



High-Grade Purification of Third-Generation HIV-Based Lentiviral Vectors by Anion Exchange Chromatography for Experimental Gene and Stem Cell Therapy Applications

Hazal Banu Olgun, Hale M. Tasyurek, Ahter D. Sanlioglu,
and Salih Sanlioglu

Abstract

Lentiviral vectors (LVs) have been increasingly used in clinical gene therapy applications particularly due to their efficient gene transfer ability, lack of interference from preexisting viral immunity, and long-term gene expression they provide. Purity of LVs is essential in in vivo applications, for a high therapeutic benefit with minimum toxicity. Accordingly, laboratory scale production of LVs frequently involves transient cotransfection of 293T cells with packaging and transfer plasmids in the presence of CaPO_4 . After clearance of the cellular debris by low-speed centrifugation and filtration, lentivectors are usually concentrated by high-speed ultracentrifugation in sucrose cushion. Concentrated viral samples are then purified by anion exchange chromatography (AEX) after benzonase treatment to remove the residual cellular DNA. Here, we describe an improved practical method for LV purification using AEX, useful for experimental studies concerning gene and stem cell therapy.

Keywords Anion exchange chromatography, Gene and cell therapy, Lentivirus

1 Introduction

Gene therapy studies involve the introduction of genetic material into cells, tissues, or organs, via gene transfer tools called vectors [1]. Vectors used in gene therapy are often broadly categorized as viral and nonviral [2]. The most important feature that a gene therapy vector is expected to have is efficient transfer of the therapeutic gene into the target cell. Various factors should be taken into consideration in selection of a suitable transfer system, such as the characteristics of the target cell and tissue, immunogenicity of the vector to be used, size of the transgene to be transferred, and intended duration of gene expression [1].

Systems where naked/plasmid DNA is transferred into the target tissue directly or via chemical/physical methods are defined as nonviral vectors. Although nonviral systems are easier to apply and more cost-effective compared to the viral vectors, they also have major disadvantages such as low gene transfer efficiency and

inadequate transcription level and stability [3]. Viruses' ability to achieve efficient gene transfer to suitable host cells opened the way to their widespread use in a high number of preclinical studies and clinical trials; today, nearly 70% of the clinical trials utilize viral vectors [4]. In this regard, more effective and safer applications of viral vectors require enhanced purity and biosafety levels.

Long-term and stable gene expression provided by the lentiviral vectors (LVs), and their relatively large cargo capacity in particular, have accelerated their increased use in gene therapy studies [5, 6]. LVs in this respect should be produced in accordance with the required quality and biosafety standards, for the clinical applicability of the potential gene therapy approaches. Our study aimed to improve LV purification process with various parameters evaluated and optimized, via use of chromatography-based techniques, known as highly productive and quality methods.

1.1 Purification of Lentiviral Vectors

Mammalian cells are widely used as host cells in production of LVs. The viral supernatant acquired at the end of the LV production process contains process-related residual media components and chemicals, along with metabolic wastes. Plasmid DNA and other free nucleic acids, and serum and other proteins are among the main sources of impurity. High molecular weight proteoglycans and DNA contaminants are quite difficult to eliminate, which are large particles that hold strong negative charges similar to LVs. At this point, downstream processing facilitates elimination of impurities contained in the harvested supernatants in terms of biosafety and provides increased vector concentration [7].

The basic principle of the viral purification process is selection of a method that will provide maximum purity with minimum number of experimental steps involved. This is because each extra step to be added has the potential to weaken the transduction ability of the vector [8]. Although virus purification processes are based on basic techniques used in protein purification, viruses' being large molecules that are difficult to distinguish makes their purification more complicated [9]. The order of the downstream processes applied in the purification of the LV vectors following cell culture procedures is as follows: prefiltration, concentration via ultracentrifugation, removal of contaminant nucleic acids, and chromatography [10].

1.1.1 Prefiltration

The viral supernatant containing the viral vectors should be clarified via prefiltration prior to the concentration and purification steps. Removal of the residual cells and cell debris from the supernatant is achieved by low-speed centrifugation and microfiltration [7]. The first centrifugation step provides elimination of large particles before filtration. For the filtration step, diafiltration is frequently used, which provides the concentration of viral particles and salt removal [11, 12]. One of the major challenges encountered during the filtration process is membrane clogging. Techniques such as tangential-flow filtration

(TFF) aim to avoid such problems, providing tangential flow of the solution across the ultrafiltration membrane instead of a direct flow [13, 14]. However, such filtration techniques primarily aim increased viral concentration, rather than enhanced purity of the vectors, which are usually used together with ultracentrifugation and chromatographic methods [15].

1.1.2 Ultracentrifugation

Ultracentrifugation is one of the most preferred methods for concentration of the viral vectors harvested from the supernatant subsequent to the filtration step. Although up to a 100-fold concentration can be acquired via ultracentrifugation at $20,000\text{--}90,000 \times g$, it is not usually accompanied by an increase in the transduction yield. Despite the fact that VSV-G pseudotyping increases resistance against mechanical force, lack of endurance for such a high centrifugal force for long durations is still expected. Furthermore, ultracentrifugation-based methods may constitute a disadvantage for large-scale processes in terms of duration and work load [12, 16, 17]. Following ultracentrifugation, the total volume decreases while the concentration of the vector increases. Use of these concentrated vectors in vivo applications requires removal of the impurities that could not be removed by filtration, but precipitated along with the vector during centrifugation [18]. The most important of these impurities in LV production is the SV40T antigen, derived from the producer 293T cell lines. Removal of this antigen must be assured for the vectors to be applicable to clinical trials in terms of biosafety. Because the ultracentrifugation process does not provide a complete purification even if the sucrose cushion method is used, such contaminant proteins can only be removed by techniques such as chromatographic purification [11].

