Production of Lentiviral Vectors by Large-Scale Transient Transfection of Suspension Cultures and Affinity Chromatography Purification

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ABSTRACT: The use of lentiviral vectors as gene delivery vehicles has become increasingly popular in recent years. The growing interest in these vectors has created a strong demand for large volumes of vector stocks, which entails the need for scalable vector manufacturing procedures. In this work, we present a simple and robust process for the production of lentiviral vectors using scalable production and purification methodologies. Lentivirus particles were produced by transient transfection of serum-free suspension-growing 293 EBNA-1 cells with four plasmids encoding the vector components using linear polyethylenimine (PEI) as transfection reagent. This process was successfully scaled-up from shake flaks to a 3-L bioreactor from which $10^{10}$ IVP were recovered. In addition, an affinity chromatography protocol designed for purification of bioactive oncoretroviral vectors has been adapted in this work for the purification of VSV-G pseudotyped lentiviral vectors. Using heparin affinity chromatography, lentiviral particles were concentrated and purified directly from the clarified supernatants. During this step, a recovery of 53% of infective lentiviral particles was achieved while removing 94% of the impurities contained in the supernatant.


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KEYWORDS: gene therapy; lentiviral vectors; large-scale production; serum-free; suspension cells; 293 EBNA-1; purification; heparin affinity chromatography

Introduction

Lentiviral vectors have become the vector of choice for many fundamental and applied research applications due to their ability to integrate in the host cell genome, thereby providing the potential for long-term transgene expression in the transduced cells and their progeny (Naldini et al., 1996a,b). Over the past decade, a series of improvements in lentiviral vector design have made them a safer and more efficient gene delivery tool (Cockrell and Kafri, 2003). Advances in vector design were accompanied by the development of novel experimental applications such as gene silencing by stable RNA interference, the generation of transgenic animals, and the construction of genomic and cDNA libraries (for review see Wiznerowicz and Trono (2005)). Perhaps the most exciting application of lentiviral vectors is human gene therapy. Lentiviral vectors have been rapidly introduced into phase I clinical trials for the treatment of HIV infection (Manilla et al., 2005; Schonely et al., 2003; Slepushkin et al., 2003). In addition, a number of studies have demonstrated their extraordinary potential to transduce slowly dividing or nondividing cells, including key targets cells such as hematopoietic stem cells and terminally differentiated neurons and glial cells (Blomer et al., 1997; Naldini et al., 1996b).

As interest in the use of lentiviral vectors for various applications continues to grow, further developments in vector production and purification strategies are required. Production of lentiviral particles can be accomplished in two different ways: by development of stable vector packaging cell lines or by transient transfection of human cells. Although the generation of several stable packaging cell lines has been achieved (Farson et al., 2001; Kafri et al., 1999; Klages et al., 2000; Kumar et al., 2003; Ni et al., 2005; Pacchia et al., 2001; Xu et al., 2001), this method is hampered by the
long time required to develop a stable cell line expressing all required vector components (typically >6 months) and the lack of compatibility with cytotoxic/cytostatic transgenes or vector components that entails the need for a tight control over gene(s) expression (Ni et al., 2005; Sinn et al., 2005). Additionally, new packaging cell lines need to be developed for each desired vector pseudotype and for each generation of improved lentiviral vectors. Moreover, these cell lines often yield relatively low vector titers and loose stability over prolonged culture periods (Cronin et al., 2005).

Production of lentivirus particles by multi-plasmid transient transfection has been the most widely used technique for the generation of lentiviral vector stocks since it constitutes a faster, simpler, and more versatile approach (Blesch 2004; Coleman et al., 2003; Geraerts et al., 2005; Koldej et al., 2005; Mitta et al., 2005; Naldini et al., 1996b). Transient transfection avoids the time-consuming, tedious and cumbersome process of developing stable packaging cell lines. In addition, it allows for the use of cytotoxic/cytostatic transgenes and/or vector components, which is the case for many HIV-1 derived proteins (Bartz et al., 1996; Konvalinka et al., 1995; Li et al., 1995; Miyazaki et al., 1995) and for the commonly used VSV-G pseudotyping envelope glycoprotein (Burns et al., 1993). Furthermore, transient transfection vector systems permit testing various transgenes of interest and envelope glycoproteins with alternative cell tropisms within a reasonable amount of time (Sena-Esteves et al., 2004).

