in the flow through and 110+/−15% in combined fractions E5-E6. One of the reasons why total recovery is higher than 100% could be due to the presence of RT activity inhibitors in the load thus underestimating its amount. Nevertheless, approximately 100% of recovery in E5-E6 is an encouraging result which suggests that the losses seen in the infectivity assays are probably due to damage imposed on envelope protein or lack of functional genome and not to the inactivation of RT. Ideally we would have looked at the functionality of envelope protein and the integrity of LV membrane but currently there are no commercially available assays that would give a quantitative answer to this question highlighting the need for quick and reliable assays.

We used a lentivirus-associate p24 ELISA kit (Cell Biolabs) to quantify viral-associated p24 and consequently recovery of viral particles. The kit uses proprietary technology to separate virus-associated p24 from free p24 thus minimizing the overestimation of LV titre characteristic in standard p24 ELISA assays. Total recovery compared to the load was 22+/6% with 2+/−0.2% in the flow through and 17+/−4% in E5-E6. Although this data is
similar to the infectivity results and previously obtained TFF results, this assay is not able to distinguish between functional and non-functional viral particles.

**IEX separation of LV with direct load**

Since the salt concentrations of PBS used in our chromatography buffers and DMEM in LCM are similar, we directly loaded LCM on to the nanofibers. By taking this approach we did not further dilute the FCS present in the load which has been reported to have a beneficial impact on LV stability although the exact mechanism of action is unclear\(^{26}\). Initially we performed two chromatography runs where we loaded clarified LCM from harvest B (batch 3dps, Supplementary Fig. S3). Total infectivity recovery was 62\(\pm\)/-0.4\% with 60\(\pm\)/-3\% in E5-E6 (elution within conductivity range 35.5-62.12 mS/cm, 0.6-0.9M NaCl) which is double that of our previous results. The recovery from other three assays, RT-qPCR, SG-PERT, and p24 ELISA, matched results obtained in the previous experiment. RT-qPCR total recovery was 72\(\pm\)/-0.4\% with 4\(\pm\)/-0.1\% in the flow through and 56\(\pm\)/-2\% in E5-E6. SG-PERT total recovery was 119\(\pm\)/16\% with 12\(\pm\)/-5\% in the flow through and 92\(\pm\)/-10\% in E5-E6. P24 total recovery was 20\(\pm\)/1\% with 1\(\pm\)/-0.2\% in the flow through and 16\(\pm\)/-1\% in E5-E6. The fact that infectivity doubled while other assays gave almost the same results from the previous experiment strongly implies that the way loading sample is prepared is relevant in preserving functionality of the envelope protein and/or the envelope itself.

Based on these initial encouraging results we attempted to recover LV from harvest C (7 and 9 dps). We performed two runs in which we loaded 400 mL of LCM directly onto the 0.1 mL RCQ nanofiber module (Fig.5a,b). Results from all four quantitative assays are shown in Table 1. Infectivity, RT-qPCR, and SG-PERT recoveries obtained in run I are very similar to our initial small scale experiment with direct loading of LCM (Supplementary Fig. S3). Recoveries obtained in run II are significantly improved compared to run I and range from 94\% to 120\% for the three assays. The difference between run II and all other previous
experiments was the fact that infectivity assay was set up immediately after chromatography and SG-PERT was performed several hours later on the same day. In experiments with LV and monolith chromatography\(^{27}\) initial infectivity recovery of 55% are reported. After optimisation, which was immediate dilution of eluted material with Tris buffer infectivity recovery was increased to >90%. We diluted our eluted LV in complete media rather than PBS or Tris due to the suggested stabilizing effect of FCS on LV\(^{26}\). Based on this report, as well as our own results, immediate stabilisation and proper storage of LV after elution is crucial for maintaining high recoveries. The exact formulation is a matter of further research and is beyond the scope of this paper. Interestingly, high LV particle recoveries were obtained in both runs with p24 ELISA.

SDS-PAGE analysis of selected elution samples (Fig.5c) revealed presence of several small protein bands with molecular weight between 10 and 20 kDa. Based on the observed pattern it can be suggested that they are histone proteins\(^{28}\) implying the presence of cellular DNA in the elution samples. Western blot analysis using a polyclonal p24 antibody (Fig.5d) revealed presence of p24 in all elution fractions with E4 and E5 having the highest amount. In addition p55 as well as other intermediates in viral maturation (e.g. p41) are detected\(^{29}\). Following overnight storage at 4°C, non-diluted aliquots of several elution fractions from run II were analysed by TEM. A representative image of E5 (Fig.5e) revealed the presence of good quality LV particles.