1.1.3 Contaminant Nucleic Acid Degradation

Although the prefiltration and concentration steps provide removal of many cell- and media-derived impurities, nucleic acid remnants from the cells and plasmids utilized in the vector production process still remain in the LV solution. The quality of the viral supernatant is negatively affected with time, as cellular lysis during LV production increases the contaminating host DNA, RNA, and free nucleic acids as well as cell debris contents. Besides constituting a biosafety issue, contaminant nucleic acids may also cause an increase in viscosity, which may lead to difficulties in the purification steps [9, 19]. The decontamination process gets further complicated with the fact that the residual nucleic acids possess a similar electrical charge as the viral vector particles themselves. Benzonase application is suggested and applied as a solution to this problem [19]. Although it is possible for the residual nucleic acids to be degraded into small fragments with this application, additional effective purification steps are required afterwards, for removal of both the benzonase and the degraded fragments. Size distribution of residual

contaminant DNA in the viral stock is given as <500 bp, while the amount of residual benzonase is defined as <100 ng/ml in a sample of release tests carried out on a clinical lot [20, 21].

1.1.4 Chromatography

Chromatography is a gold standard technique in purification of not only the viral vectors but also other biological substances to be applied in in vivo applications. Chromatographic methods are basically defined as a group of separation techniques of the components of a mixture according to their sizes, electrical charges, specific affinities, or hydrophobicity, between two immiscible phases defined as mobile and stationary phases [11, 22]. Various chromatographic methods exist depending on the characteristics of the stationary and mobile phases, and mechanism of separation and related properties, utilized also in viral purification processes.

Gel Filtration (Size Exclusion) Chromatography

Size exclusion chromatography (SEC) separates the molecules in solutions primarily by their sizes. Molecules do not bind to a ligand. Viral solutions put through SEC result with fast flow of the viral particles without getting inside the pores of the beads, while the smaller proteins flow slower through the pores. Gel filtration is often used in changing of the buffer the viral vectors are suspended in, for salt removal, and as an additional step to increase the quality of the end product [11, 12].

Ion Exchange Chromatography

Ion exchange chromatography (IEX) is a simple and cost-effective technique that is also made use of in LV purification, providing separation of the vectors on the basis of their net charges under neutral pH. IEX utilizes anion or cation exchange columns depending on the charge of the vector [23]. Anion exchange chromatography (AEX) is used for purification of LVs which are negatively charged under neutral pH [12]. In this technique, while the solution containing the viral vectors flows through the stationary phase, the negatively charged particles bind tightly to the positively charged stationary phase. The sources of impurity are thus removed with the solution flowing through the column. LVs that are tightly bound to the column are then eluted and collected with a high concentration salt solution (0.5–1 M NaCl). Various different studies define AEX-mediated purification of LVs, where two major types of chromatography columns are defined. Of these, the Q (quaternary ammonium) column is a strong anion exchange column, while DEAE (diethylaminoethyl) is a weak anion exchange column [8]. However, the high salt concentration used in both techniques is thought to have a negative effect on the viral transduction efficiency [24].

Besides investigation of the functional and therapeutic efficiencies of the LVs to be used in treatment of various different diseases, efficient and optimized purification methods are also essential for use of these vectors in clinical applications.

The protocol described in this chapter is an optimized downstream procedure that summarizes the methodological algorithm for optimization of the third-generation HIV-based LV purification process, where many different parameters are evaluated, and different purification steps are applied in combination besides the basic chromatographic processes, for obtaining highly purified LVs with a high level of biosafety.

2 Materials

- Benzonase endonuclease (Thermo Pierce™ Universal Nuclease for Cell Lysis, 88700)
 - Anion exchange column; HiTrap™ Q HP (17115301, GE Healthcare Inc.)
 - Desalting column; HiTrap™ Desalting (17140801, GE Healthcare Inc.)
 - Polishing column; HiTrap™ Capto Core 700 (17548151, GE Healthcare Inc.)
 - Tris(hydroxymethyl)aminomethane (252859, Sigma Aldrich®)
 - Sodium chloride (NaCl) (S9888, Sigma Aldrich®)
 - HT1080 cell line (ATCC® CLL-121™)
 - Hexadimethrine bromide (Polybrene) (107689, Sigma Aldrich®)
 - DMEM (Dulbecco's modified Eagle's medium) (Sigma Aldrich®, D5648)
 - WPRE primers (Fwd: 5'-CCGTTGTCAGGCAACGTG-3'; Rev: 5'-AGCTGACAGGTGGTGGCAAT-3')
 - Albumin primers (Fwd: 5'-GCTGTCATCTCTTGTGGGCTGT-3'; Rev: 5'-ACTCATGGGAGCTGCTGGTTC-3')
 - QuantiTect SYBR Green PCR Kit (Qiagen, 204143)
 - HIV p24 protein ELISA (QuickTiter HIV Lentivirus Quantitation Kit, Cell Biolabs, VPK-108-H)
 - TritonX-100 buffer (X100, Sigma Aldrich®)
 - MTT (475989, Merck Millipore)
 - Bradford Protein Colorimetric Assay Kit (5000002, Biorad)
 - Opti-MEM (Gibco, 26600134)
 - Coomassie blue (Coomassie Brilliant Blue R-250, 1610436, Biorad)
- 2.1 Recipes**
- 1×PBS (phosphate buffered saline):
 - 137 mM NaCl
 - 2.7 mM KCl

4.3 mM Na₂HPO₄

1.47 mM KH₂PO₄

Dissolve the reagents listed above in 800 ml dH₂O

Adjust the pH to 7 or 7.5

Add distilled water to a total volume of 1 L

Sterilize solution by autoclaving at 121 °C for 15 min on liquid cycle

Store at +4 °C

- Benzonase endonuclease (BE):

Dilute benzonase endonuclease from main stock with RNase-free dH₂O at a 1/100 final concentration.