One key aspect to achieve optimal large-scale transient transfection is the selection of the producer cell line. The human embryonic kidney 293 cell line (HEK293 or 293) is widely used for the production of recombinant proteins and viral vectors since it is highly transfectable and it can be adapted to grow in suspension culture and serum-free media. These characteristics make this cell line ideal for large-scale production of biological products since suspension growth is required for productions in controlled bioreactors and serum-free manufacturing conditions are highly desirable to reduce the complexity, duration, and cost of downstream processing operations while eliminating the risk of introducing adventitious agents. In addition, the 293 cell line has two genetic variants, the 293T (expressing the EBV nuclear antigen-1, EBNA-1) cell lines that allow increasing expression levels by permitting plasmid replication or episomal persistence, respectively, in the transfected cells throughout the production phase. Essential for the plasmid replication or episomal maintenance is the presence of SV40 ori and EVB-ori replication origins, respectively, in the plasmids backbones. The EBV oriP-EBNA1 system also serves as a strong cis transcriptional enhancer for many viral and nonviral promoters. To date, the vast majority of lentiviral vector production protocols have used 293 T-cells. These cells are typically transiently transfected in small monolayer cultures in the presence of 10% FBS.

Another important aspect for successful large-scale transient transfection is the choice of transfection reagent. There are various chemical compounds in the market commonly used to efficiently transfect mammalian cells. Calcium phosphate co-precipitation of DNA was first described over 30 years ago (Graham and van der Eb, 1973) and continues to be used since it offers good transfection efficiencies at low cost. However, this method is largely being replaced by more modern, easier to use methods that employ transfection reagents such as cationic lipids and polymers. These chemical compounds can complex nucleic acids favoring cell binding and uptake (Boussif et al., 1995; Felgner et al., 1987). Although costly, lipid-based approaches are widely used in vitro because they consistently result in higher transfection efficiencies in a wide variety of cell types. However, for large-scale manufacturing of biological products by transient transfection, the use polyethylentimine (PEI) is the most cost-effective option (Durocher et al., 2002). The method typically results in high transfection efficiencies in many cell types including 293 suspension-growing cells while showing minimal cytotoxic effects. Despite the advantages offered by the cationic transfection compounds, in the case of lentiviral vectors, calcium phosphate co-precipitation continues to be by far the most widely used transfection technique (Follenzi and Naldini 2002).

An important limitation with lentiviral vectors is that the gene transfer rates achieved using unprocessed vector supernatants are usually too low to elicit the desired biological effect, particularly for most in vivo applications (typical vector titers range from $10^6 - 10^7$ IVP/mL). Moreover, elimination of serum proteins and producer cell-derived contaminants from vector supernatants has shown to prevent local inflammatory and immune responses in vivo (Baekelandt et al., 2003; Scherr et al., 2002) and to increase transduction efficiencies ex vivo (Yamada et al., 2003). For this reason, harvested vector supernatants must undergo a series of processing steps aimed at improving the potency and purity of the vector preparation (for review see Segura et al. (2006a)). Although at laboratory scale this is typically achieved using conventional centrifugation techniques, for the large-scale manufacturing of clinical-grade lentivirus vectors, the use of scalable purification strategies such as membrane filtration and adsorptive chromatography is highly desirable.

In an attempt to improve the current lentivirus production procedure, a 293E cell line (clone 6E) was used for the first time for lentivirus vector production purposes. This cell line has been successfully employed in our laboratory for the fast production of large amounts of various recombinant proteins by large-scale transient transfection (Durocher, in preparation). Lentiviral particles were generated by PEI-mediated transient transfection of suspension cultures in serum-free medium. We demonstrate the scalability of this process in a 3-L bioreactor. Moreover, we show that lentiviral particles can be successfully concentrated and purified directly from supernatants using...
scaleable membrane filtration and/or chromatography technologies.

Materials And Methods

Cell Line and Medium

The cell line used for lentivirus vector production and titration is the 293E cell line (clone 6E) stably expressing a truncated functional form of the EBV nuclear antigen-1. This cell line grows in suspension culture in a serum-free chemically defined culture medium (FreeStyle™, Invitrogen, Grand Island, NY) supplemented with 0.1% Pluronic F-68 (Invitrogen) and 50 μg/mL Geneticin G418. Cells were maintained in exponential growth in 125-mL disposable polycarbonate Erlenmeyer flasks shaken at 110 rpm using an orbital shaker placed in an incubator with a 37°C, humidified, 5% CO₂ atmosphere.