**Host cell protein and DNA**

Fractions from three representative runs from three sets of experiments (Fig.4, Fig.5, and Supplementary Fig. S3) were analysed for host cell proteins (HCP) and host cell DNA (HC DNA) content (Fig.6, Supplementary Table S1). LCM used in the three experiments originated from different LCM harvests and batches, which is reflected in HCP and HC DNA absolute content (two log reduction value (LRV) of HCP from elution fractions (E5-E6)).
IEX chromatography step on RCQ nanofibers provides LV capture and 100 fold concentration directly from LCM and a commensurate removal of HCP. Due to the fact that HCP are a part of LV membrane composition, an absolute removal of HCP is not an expected outcome. Unfortunately host cell DNA is also being captured in LV elution fractions therefore; further development of additional LV DSP steps such as endonuclease treatment prior to the nanofiber IEX step would be beneficial to the overall purification process.
DISCUSSION

In this work, we explored the application of a new type of stationary phase as a capture step for LV purification where we achieved up to 90% recovery of biologically active/infective material. Unlike particle based columns with intraparticle pore sizes designed for proteins, such a platform paves the way for scalable viral vector production to meet current and future market demands.

Since our harvest titres were in the low range (low to mid $10^5$ TU/mL), we attempted to concentrate our LV material prior to chromatography by using TFF. This is a scalable technique that provides concentration as well as partial purification and encouraging results were reported for LV vectors with VSV-G envelope proteins\textsuperscript{21,22}. LVs with RDpro envelope proteins have been concentrated by TFF with recovery up to 30%\textsuperscript{12}. In our TFF concentration experiments we did see a similar titre increase but 80% of infective material was lost which was confirmed with additional analytics (RT-qPCR and p24 ELISA). Based on these three independent assays we can assume that the loss of infectivity was probably due to LV particle entrapment within the hollow fibre pores, therefore, we decided to by-pass this step and use nanofibers for concentration of LV instead. Encouraging results were obtained with non-derivatised regenerated cellulose nanofiber where we did not see any losses due to entrapment (Fig.3). This may be a significant advantage compared to other non-traditional stationary phases such as monolith and losses we experienced in TFF. Monoliths are cast as a single piece of material with interconnected channels and large enough diameters to accommodate viral particles which provide mass transfer based on convection\textsuperscript{30}. Research on Adenovirus type 5 (Ad5) vectors has shown that viral particles can be lost in the monoliths due to entrapment in the dead end channels\textsuperscript{31}. We then performed experiments with nanofibers derivatised with quaternary (Q) amine ligands to create ion exchange functionality. Although infectivity results were initially quite low, once we started applying
undiluted LCM infectivity doubled and reached ~63% which is similar to other LV chromatography recoveries reported\textsuperscript{27,32}. In addition to that, nanofiber performed better in removing process related impurities compared to TFF.

We were able to increase the recovered infectivity by immediately diluting the LV containing fractions with media which is similar to optimisation strategies previously reported\textsuperscript{20}. This implies that LV stabilisation is crucial in the first steps after capture by ion-exchange chromatography. Using this process we were able to obtain LV particles with typical morphologies as seen in TEM analysis of the elution fraction containing the highest amount of LV (Fig.5e) and achieve two log removal of HCP (Fig.6). HCPs are an integral part of retroviruses and are detected in chromatography purified viral vector particles\textsuperscript{33}, therefore complete removal of HCPs is not possible. Unfortunately, unlike other reports\textsuperscript{27,32,34} we did not achieve DNA removal from our LCM thus further development would require an endonuclease step. The papers in question all used LV with VSV-G envelope protein and the impact of the different envelope proteins on LV chromatographic behaviour has not been investigated. We know today that different viral serotypes and small changes in the virus structure can have a significant impact on ion-exchange elution profile\textsuperscript{35,36} and it is highly likely that VSV-G and RDpro, as well as different HCP composition in the LV membrane, contribute to different strengths of interaction with ion-exchange ligands.