Store at -20 °C.

Before chromatography, add appropriate amount of BE to a final concentration of 1/1000.

- 100 mM Tris buffer + 1 M NaCl:

Dissolve 58.4 g NaCl.

Dissolve 12.114 g Tris in 800 ml dH₂O.

Adjust pH to 7 or 7.5.

Add distilled water to a total volume of 1 L.

- HBSS (Hanks' balanced salt solution):

1. Dissolve 8 g NaCl, 0.4 g KCl, and 1 g glucose, in 100 ml dH₂O.

2. Dissolve 0.358 g Na₂HPO₄ (anhydrous) and 0.6 g KH₂PO₄, in 100 ml dH₂O.

3. Dissolve 0.73 g CaCl₂ in 50 ml dH₂O.

4. Dissolve 1.23 g MgSO₄·7H₂O in 50 ml dH₂O.

5. Dissolve 0.35 g NaHCO₃ in 10 ml dH₂O.

PREMIX: Mix 10 ml #1, 1 ml #2, 1 ml #3, 1 ml #4, and 86 ml dH₂O.

Add 0.1 ml #5 solution to 9.9 ml *PREMIX*.

Sterilize solution by 0.22 µm bottle-top filter.

Store at +4 °C.

2.2 Instruments

- GE Healthcare ÄKTA Purifier™ UPC 10
- Thermo HeraCell240i CO₂
- ABI 7500 Fast Real-Time PCR
- Multiskan Spectrum Spectrophotometer
- Thermo Class II Laminar Flow Cabin
- Thermo Multi RF Centrifuge
- Olympus IX-81 fluorescence microscopy

3 Methods

1. Following completion of the LV production [6], viral supernatants were centrifuged at $2000 \times g$ for 15 min for removal of the cellular debris.
2. The viral supernatants were prefiltered through a 0.45- μm pore size filter following centrifugation.
3. The prefiltered supernatants were transferred to ultracentrifuge tubes that can withstand high speeds, and 5 ml 10% sucrose solution was added to the bottom of the tubes to form a sucrose cushion.
4. Ultracentrifugation was carried out in Beckman Coulter Optima L-90K ultracentrifuge via use of Beckman SW28 rotor, at $\sim 82,000 \times g$ speed and $+4^\circ\text{C}$ temperature for 2,5 h.
5. The viral pellets were resuspended and kept in PBS and HBSS buffers for a day, to choose the more suitable storing solution.
6. The pre-concentrated LVs for the chromatography-based purification were dissolved in two different buffers as HBSS and PBS, to determine their effects on LVs.
7. LVs dissolved in different buffers were treated with benzonase endonuclease (Thermo Pierce Universal Nuclease for Cell Lysis, 88700) at a final concentration of 1/1000 for 15 min at RT for nucleic acid degradation (*see Note 1*).

In planning of the experimental studies, optimizations were performed for the AEX, with alterations in several parameters such as feed rates, elution pattern (*see Note 2*), equilibration and elution buffer types, and the pH values of these solutions. Optimum values given in Table 1 were reached through variable parameters listed in Table 2.

Following these studies, the acquired chromatograms were examined and parameters such as process duration and resolutions of the lentiviral peaks were evaluated. The optimum feed rate for AEX was defined as 1 ml/min (*see Note 3*), where the optimal solution pH value was observed as 7.5 (*see Note 4*). The optimal equilibration and elution buffers to use were determined as 100 mM Tris and as 100 mM Tris + 1 M NaCl, respectively. Desalting and polishing processes were carried out following AEX, for further purification purposes. Optimal buffers and flow rates for these steps were applied according to the manufacturer's specifications.

3.1 Chromatographic Purification of the LVs

1. All experiments were carried out separately in two different stocks, for determination of the effects of the two different dissolving buffers.

Table 1
Optimized feed parameters and solution characteristics for different column types for chromatographic purification

	Flow rate (ml/min)	Buffer concentrations	Buffer pH
AEX	1	EQ: 100 mM Tris EL: 100 mM Tris + 1 M NaCl	7.5
Desalting	5	1 × PBS	7.5
Polishing	1	1 × PBS	7

Table 2
Different conditions for chromatographic lentiviral purification optimization

AEX	Flow rate (ml/min)	Buffer concentrations	Buffer pH
Exp A	0.5	EQ: 1 × PBS EL: 1 × PBS + 1 M NaCl	7
Exp B	0.5	EQ: 100 mM Tris EL: 100 mM Tris + 1 M NaCl	7
Exp C	1	EQ: 1 × PBS EL: 1 × PBS + 1 M NaCl	7.5
Exp D	1	EQ: 100 mM Tris EL: 100 mM Tris + 1 M NaCl	7.5

2. For both stock solutions, four different experimental setups were implemented following studies at two different pH values and flow rates.
3. The column was washed with ten column volumes (CV) of equilibration buffer for 5 min before the viral samples were loaded, according to the flow rates given in Table 2.
4. Following these steps, 200 times concentrated LVs were treated with benzonase endonuclease at 1/1000 final concentration for 15 min at RT, for genomic DNA degradation. Subsequently, samples that were completed to a final volume of 1 ml with the equilibration buffer were loaded onto the injection loop.
5. Linear gradient elution was selected in the UNICORN™ software, and virus elution was carried out by elution buffer, via use of the parameters specified in Table 2.
6. Protein elution was followed by UV absorbance at 280 nm.
7. Elution peak fractions and fixed fractions were collected by a Frac-950 fraction collector.
8. Following linear gradient elution, AEX column was reequilibrated with the equilibration buffer, and stored at +4 °C after sterilization with 20% ethanol.