Plasmids

Lentivirus particles were produced by simultaneous co-transfection of 293E cells with four plasmids. The lentivirus vector plasmid used in this study is a self-inactivating (SIN) transfer vector (CSII-CMV5) containing the green fluorescent protein (GFP) reporter gene. This vector was derived from CSII-CMV (Miyoshi et al., 1998) containing the GFP reporter protein by replacing the CMV promoter with CMV5 (Brousseau et al., manuscript in preparation). Helper functions for lentivirus production were provided by the envelope protein plasmid SVCMV-IN (Kobinger et al., 2001) that codes for the VSV-G envelope glycoprotein and the third generation packaging plasmids pMDLg/pRRE and pRSV-Rev (Dull et al., 1998) that contain the Gag/Pol and Rev coding sequences, respectively. Constructs were kindly provided by Dr. Inder Verma (Salk Institute, CA), Dr. Luigi Naldini (University of Torino, Italy), and Dr. Bernard Massie (Biotecnology Research Institute of Montreal, Canada). Plasmids were amplified in Escherichia coli, DH5α strain for helper plasmids, and SURE® strain (Stratagene, La Jolla, CA) for the transfer vector, grown in CircleGrow media (Qbiogene, Carlsbad, CA) supplemented with ampicillin (100 μg/mL). DNA purification was carried out using Gigaprep columns (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer’s instructions. Plasmids were produced in large quantities (~8–10 mg of each) in order to have homogeneous plasmid stocks to conduct all experiments. DNA concentration and purity was measured by UV absorbance at 260 and 280 nm in 50 mM Tris-HCl, pH 8. DNA integrity was assessed by agarose gel electrophoresis. Purified plasmids had an A260/A280 ratio of 1.87–1.89 and showed to be in supercoiled form free of RNA contamination.

PEI-DNA Complex Preparation

PEI transfection reagent (25-kDa linear PEI, Polysciences, Warrington, PA) was prepared as previously described (Pham et al., 2005). All transfections were performed using a total plasmid DNA amount of 1 μg/mL of culture and a DNA to PEI mass ratio of 1:3. Transfection complexes (DNA–PEI) were prepared by adding PEI to plasmid DNA diluted in culture media (10% of the total volume of the culture to be transfected). This mixture was vortexed and incubated for 15 min at room temperature (RT) prior to its addition to the cell culture.

Transient Transfection Small-Scale Studies

Transient transfection experiments to select optimal plasmid ratios, harvesting times, and cell densities at the time of transfection were conducted in 6-well plates or shaker flasks. The day before the experiments cells were inoculated at 0.5 × 10⁶ cells/mL in 125-mL shake flasks containing 20 mL of culture medium. Typically cells reached a density of 1 × 10⁶ cells/mL with a viability >95% within 24 h.

The optimization of the four plasmid ratios was performed in 6-well plates. Cell suspensions (1 × 10⁶ cells/mL) were transferred into 6-well plates (1.8 mL/well) and transfected with various [VSV-G: Gag-Pol: Rev: transfer vector] plasmid DNA mass ratios. At 45 h post-transfection (hpt), lentivirus particles were harvested by centrifuging cell suspensions at 350 × g during 5 min. Lentivirus-containing supernatants were filtered through 0.45 μm pore size Acrodisc syringe-mounted filters (Pall Gelman Sciences, Montreal, QC, Canada) and stored at −80°C or immediately titrated. The kinetics of lentivirus production and the effect of cell density at the time of transfection were investigated by transfecting high and low density cell cultures (1 × 10⁶ and 0.5 × 10⁶ cells/mL, respectively) in 125-mL shaker flasks containing 20 mL of culture. The [VSV-G: Gag-Pol: Rev: transfer vector] plasmid ratio used was 1:1:1:2. Total media replacement by centrifugation of the batch cultures was performed every 24 h for 7 days starting on day 3 post-transfection. Viable and total cells were counted using a hemacytometer prior to centrifugation. Supernatants were filtered through 0.45 μm membranes and stored at −80°C for subsequent analysis. All small-scale experiments were carried out in duplicate.