The absence of reverse transcriptase activity, lack of RNA genome, and/or envelope proteins causes formation of deficient/non-infectious vectors and contributes to heterogeneity of LV harvests in their production. Although minimizing the formation of these product related impurities is part of the upstream optimisation, ion-exchange chromatography has the potential to remove soluble retroviral envelope proteins and particles lacking the envelope protein from the biologically active retroviral particles with the envelope proteins\textsuperscript{37}. With the expansion of ligand types and chromatography modes additional opportunities for application
of nanofibers in other viral vector DSP steps (e.g. polishing) are possible.
Materials and Methods

Cell line and culture media

All cell lines were cultured at 37°C and 5% CO₂. HEK293T cell line was maintained in Dulbecco’s modified Eagle medium (DMEM + GlutaMAX™-I; Gibco), supplemented with 10% (v/v) foetal bovina serum (FBS) (Sigma) and 1x Antibiotic-Antimycotic (AntiAnti, Gibco). Lentivirus producing cell line WinPac-RD-HR was maintained in DMEM + GlutaMAX™-I supplemented with 10% (v/v), 1x AntiAnti, and antibiotics (1 µg/mL Puromycin, 100 µg/mL Hygromycin, 30 µg/mL Phleomycin, 10 µg/mL Blasticidin). WinPac-RD-HR cells were expanded in T175 flasks under antibiotic selection and seeded in 10 layer HYPER Flask Cell Culture Vessels (Corning) to produce higher LV quantities necessary to carry out further experiments.

Lentiviral vector production, storage, and clarification

WinPac-RDpro cells were seeded in a single HYPER Flask using 560 mL of DMEM + GlutaMAX™-I supplemented with 10% (v/v) FCS and 1x AntiAnti. In harvest A 7.9x10⁷ WinPac-RDpro cells were seeded in a HPER Flask under additional antibiotic selection (1 µg/mL Puromycin, 100 µg/mL Hygromycin, 30 µg/mL Phleomycin, 10 µg/mL Blasticidin). Three days post seeding (dps) antibiotic containing media was discarded and fresh antibiotic-free complete media was added to the cells. Twenty four hours later (4 dps), LV containing media (LCM) was collected and stored at -80°C in 500 mL bottles. This process was repeated every 24 hours for the next 3 days. Aliquots from each day were stored at 4°C and the infectivity assay was performed at the last day of collection (7 dps). Batch from harvest A collected on the 7th dps was defrosted at 4°C overnight followed by several hours at room temperature. This batch was then clarified by centrifugation (4500 rpm 15 min) and filtration (Millex HP PES Express 0.45um) at room temperature and used in diafiltration experiments.

Since detectable titres were obtained in harvest A batches without antibiotic selection, we removed antibiotics from further LV production runs in order to keep the cost down. In harvest B 1.29x10⁸ WinPac-RDpro cells were seeded in one HYPER Flask with complete antibiotic-free media (DMEM + GlutaMAX™-I supplemented with 10% (v/v) FCS). After 72 h incubation (3 dps) LCM was collected and fresh antibiotic-free complete media was added to the cells. LCM was aliquoted in 150 or 250 mL
bottles and stored at -80°C. This process was repeated every 24 hours for the next 4 days. There was a break in collection on 8th and 9th dps which was resumed at 10th and 11th dps. One mL aliquots from each batch were stored at 4°C and the infectivity assay was performed several days later. LCM batches stored at -80°C were subjected to a quick defrost at 37°C in a water bath followed by centrifugation (4500 rpm 15 min), and filtration (Millex HP PES Express 0.45um). Aliquots were taken after each step and analysed by an infectivity assay. Batches from harvest B were used in TFF concentration experiments (5-7 dps), regenerated cellulose (RC) (5 dps), and initial RCQ experiments (6 dps).

In harvest C 2.37x10^8 WinPac-RDpro cells were seeded in one HYPERFlask with complete antibiotic-free media (DMEM + GlutaMAX™-I supplemented with 10% (v/v) FCS). After 48 h incubation period (2 dps) LCM was collected and fresh media was added to the cells. LCM was immediately clarified by centrifugation (4500 rpm 15 min), and filtration (Millex HP PES Express 0.45um) after which 150 or 250 mL aliquots were stored at -80°C. At each step 1 mL aliquots were taken and stored at -80°C. This process was repeated after 24 hours for the next 9 days without any interruptions in collection. At the end of the collection infectivity assays were performed with 1mL aliquots which were defrosted in thermal heat block at 37°C. Batches from harvest C were used in the final RCQ experiments.