9. The peaks acquired for the LV samples dissolved in each buffer (Fig. 1) were first loaded to the HiTrap™ Desalting (17140801, GE Healthcare Inc.) column with phosphate buffer (PBS, pH 7.5) to perform isocratic elution at 5 ml/min feed rate, for removal of salts (*see Note 5*). Following this step, the column

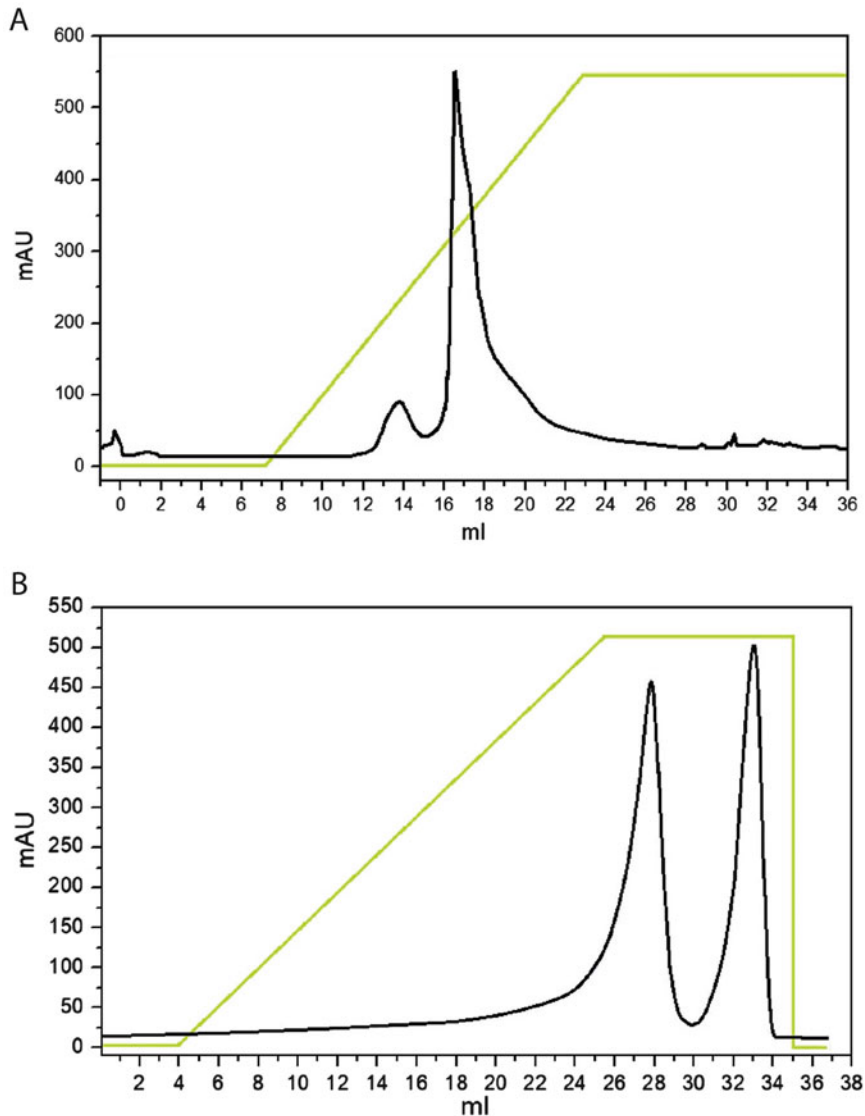


Fig. 1 (a) Chromatogram image acquired following purification process of ultracentrifuge-concentrated LVs dissolved in phosphate buffered saline (PBS) [Green: conductivity curve (Linear Gradient Elution), black: UV curve, y axis; mAU (milli Absorbance Unit) values, measured at 280 nm, x axis; changes in volume during elution (ml)]. (b) Chromatogram image acquired following purification process of ultracentrifuge-concentrated LVs dissolved in Hanks' balanced salt solution (HBSS) [Green: conductivity curve (Linear Gradient Elution), black: UV curve, y axis; mAU (milli Absorbance Unit) values, measured at 280 nm, x axis; changes in volume during elution (ml)]

was sterilized with first ten CV of PBS followed by ten CV of 20% ethanol, and stored at +4 °C.

10. The LVs acquired following the desalting process were loaded to the HiTrap™ Capto Core 700 (17548151, GE Healthcare Inc.) column with phosphate buffer (pH 7.0) to perform isocratic elution at a flow rate of 1 ml/min, for further purification purposes (polishing). Following this step, the column was cleaned at 1 ml/min reverse flow rate for an hour in 1 M NaOH, with 30% 2-propanol, sterilized with ten CV of 20% ethanol, and stored at +4 °C.
11. HIV p24 protein ELISA assay (QuickTiter HIV Lentivirus Quantitation Kit, Cell Biolabs, VPK-108-H) was used for quantitation of the LV particles in the final product, while quantitation of the integrated viral genomes was determined via quantitative real-time PCR. Functional efficiency was analyzed via HT1080 cell transduction.
12. SDS-PAGE analysis was performed for determination of the efficiency of the residual protein removal processes.