Bioreactor-Scale Lentivirus Vector Production

A 3-L Chemap CF-2000 bioreactor (Mannedorf, Switzerland), equipped with a helical-ribbon impeller (HRI) and three vertical surface baffles, was used for the lentivirus productions (Kamen et al., 1991). The agitation was set at 73 rpm. Temperature was maintained at 37°C. The pH was controlled at 7.2 by CO₂ supplementation to the gas mixture and base addition. The dissolved oxygen (DO) was
controlled at 40% of air saturation by supplementing oxygen in the inlet gas using computer-controlled mass-flow controllers and FIX-MMI software (Intellution, Norwood, MA). The partial pressure of oxygen and other critical operating parameters were monitored online to allow supervision of the culture (Fig. 3A). Large-scale transient transfection was performed using DNA concentration of 1 mg/L, a plasmid [VSV-G: Gag-Pol: Rev: transfer vector] ratio of 1:1:1:2 and PEI to DNA ratio of 1:3. Bioreactors were inoculated with $0.25 \times 10^6$ cells/mL and transfected with DNA-PEI complexes 48 h post-inoculation when cells reached a density of $0.9 \times 10^6$ cells/mL (97% viability). A total media replacement was performed at day 3, 4, and 5 post-transfection by centrifugation of the bioreactor culture (420g x 10 min.). Cell pellets were resuspended in fresh medium and re-introduced into the bioreactor. The clarified culture supernatant from day 3 post-transfection was subjected to further processing by ultra/diafiltration and heparin affinity chromatography. Bioreactors were sampled daily for cell counts and culture supernatants samples were stored at $-80^\circ$C for subsequent analyses for titration analyses.

**Infective Vector Titer Determination**

Infective virus particle titers were determined by transduction of suspension-growing 293E cells (clone 6E) followed by GFP gene expression analysis by flow cytometry. For this purpose, exponentially growing cells were diluted to a density of $\sim 5.5 \times 10^5$ cells/mL in fresh FreeStyle™ medium supplemented with 0.1% Pluronic F-68 and 50 μg/mL Geneticin G418. Polybrene was added to the cell suspension to achieve a final concentration of 8 μg/mL. Cells were plated in 12-well plates (0.9 mL/well) and subsequently exposed to 0.1 mL aliquots of virus (nondiluted or diluted in fresh culture media). Plates were incubated on an orbital shaker set to 110 rpm during 48 h at 37°C in a humidified 5% CO$_2$ atmosphere. Transduced cell suspensions were transferred to 1.5 mL Eppendorf tubes, centrifuged at 350 g for 5 min and resuspended in 0.5 mL of PBS. Samples were fixed by addition of 0.5 mL of 4% formaldehyde, incubated 30 min at 4°C and filtered through Nitex fabric prior to fluorescent activated cell sorting (FACS) analysis. Viral titers were calculated as follows: Titer (IVP/mL) = (% GFP + cells) x (number of cells at time of exposure) x (dilution factor)/(sample volume). Samples from a given experiment were analyzed in a single titration assay to avoid inter-assay variability and all samples were processed in duplicate to assess intra-assay variability. Virus dilutions that resulted in %GFP + cell values ranging from 3 to 25% were selected for titer determination. It should be noted that the titration assay is basically the same as the traditional reporter gene expression assay with the exception that instead of using adherent target cells we have successfully employed suspension-growing target cells. No overnight cell attachment and trypsinization steps are required, thus, reducing the time and complexity of the assay.

**Ultrafiltration**

Lentiviral particles were concentrated using a 76 mm diameter membrane disc filter (effective filtration area = 41.8 cm$^2$) with a molecular weight cut-off (MWCO) of 100,000 (YM-100; Millipore, Etobicoke, ON, Canada) in a 400 mL stirred cell ultrafiltration unit (Amicon 2000; Millipore). The membrane was prewashed with 200 mL of Milli-Q water followed by 200 mL of phosphate buffer saline (PBS). Supernatants (~200 mL) were concentrated ~20-fold under constant nitrogen pressure (30 psig) and tip speed (33.5 cm/sec). Lentivirus enriched retentate was diafiltered against cold PBS (35 mL) twice in discontinuous mode. Virus was aliquoted and stored at $-80^\circ$C. Duplicate runs were processed.