Tangential flow filtration (TFF)
TFF experiments were performed using a KrosFlo Research IIi System and mPES hollow fiber modules (Spectrum Labs). Four different MWCO sizes (100kDa, 300kDa, 500kDa, and 750kDa; D02-E100-05-N, D02-E300-05-N, D02-E500-05-N, D02-E750-05-N respectively, Spectrum Labs) were tested in triplicate in diafiltration (DF) experiments against 20 mM Tris pH 7.4 with flow rate of 20 mL/min and manually controlled transmembrane pressure (TMP) of 1 psig. mPES hollow fibre with 500kDa MWCO (D02-E500-05-N) was selected for further concentration experiments where flow rate of 30 mL/min and TMP of 1 psig were set.

Chromatography
In order to determine whether AKTApure (GE Healthcare) itself (flow rate 100 CV/min, 10 mL/min) and nanofiber membrane consisting of a non-derivatized regenerated cellulose (RC) (Puridify, now part of GE Healthcare) could have an effect on LV stability, flow through experiments where performed. Runs were done in triplicate and membrane was washed with PBS (Gibco PBS tablets, cat no 18912014) + 0.0001% Tween 20, pH 7.45. RC column volume (CV) was 0.1 mL. Harvest B 6 dps batch was 4 times diluted with loading buffer (PBS + 0.0001% Tween 20) and in total 100 mL was loaded to the 0.1 mL regenerated cellulose quaternary amine (RCQ) nanofiber membrane (0.1 mL CV, Puridify). Elution was done with a linear gradient over 120 CV with elution buffer (0-100%) containing PBS (Gibco) and 1M NaCl (Sigma). Runs were done in quadruplicate. Flow rate was 100 CV/min (10 mL/min). Undiluted 125 mL of harvest B 3 dps batch was loaded onto the RCQ nanofiber under the same conditions in duplicate.

In two independent runs (run I and run II), undiluted 400 mL from harvest C batches (7 and 9 dps) were loaded onto the RCQ nanofiber and eluted using a linear gradient (0-50% elution buffer) over 100 CV followed by a step gradient over 60 CV (100% elution buffer). Elution buffer contained PBS and 2M NaCl. Flow rate was 200 CV/min (20 mL/min). Aliquots from elution samples from run I were diluted five times with complete media and stored at 4°C until infectivity assay and SG-PERT were performed three days later. Aliquots from elution samples from run II were also diluted five times with complete media and infectivity assay was immediately started while an SG-PERT was performed several hours later. Aliquots of samples from both runs were stored at -20°C until the RT-qPCR and p24 ELISA assays were performed.

Infectivity assay
Functional viral titre (TU/mL) of harvest batches, clarification and TFF samples, and chromatography fractions was determined by transduction of HEK293T cells on 12-well plate followed by flow cytometric analysis of GFP expression by BD Accuri (BD Biosciences). Briefly, 3x10^5 HEK293T cells were transduced with neat LV or diluted LV samples (2 fold or 5 fold dilutions) in presence of 8 µg/mL polybrene in total of 500 µL. After 24 h additional 1 mL of complete antibiotic-free media was added to the cells and after another 48 h (72h in total) cells were analysed for GFP expression after
fixation in 4% PFA and 30 min incubation at 37°C to inactivate LV. Titres were calculated from virus dilutions where 1-20% of the cell population was GFP positive according to the following formula:

\[
\text{Titer (TU/mL)} = \frac{[\text{No of cells at transduction} \times (\% \text{ of GFP positive cells/100})]}{\text{vector input volume}} \times \text{dilution factor}
\]

**Vector genome recovery determination using one step RT-qPCR**

A QIAamp Viral RNA Kit (Qiagen) was used to isolate total RNA from the fractions. Two nanograms of luciferase control RNA (LUC) were added per sample immediately prior to isolation to account for discrepancies between samples during the isolation process and/or RT-qPCR. RT-qPCR reactions were performed with iTaq™ Universal SYBR® Green One-Step Kit (BioRad). Five microliters of neat or diluted RNA were applied to the LV-GFP specific 12 RT-qPCR assays in duplicate, as well as to the LUC-specific 38 RT-qPCR assay in 20 µL final volume. The primers’ final concentrations were 300 nM and cycling conditions were defined according to the manufacturer’s instructions. The RT-qPCR was performed on a CFX Connect™ Real-Time PCR Detection System (BioRad). Average Ct values for LV-GFP were determined for each fraction and used to calculate LV genome concentrations via previously generated standard curves using StemMACS™ eGFP mRNA (Miltenyi Biotec). Recoveries were calculated as the percentage of virus genome present in the fractions in relation to the load. A buffer control and a non-template control (NTC) were included on each plate. Samples with Ct > 30 were considered to be negative. Normalization was performed according to the following formula:

\[
\text{LV-GFP Ct}_{\text{sample normalized}} = (\text{LUC Ct}_\text{buffer control} - \text{LUC Ct}_\text{sample}) + \text{LV-GFP Ct}_\text{sample}
\]