3.2 Calculation of the LV Particle Numbers

HIV p24 protein ELISA assay (QuickTiter HIV Lentivirus Quantitation Kit, Cell Biolabs, VPK-108-H) was performed for determination of the LV particle contents and multiplicity of infection (MOI) of the samples that were put through chromatographic purification, concentrated via ultracentrifugation, or left as unconcentrated supernatant samples. Absorbance was measured at 450 nm by Thermo Multiskan Spectrum equipment, and optical density (OD) values were recorded. At the end of the calculations, the number of LV particles of the supernatant, ultracentrifuge-concentrated vectors, and chromatography-purified samples were determined. MOI values were calculated based on these inputs.

Results of the p24 analysis revealed that the number of viral particles varied depending on the type of the dissolving buffer used (Fig. 2a). Statistically significant viral particle loss was observed in viruses dissolved in PBS solution, compared to those dissolved in HBSS (unpaired *U* test; $p = 0.0381$).

Two chromatographic peaks were acquired from the viral samples dissolved in HBSS, while a single peak was evident for the viral samples dissolved in PBS (Fig. 2b). Although viral particle loss may seem to exist in chromatography results performed following ultracentrifugation of the HBSS samples, the fact that each two peaks reflects the same viral sample should be taken into consideration (*see Note 6*). Evaluation of the particle numbers from both peaks made it evident that the viral particles could be chromatography-purified following ultracentrifugation without significant loss (Fig. 2c). With the amounts obtained following ultracentrifuge concentration taken as a reference in numerical analysis (100%), the viral particle

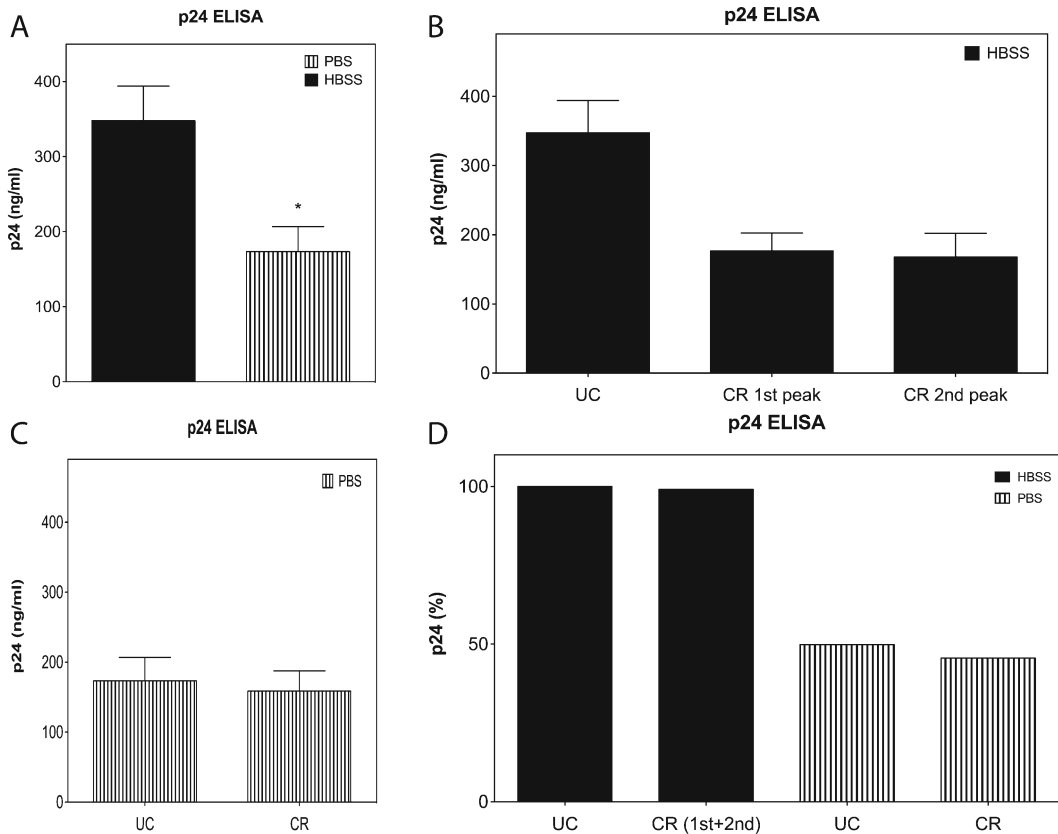


Fig. 2 (a) Detection of the viral particle quantities in different groups via HIV p24 protein ELISA test. (b–d) Products following UC: ultracentrifugation, and CR: chromatography (Dark column: LVs dissolved in HBSS and textured column: LVs dissolved in PBS)

quantities were observed to be maintained at a very high percentage throughout the chromatographic purification processes (Fig. 2d) (*see Note 7*).

3.3 Calculation of the Infectious Viral Titers

Viral supernatant fractions, the chromatography-purified vectors, and the ultracentrifuge-concentrated viruses were transduced into HT1080 cells, for quantitation of the functional viral particle contents. Vector copy numbers (TU/ μ l) of each sample were detected in ABI 7500 Fast Real-Time PCR (Applied Biosystems Inc.) via QuantiTect SYBR Green PCR Kit (Qiagen, 204143), by use of WPRE and albumin primers as internal controls, in all samples and standards. Integrated viral copy numbers of the viral samples taken from the supernatant fractions, the ultracentrifuge-concentrated viruses, and the chromatography-purified vectors were compared in terms of TU/ μ l for significance (Fig. 3), according to the quantitative real-time PCR results (Table 3).