**Heparin Affinity Chromatography Purification**

Virus purification experiments were carried out at RT using a low-pressure liquid chromatography system (Gradi-Fract™; GE Healthcare, Uppsala, Sweden) and an HR 5/5 column packed to a final volume of 1 mL with tentacle-type Fractogel® EMD Heparin (S) gel. Protein elution was monitored by UV absorbance at 280 nm. Virus stocks were thawed at 37°C and filtered with a 0.45 μm GHP Acrodisc filter membrane (Pall Gelman Sciences) prior to chromatography. The ability of lentivirus particles to bind immobilized heparin ligands was investigated using a linear NaCl gradient elution strategy. Briefly, clarified lentivirus supernatant (35 mL) was loaded onto the heparin column pre-equilibrated with 20 mM Tris-HCl buffer, pH 7.5. A linear salt gradient from 0 to 1,200 mM NaCl (30 mM NaCl/min) was applied at a linear flow rate of 153 cm/h. Fractions of 5 mL were collected throughout the run and immediately titrated. Optimal salt concentrations for virus binding and elution were defined. The performance of heparin affinity chromatography using a previously described scaleable step elution strategy for oncoretroviral vector purification was also investigated in this work (Segura et al., 2005, 2006b). Both supernatant and concentrated lentivirus stocks were used as feed for chromatography. Briefly, the heparin column was pre-equilibrated with 150 mM NaCl in Tris-HCl buffer, pH 7.5 and loaded with either 35 mL of clarified supernatant or 5 mL of 20-fold concentrated virus stock. Following sample loading, a step NaCl elution strategy consisting in a wash step at 150 mM NaCl (15 column volumes (CV)), a virus elution step at 350 mM NaCl (13 CV) and a high stringency final wash step at 1200 mM NaCl (6.5 CV) was applied. The running linear flow rate was 153 cm/h. Fractions of 2.5 mL were collected throughout the run and peak fractions were pooled and immediately titrated or stored at $-80^\circ$C. Duplicate runs per feed type were...
carried out. Total protein concentrations were determined by the Bradford Protein Assay (BioRad, Hercules, CA) and double-stranded DNA concentrations were quantified using the PicoGreen \textsuperscript{R} dsDNA Quantitation Kit (Molecular Probes, Eugene, OR) according to the manufacturers’ instructions.

Results

Optimizing Plasmid Ratios

To establish optimum plasmid mass ratios for lentivirus vector production, several plasmid ratios were tested in 6-well plates by varying individual plasmid quantities while maintaining the total amount of DNA at 2 μg/well. Additionally, two negative controls were included where either the transfer vector or the Gag-Pol plasmid was omitted. Results are shown in Figure 1. The higher vector titers (~1.2 x 10⁶ IVP/mL) were obtained using high amounts of transfer plasmid either alone (1:1:1:2) or in combination with high Rev plasmid (1:1:2:2) or high Gag-Pol plasmid (1:2:1:2). Further increasing the amount of transfer vector (1:1:1:3) did not result in improved vector titers. Poor viral titers were obtained using high amounts of VSV-G plasmid, most likely due to its known cytotoxic effect. As expected, the omission of the transfer vector or Gag-Pol plasmid resulted in undetectable vector titers.

![Figure 1](image)

**Figure 1.** Optimizing plasmid ratios. Effect of various plasmid ratios on lentiviral vector production. The solid bars represent the virus titers obtained by transfecting cultures using various plasmid DNA mass ratios of Env-protein protein plasmid (VSV-G): packaging plasmid I (Gag-Pol): packaging plasmid II (Rev) and SIN transfer vector plasmid (Transfer Vector) into 293 6E with a total DNA amount of 2 μg/well.