**LV reverse transcriptase recovery determination using SYBR Green 1-based product-enhanced reverse transcriptase assay (SG-PERT)**

The SG-PERT assay was performed as described previously 13,14 with minor modifications. iTaq™ Universal SYBR® Green One-Step Kit (BioRad) components were used to perform the assay minus the iScript™ Reverse Transcriptase component of the kit. HIV Reverse Transcriptase (Merck) was used to build a standard quantification curve and MS2 RNA and MS2 specific primers were used 14.
Prior to performing the assay, chromatography elution fractions were five times diluted in DMEM + GlutaMAX™-I supplemented with 10% (v/v) FBS. Otherwise, neat samples were used.

**LV particle recovery determination**
Recovery of LV particles was determined with QuickTiter™ Lentivirus Titer Kit (Lentivirus-Associated HIV p24, Cell Biolabs, Inc) according to the manufacturer’s instructions.

**Protein and host cell DNA analysis**
The protein composition of TFF samples and chromatographic fractions was analysed by SDS-PAGE using NuPAGE™ 4-12% Bis-Tris Protein Gels (Invitrogen) which were stained with SimplyBlue SafeStain Coomassie Brilliant Blue solution (Invitrogen). Selected gels were further stained with ProteoSilver™ Silver Stain Kit (SigmaAldrich). Western blots were performed using iBLOT™ 2 Dry Blotting System (Invitrogen) and iBlot™ 2 Transfer Stacks, PVDF (Invotrogen). Membranes were blocked in SuperBlock™ T20 (PBS) Blocking Buffer (Invitrogen). Primary antibody was Rabbit polyclonal to HIV p24 (abcam, ab63913, 1/2500) and secondary antibody was Goat Anti-Rabbit IgG H&L (HRP) (abcam, ab205718, 1/20000). Membranes were developed using SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific). Stained gels and membranes were documented using Amersham Imager 600 (GE Healthcare). DC protein assay (BioRad) and HEK 293 HCP ELISA kit (Cygnus) were used for protein quantification according to manufacturer’s instructions. Host cell DNA was quantified using Femto™ Human DNA Quantification Kit (Zymo Research) according to the manufacturer’s instructions.

**Transmission electron microscopy (TEM)**
Selected elution fractions together with the load material used for separation were examined under TEM using the negative staining method. Samples stained with 1% aqueous uranyl acetate using the sequential drop method (2 minutes adsorption on to a plasma cleaned carbon/formvar TEM grid, 2 x 30 second washes in dH2O and 1 minute in stain). Excess stain was removed from grid and samples were air dried. Grids were images in a JEM2100 electron microscope (JEOL, UK) at 200Kv under normal imaging conditions. Images were captured on a Gatan US4000 camera running digital
micrograph 2 (GMS2) (Gatan, USA) at 10k, 12k, 25k and 50k magnifications with exposure times of 1-4 seconds.

Statistics
Statistical analyses were performed using one-way analysis of variance (ANOVA) using OriginPro 2017 software. Tukey, Bonferroni, Dunn-Sidak, Fisher LSD, Scheffe’, Holm-Bonferroni and Holm-Sidak tests were used with significance level set at 0.05.
Acknowledgments
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Author Contributions
DGB conceived the study. JR designed and executed experiment and drafted the manuscript. YT provided cell lines used in the study and protocols for SG-PERT and infectivity assay. CP assisted with assay and material transfer between labs and executed final protein composition experiments. Research was performed in the laboratory of DGB and TM with their oversight. JR and DGB wrote the manuscript with contributions from all other authors.