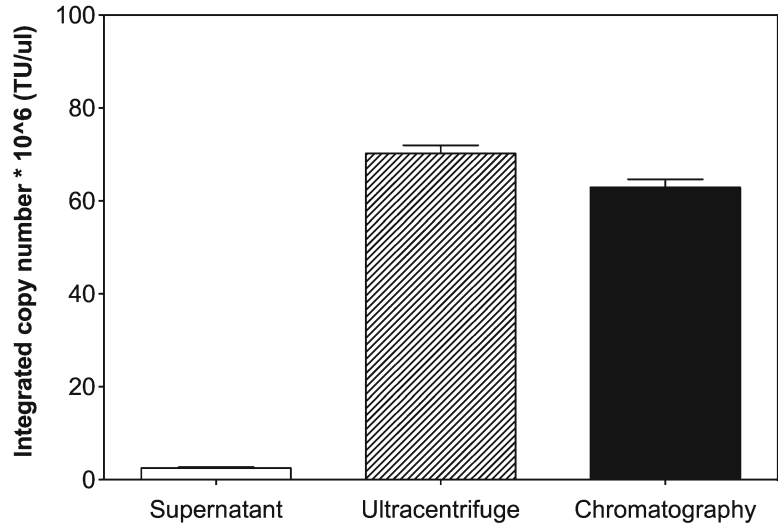


Fig. 3 Quantitative real-time PCR analysis of LVs dissolved in HBSS as bulk supernatants (white column), following ultracentrifugation (textured column), or after AEX chromatography (dark column)

Table 3
Quantitative real-time PCR results

	Vector copy number ($\times 10^6$ TU/ μ l)		
	Supernatant	Ultracentrifugation	Chromatography
First sample	2.5	70.25	62.83
Second sample	2.9	73.25	65.97
Third sample	2.1	67.25	59.93
<i>Mean</i>	2.5	70.25	62.91

According to the results obtained (Table 3), the viral titer was calculated as 2.5×10^9 TU/ml for the supernatant, as 7.02×10^{10} TU/ml for ultracentrifuge-concentrated samples, and as 6.2×10^{10} TU/ml for the chromatography-purified vectors. Low copy numbers in the supernatant may be due to the diluted viral sample in a large non-concentrated volume. Because equal amounts of samples were compared, viral particle count per microliter supernatant was quite low compared to the concentrated samples. Thus, direct comparison of the genome-integrated copy numbers acquired from the supernatant samples with those in the ultracentrifuged and chromatography-purified samples is not reliable (*see Note 8*). Evaluation of the calculated genome-integrated copy numbers revealed that 89.6% of the ultracentrifuge-concentrated viruses were recovered at the end of the chromatographic purification (*see Note 9*).

3.4 Analysis of the Effect of LV Transduction on Cell Viability

MTT Cell Growth Kit (Merck Millipore, 475989) was used for testing of the possible negative effects of LV transduction on cell viability. Absorbance measurements were performed via Thermo Multiskan Spectrum equipment at 595 nm.

Analyses pointed out to certain effects of the viruses purified with either method, on the metabolic activities of the cells following transduction at equal MOIs. When the control groups were taken as references, 82% cell viability was observed at 250 MOI, whereas 86% viability was evident at 150 MOI (Fig. 4a).