Kinetics of Lentivirus Production at Two Different Cell Densities

The kinetics of viral vector production was initially evaluated by transfecting batch cultures at 1 x 10⁶ cells/mL in 20-mL shake flasks. These experiments revealed that vector activity could be reproducibly detected in the culture supernatant starting 48 hpt. Vector titers were sustained at maximum levels from day 3 to 5 post-transfection and decreased at day 6 post-transfection (data not shown). Therefore, 20-mL batch culture experiments with total media replacement and virus harvest starting day 3 post-transfection were conducted. The effect of cell density at the time of transfection was investigated by transfecting high (1 x 10⁶ cells/mL) and low (0.5 x 10⁶ cells/mL) density cultures. The kinetics of virus production was reproduced in batch replacement mode (Fig. 2A). The highest vector titers were obtained by transfecting high-density cultures (black bars, Fig. 2A). As previously observed, vector titers were kept high in harvested supernatants of day 3, 4, and 5 days post-transfection for which the average vector titer was ~2.3 x 10⁶ ± 0.1 x 10⁶ IVP/mL. In contrast, the maximum vector titer achieved by transfecting low-density cultures was 1.2 x 10⁶ IVP/mL on day 3 post-transfection but continuously decreased thereafter (hatched bars, Fig. 2A). Importantly, specific virus productivities were similar for both transfection densities tested (Fig. 2B) indicating no nutrient limitations at the higher cell density tested. Producer cells reached their highest cellular density at day 5 post-transfection. While cell cultures transfected at 1 x 10⁶ cells/mL doubled their concentration, cultures transfected at 0.5 x 10⁶ cells/mL only reached a maximum of ~0.75 x 10⁶ cells/mL (Fig. 2C). Cell viability at the time of transfection was 98% and decreased to 81% in cell cultures transfected at high density (Fig. 2C). The drop in cell viability was more abrupt for cell cultures transfected at low density (98–59%). A total of 1.7 x 10⁶ IVP were collected from the 20-mL high-density culture flasks during days 3 to 7 of production. Virus production was reduced to 1/3rd (0.6 x 10⁶ IVP) when transfecting low-density cultures.

Production of Lentiviral Vectors in 3-L Bioreactors

To demonstrate the scalability of the lentiviral vector production process, a 3-L bioreactor culture was transiently transfected with the four plasmids. The culture was maintained under controlled conditions: temperature at 37°C, pH at 7.2, and stirring rate at 73 rpm (Fig. 3A). The DO was maintained at 40% by aeration until the oxygen molar fraction in the inlet gas (yo₂) reached 100% at day 5 post-transfection (127 hpt) (Fig. 3A). Infectious lentiviral vector particles were detected in harvested supernatants from day 1 to 6 post-transfection (Fig. 3B). The highest vector titers were achieved from day 2 and 3 post-transfection that presented an average vector titer of 1.1 x 10⁶ ± 0.2 x 10⁶ IVP/mL. The highest specific vector productivity was observed at day 2 post-transfection.
Figure 2. Kinetics of lentivirus production at two different cell densities. Analyses of a 20-mL scale lentivirus vector production in batch replacement mode by transient transfection at two different cell densities. A: Infectious viral titer and B: virus specific productivities by transfection at high (solid bars) and low (hatched bars) cell densities. C: Cell density (triangle) and cell viability (circle) for transfection at high (triangles) and low (circles) cell densities. Values presented are the mean ± standard deviation of duplicate experiments. Abbreviation: IVP, infective viral particles.

Figure 3. Production of lentiviral particles in a 3-L bioreactor. Analyses of a 3-L scale lentivirus vector production in batch replacement mode. A: Bioreactor online monitoring: $y_{CO2}$ (green), DO (blue), $y_{O2}$ (black), temperature (red), and pH (orange). B: Viral titer in the supernatant and accumulated lentiviral viral vector production. C: Cell density and cell viability. Values presented are the mean ± standard deviation of duplicate or triplicate determinations. Abbreviation: IVP, infective viral particles; VV, viral vector; $y_{CO2}$, CO$_2$ molar fraction in the inlet gas; DO, dissolved oxygen; $y_{O2}$, O$_2$ molar fraction in the inlet gas.
(0.6 IVP/cell per day). In the 3-L bioreactor, a total of $\sim 1 \times 10^{10}$ IVP were produced during this process (Fig. 3B). The cell density increased from $0.9 \times 10^6$ cells/mL on day of transfection to $7 \times 10^6$ cells/mL on day 6 post-transfection (Fig. 3C). The cell viability was >90% throughout the whole process.

Concentration of Lentiviral Particles by Ultrafiltration

Supernatants from bioreactor cultures harvested on day 3 post-transfection were successfully concentrated 20-fold, partially purified and conditioned in appropriate buffer using a stirred cell ultrafiltration unit. The ultra/diafiltration methodology used was adapted from a previously reported method used for the concentration of oncoretroviral particles produced in the presence of 10% fetal bovine serum (Segura et al., 2005, 2006b). However, the concentration of lentiviral particles produced in serum-free conditions required the use of a lower MWCO membrane filter (100,000) for optimal recovery of infective particles. Using this filter, the recovery of infective viral particles in the retentate fraction was 56 ± 2%. The use of 300,000 MWCO membranes was also tested but resulted in lower recoveries of infective viral particles (7.5%). The ultra/diafiltration process time was 1 h. In addition, removal of 67 ± 14% of the protein content and 64 ± 10% of small DNA fragments was achieved during this step.