Competing Interest
The authors declare no competing interest.
REFERENCES


purification-of-a-lentiviral-vector-by-size-exclusion-chromatography-or-mustang-q-ion-exchange-capsule


**Fig. 1** | **a**, Optimization of LV production. In total $7.9 \times 10^7$, $1.29 \times 10^8$, $2.37 \times 10^8$ WinPac-RD cells were seeded in one HYPERFlask in harvest A, harvest B, and harvest C, respectively. Standard deviations for harvests A and B represent duplicates and triplicates while in harvest C all data points are all triplicates. **b**, The effect of defrosting and clarification (low speed centrifugation and filtration with Millex HP PES Express 0.45 µm) on infectivity of LV batch from harvest A (7 dps). At collection n=3, after defrosting and clarification n=12 **c**, Infectivity recovery after defrosting and clarification (low speed centrifugation and filtration with Millex HP PES Express 0.45 µm) in comparison to infectivity at harvest. Batches from harvest B were tested in defrosting experiments (n=4) and batches from harvests B and C were tested in low speed centrifugation (n=14) and filtration with Millex HP PES Express 0.45 µm (n=12) experiments. There are no statistically significant differences between samples obtained after defrosting and clarification compared to samples taken at harvest. Statistical analyses were performed using one-way analysis of variance (ANOVA). * p < 0.05 Error bars represent mean ±1SD

**Fig. 2** | Recovery from TFF concentration experiments. Batches from harvest B (5-7 dps) and mPES hollow fibre with 500kDa MWCO were used (n=5) where permeate and retentate samples were subjected to infectivity assay (n=5), RT-qPCR (n=5), p24 ELISA (n=3), DC proteins assay (n=2), and HEK293 HCP ELISA (n=2). Error bars represent mean ±1SD

**Fig. 3** | LV recoveries flow through experiments on regenerated cellulose (RC) nanofibers. Runs were done in triplicate and 5 dps batch from harvest B was used. LV recovery was monitored by four assays: infectivity, RT-qPCR, SG-PERT, and p24 ELISA. Error bars represent mean± 1SD. Statistical analyses were performed using one-way analysis of variance (ANOVA) and there were no statistically significant differences between results
obtained with the four methods.

**Fig. 4** | **a**, Representative LV ion-exchange chromatographic profile performed using RCQ nanofiber. LCM from harvest B (6 dps) which was diluted four times with loading buffer and 100 mL was loaded onto the nanofiber. **b**, A closer look at the elution profile from all four runs with corresponding **c**, SDS-PAGE visualized with silver staining and **d**, p24 Western blot analysis of selected fractions from a single run using a polyclonal p24 primary antibody. **e**, LV recovery analysis was performed for all four runs. L – load, FT – flow through, E – elution fraction. Error bars in e) represent mean ±1 SD

**Fig. 5** | **a**, Overlapping chromatograms of 400 mL LCM (harvest C, 7 and 9 dps) directly loaded onto a 0.1 mL RCQ nanofiber adsorbent module, **b**, the elution profile, corresponding **c**, SDS-PAGE visualized by silver staining and **d**, p24 Western blot analysis of selected fractions from run II. **e**, TEM image of elution fraction 5 (E5) from run II. L - load, FT - flow through, E - elution fraction.

**Fig. 6** | Removal of host cell proteins (HCP) and host cell DNA (HC DNA) determined by HEK 293 HCP ELISA kit (Cygnus Technologies) and Femto™ Human DNA Quantification Kit (Zymo Research), respectively. Fractions from three chromatographic runs were analysed: one representative run from Supplementary Fig. 4, one from Supplementary Fig. 6, and run I. LRV – Log10 reduction value. Error bars represent mean ±1SD
Table 1 Recoveries obtained in two chromatographic runs performed by loading 400 mL of harvest C material to a 0.1 mL RCQ nanofiber adsorbent module.

Recovery (%)

<table>
<thead>
<tr>
<th>run</th>
<th>Infectivity</th>
<th>RT-qPCR</th>
<th>SG-PERT</th>
<th>p24 ELISA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>FT</td>
<td>1.7</td>
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<td>11.4</td>
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<td>E3</td>
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<td>0.7</td>
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<td>3.6</td>
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<tr>
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<td>6.1</td>
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</tr>
<tr>
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<td>85.4</td>
<td>40.8</td>
<td>62.7</td>
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<tr>
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<td>0.9</td>
<td>1.3</td>
<td>0.6</td>
<td>1.7</td>
</tr>
<tr>
<td>E8</td>
<td>0.3</td>
<td>0.0</td>
<td>0.2</td>
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</tr>
<tr>
<td>Total</td>
<td>49.7</td>
<td>93.7</td>
<td>67.4</td>
<td>101.2</td>
</tr>
</tbody>
</table>
1.0 \times 10^5
5.0 \times 10^5
1.0 \times 10^6
Infectivity (TU/mL)

Harvest A
Harvest B
Harvest C

1 - at collection
II - after defrosting and clarification

I - after defrosting
II - after centrifugation
III - after filtration

1 3 5 7 9 11

Infectivity (TU/mL)