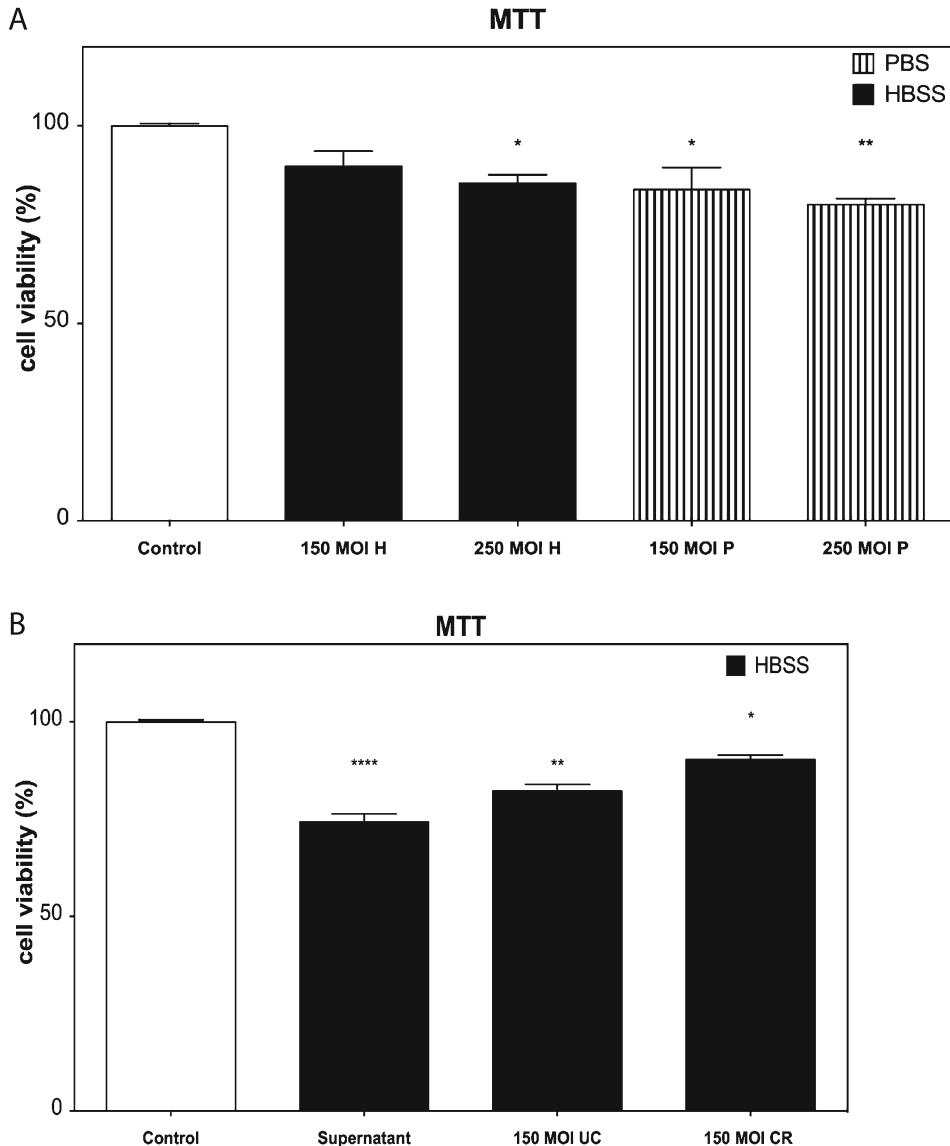


Fig. 4 (a) Detection of cell viability rates via MTT cell viability assay following viral transductions. (b) Detection of cell viability by MTT test following transduction with chromatography-purified vectors and viruses directly acquired from the supernatant (white column: (+) control, dark column: LVs dissolved in HBSS, and textured column: LVs dissolved in PBS)

Furthermore, metabolic activities of the cells transduced with varying MOIs of LVs dissolved in HBSS buffer followed by chromatographic purification were evaluated with non-transduced control groups taken as reference. Cells infected with purified viruses displayed 90% metabolic activity; whereas this rate was 82% for cells transduced with ultracentrifuge-concentrated viruses, and 74% for cells infected with the supernatant samples. These results revealed significantly higher cell viability rates in cells transduced with chromatography-purified vectors (Fig. 4b).

This result reflects the toxic effects of the cell-derived contaminants and impurities contained in the non-purified supernatant samples. Results of the cell viability analyses correlated with the SDS-PAGE results, with no negative effect of transduction with chromatography-purified viruses on cell viability (*see Note 10*).

3.5 Testing of Impurity Removal Efficiency Following Purification of LVs

For detection of the purified protein amounts at the end of the ultracentrifugation and chromatographic purification processes following LV production, protein amounts were defined as milligrams per microliter sample via Bradford Protein Colorimetric Assay Kit (Biorad, 5000002, 10 µg/ml sensitivity).

The protein content of the supernatant was taken as a reference in Bradford assay and considered as 100%. As expected, the ultracentrifuged samples displayed a total protein purification rate of 60%. Yet, it should be taken into consideration that an important amount of protein content precipitates with the viral pellet via ultracentrifugation. More efficient removal of the protein impurities was observed following chromatographic purification, where a nearly 85% decrease in the total protein amount was detected (Fig. 5a).

SDS-PAGE analysis carried out following Bradford assay revealed that majority of the viral protein bands could not be removed, including the SV40T antigen band derived from the 293T cell lines, particularly in supernatant samples and ultracentrifuged viruses (*see Note 11*). In contrast, elimination of all the contaminant protein bands was evident in the chromatography-purified samples, resulting in purer viral vectors (Fig. 5b) (*see Note 12*). These results are in correlation with the Bradford assay outcomes, where the contaminant protein removal rates were 90% for the chromatography-purified samples.

3.6 Functionality Tests of the Purified Vectors

For determination of whether the acquired viral samples retained the ability of efficient *in vitro* transduction following chromatography, HT1080 cell transductions were carried out. RFP signaling recorded via Olympus IX-81 fluorescent microscope verified the efficient transduction abilities of the chromatography-purified Lenti-RFP vectors (Fig. 6).

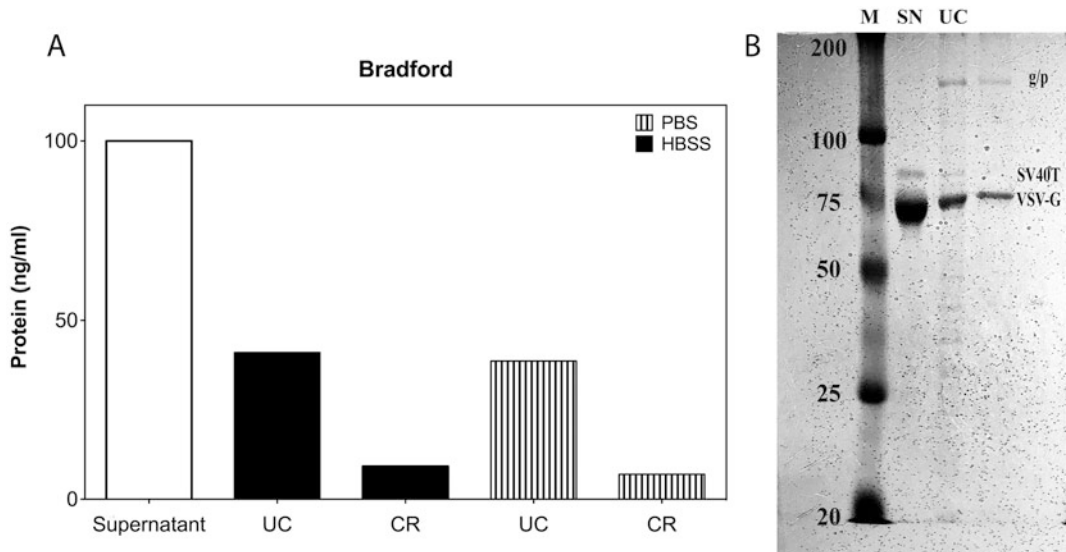


Fig. 5 (a) Analysis graph of total protein quantities of all groups detected by Bradford protein assay (white column: supernatant, dark column: LVs dissolved in HBSS, and textured column: LVs dissolved in PBS). **(b)** SDS-PAGE electrophoresis banding patterns of viral samples from different groups loaded in equal amounts (30 μ g) to 10% gel. *M* marker, *SN* supernatant, *UC* ultracentrifuge, *AEX* anion exchange chromatography