Purification of Lentiviral Particles by Heparin Affinity Chromatography

We have previously demonstrated the usefulness of heparin affinity chromatography for the purification of various oncoretroviral pseudotypes (Segura et al., 2005, 2006b). A preliminary chromatography run using a linear NaCl elution gradient indicated that lentivirus particles could also successfully bind heparin ligands and be eluted from the chromatographic column at a NaCl concentration of $\sim 300$ mM (data not shown).

Two heparin affinity chromatography purification modalities were tested. The first one involved direct loading of lentivirus supernatants (35 mL) onto a heparin column (Fig. 4A). Contaminants were successfully removed during the virus loading and washing step at 130 mM NaCl. Viral particles were eluted in a well-defined peak by addition of 350 mM NaCl into the mobile phase. Virus stocks were concentrated fivefold during this procedure and a good recovery of infective virus particles (53 ± 1%) was achieved.

Figure 4. Heparin affinity chromatography step gradient elution profiles. A: Nonconcentrated lentivirus supernatant (35 mL) or (B) 20-fold concentrated lentivirus stocks (5 mL) were used as feed for heparin affinity chromatography. Samples were loaded onto a 1 mL Fractogel® EMD Heparin (S) column. The virus was eluted by addition of 350 mM NaCl into the mobile phase using a previously designed elution strategy for the purification of oncoretroviral particles (Segura et al., 2005).
Using this approach, 94% of the protein impurities (purification factor of 8.4) and 56.3% DNA contaminants were removed. Chromatography was completed within 2.5 h. The second purification modality tested was identical to that previously reported for oncoretroviral particles in which 20-fold concentrated and partially purified vector stocks (5 mL) were loaded onto the heparin affinity chromatographic column. The chromatography profile is very similar to that observed for VSV-G pseudotyped particles with the exception that the concentrated lentivirus stocks obtained in serum-free conditions were much less contaminated, as indicated by the low amount of contaminants being washed off the column at 150 mM NaCl (Fig. 4B). The recovery of infectious viral particles in the peak eluting at 350 mM salt was 33 ± 2% (Table IB). Overall, using this two-step purification strategy the final purity achieved was slightly higher, since 98% of the contaminating proteins (final purification factor of 11.1) and 78.4% of contaminating DNA species were removed. The recovery of infective viral particles was 19% (Table IB).

### Discussion

Production of lentiviral vectors by transient transfection clearly offers a number of advantages over stable packaging cell lines. Despite the simplicity and speed of this approach, relatively little progress has been made at large-scale. Only minor modifications of the protocol or optimization of the transient transfection parameters originally published by Naldini et al. (1996b) have been reported (Coleman et al., 2003; Geraets et al., 2005; Koldej et al., 2005; Mitta et al., 2005), which contrasts the many efforts devoted to the development of stable packaging cell lines (Farson et al., 2001; Kafri et al., 1999; Klages et al., 2000; Kumar et al., 2003; Ni et al., 2005; Pacchia et al., 2001; Xu et al., 2001). This may be due to the fact that transient transfection is still commonly regarded as a nonscaleable technology. However, it should be noted that in recent years transient transfection of suspension-growing cells has emerged as a powerful technology for the large-scale production of biopharmaceuticals (Durocher et al., 2002; Geisse and Henke 2005; Muller et al., 2005; Pham et al., 2003, 2005; Rosser et al., 2005; Sun et al., 2006). Additionally, this technology has recently shown great potential for the large-scale manufacturing of adeno-associated viral vectors (Park et al., 2006; Reed et al., 2006). So far, the most widely used cell line in large-scale transient transfection protocols has been the 293E cells and the most widely used transfection reagent has been PEI (for review see Pham et al. (2006)). To the best of our knowledge, production of lentiviral particles by transient transfection of suspension-growing cells has not been reported to date, nor has the use of 293E cells or bioreactors for lentiviral production purposes.