Fig. 6 RFP signals from HT1080 cells reflecting functional efficiency of the chromatography-purified Lenti-RFP vectors

4 Notes

1. Cell-derived contaminant nucleic acids not only constitute a biosafety issue in LV production but also affect viscosity and complicate the purification steps. Thus, nucleic acid degradation steps by endonucleases such as benzonase are of great importance.
2. Separation and elution of the viral sample from the column is usually achieved by linear pH or ionic concentration changes in the buffer. These linear changes selectively decrease the affinities of the sample molecules towards the charged groups that normally provide their adsorption to the column. Thus, this selective decrease leads to elution of the samples from the column at different times. Such gradient linear elution defined here is frequently preferred in lentiviral purification.
3. The optimum feed rates should be optimized based on duration, as no changes in the elution pattern was observed at 0.5 ml/min or 1 ml/min feed rates in chromatographic purification processes of either of the samples dissolved in PBS or HBSS, and the fact that feeding at lower rates leads to longer elution times.
4. The pH of the starter buffer should be chosen between values that will assure that the molecules in the sample have the right charge for binding to the ion exchange column. In cases where AEX columns are used, the pH of the buffer should be at least one pH unit above the isoelectric point of the compound to be separated. Solubility of a protein is minimum at its isoelectric point, and equal numbers of positive and negative electrical charges are transferred at this pH. Thus, a minimum repulsion force between molecules results in maximum intramolecular interaction, which leads to the formation of insoluble protein aggregates. For the LVs to be purified without aggregate formation, solution pH levels should be between 7 and 7.5, as opposed to the pI value between 6 and 6.5.
5. The pH value, temperature, and salt concentrations of the solutions used in the purification processes are of great significance in terms of quality of the vector. The basic principle in viral purification is to use a method that will provide maximum purity through a minimum number of steps. Each extra step to be applied will weaken the transduction ability of the vector. Although viral activity decreases with the increased number of steps in chromatography due to the high salt content of the solutions, it is possible to solve this problem with a desalting process.
6. In some instances, vectors can be obtained consecutively in two peaks during LV chromatography. The reason for this is that

LVs initially bind very strongly to the column following loading of the samples, due to their strong negative charges. However, as the column starts to get full in time, all the viral particles in the sample cannot bind to the column with the same strength. Thus, when the elution buffer starts to go through the column, those LVs that bind with less strength dissociate from the column earlier and are collected in the tube, which is reflected as the first peak in the chromatogram. As the elution process continues, LVs that cannot stay binded to the column are isolated, and the second peak appears in the chromatogram [24]. These two peaks can be evaluated together, as they are two different peaks of the same virus.

7. Viral particle numbers can be detected in the purified samples following buffer selection, by HIV p24 protein ELISA test. Mean number of LV particles obtained from both the ultracentrifuge-concentrated and chromatography-purified samples was calculated as 5.2×10^{10} LP/ml. However, it is known that p24 ELISA test does not always reflect the transductionally efficient viral load. Thus, this test provides only a coarse analysis regarding the viral particle numbers, and viral transduction units can only be evaluated by analysis of genome-integrated copy numbers.
8. According to the quantitative real-time PCR results, it should be taken into consideration that the supernatant samples are not concentrated, thus will have larger volumes with lower viral concentrations. For this reason, viral particle counts per microliter supernatant appear much lower compared to the concentrated samples, as expected.
9. The reason for the decrease in the genome-integrated copy numbers of the concentrated viruses following additional chromatography steps is exposure of the vectors to high salt concentrations throughout chromatography. This issue should not be disregarded [22, 25]. Although calculated genome-integrated copy numbers reveal that 89.6% of the ultracentrifuge-concentrated viruses are recovered at the end of chromatographic purification, total vector recovery after all the downstream processes was detected as 53%. Thus, total recovery following all the downstream processes should be taken into consideration, instead of the recovery rates following individual procedures.
10. Cells transduced with chromatography-purified vectors are detected to have a much more healthy morphology compared to the other methods, in accordance with the numerical data. A qualitative evaluation of the effects of the purified vectors on the cells may also be carried out along with the cell viability tests, with various parameters taken into consideration, such as morphological criteria, cell growth rates, and adhesion abilities of the cells transduced with the purified vectors.

11. One of the most important issues to be taken into consideration in elimination of impurities is the absolute requirement for removal of the contaminant cell-derived SV40T antigen for applicability of the vector in in vivo applications. The success of the LV chromatography purification should thus be evaluated in terms of the absence of a band corresponding to the SV40T antigen.
12. A thick VSV-G band appears particularly in SDS-PAGE analysis of the ultracentrifuged samples. The main reason for this is that viral proteins with similar densities form aggregates when rotated at high speeds. Thus, advanced purification steps are of great importance.

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