In this work, we describe a simple protocol for the production of lentiviral vectors by transient transfection of

### Table 1. Purification Table.

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<th>DNA (μg/mL)</th>
<th>SVA (IVP/mg)</th>
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<td>17.0</td>
<td>53</td>
<td>100</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Heparin affinity</td>
<td>10.6</td>
<td>2.79E+06</td>
<td>0.008</td>
<td>10.2</td>
<td>33</td>
<td>53</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Overall</td>
<td>19</td>
<td>20</td>
<td>98</td>
<td>78.4</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

(A) Nonconcentrated lentivirus supernatant or (B) 20-fold concentrated lentivirus stocks were purified by heparin affinity chromatography. All values shown are mean of two independent runs. Abbreviation: SVA specific virus activity.
The highest vector titers were obtained when the amount of transfer vector plasmid exceeded that of the packaging and VSV-G Env-protein plasmids in the transfection mixture. The [VSV-G: Gag-Pol: Rev: transfer vector] plasmid ratio of 1:1:1:2 was selected for optimal production. This result is consistent with the work published by Mitta and collaborators who have recently reported a detailed optimization study of various transient transfection parameters (Mitta et al., 2005). Additionally, it substantiates empirical findings by other research groups who also use higher relative amounts of transfer vector for transient transfection (Dull et al., 1998; Follenzi and Naldini 2002; Mitta et al., 2005; Reiser 2000). Production of lentiviral vectors in lower serum content as well as serum-free media has been achieved. Although successful for some researchers (Mitta et al., 2005; Reiser 2000), in other cases it resulted in decreased viral titers (Baekelandt et al., 2003; Ballas et al., 2005). In this work, lentivirus particles were produced in serum-free medium resulting in average viral titers similar to those reported using the standard transfection protocols. In terms of cell-specific productivities, our results are in line with those reported for oncoretroviral vectors for which cell specific productivities in the order of 1–3 infectious particles per cell per day are typically achieved (Ghani et al., 2006; Le Doux et al., 1999).

Chromatography is deemed the most promising technology for large-scale purification of viral vectors. Heparin is a relatively inexpensive and stable affinity chromatography ligand widely used in the pharmaceutical industry for the isolation of various licensed therapeutic plasma products (Burnouf and Radasevich, 2001). In previous work, we have shown that heparin affinity chromatography was a convenient method for the purification of oncoretroviral vectors yielding good recoveries of active viral particles (61%). The method proved to be useful for the purification of oncoretroviral vectors derived from different cellular origins carrying alternative Env-proteins, including VSV-G pseudotyped oncoretroviral vectors derived from 293 producer
cells (Segura et al., 2005, 2006b). Since in principle these oncoretroviral vectors should display the same external composition as VSV-G pseudotyped lentiviral vectors derived from a 293 cell line, it was reasonable to assume that heparin affinity would be useful for the purification of the latter as well. Indeed, lentiviral particles bound the heparin ligands with similar affinity and efficiency as oncoretroviral particles and were successfully purified by heparin affinity chromatography.

Lentiviral vector stocks produced in serum-free media are free of the heparin-binding impurities contained in serum that can compete for the heparin-binding sites with the vector reducing the column binding capacity. In addition, these heparin-binding serum contaminants can elute together with the virus at 350 mM NaCl contaminating the vector preparation and need to be removed in subsequent purification steps to achieve a highly purified product (Segura et al., 2005). In the presence of these contaminants, direct loading of retroviral vector supernatants onto the chromatography column was not possible due to inefficient virus binding (Segura et al., 2005). Conversely, we were able to concentrate and purify lentiviral particles in a single step directly from vector supernatants using serum-free media. Moreover, omitting the use of serum in the culture media eliminates the risk of contamination with adventitious agents and minimizes regulatory concerns. The recovery of infective viral particles (53%) by heparin affinity chromatography was comparable to those reported for lentiviral vectors using anion exchange chromatography (Scherr et al., 2002; Slepushkin et al., 2003; Yamada et al., 2003) and to those obtained with oncoretroviral vectors produced in the presence of 10% FBS (Segura et al., 2005, 2006b). As with size exclusion chromatography and anion exchange chromatography, treatment of lentiviral vector stocks with DNase will be required to remove residual contaminating nucleic acids (Slepushkin et al., 2003).

In conclusion, this work demonstrates that lentiviral vectors can be produced using the same state-of-the-art technology currently used for the production of biopharmaceuticals: large-scale transient transfection of suspension-growing cells in serum-free media. The method is suitable for both laboratory and industrial scale. This approach is routinely operated in our laboratory at 60 L-scale for recombinant protein production. In addition, we showed that harvested supernatants can be easily purified using heparin affinity chromatography. Media optimization work is underway in an attempt to further improve vector titers.

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References


