# Lentivirus Vector Purification Using Anion Exchange HPLC Leads to Improved Gene Transfer

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**ABSTRACT**

*Recombinant lentiviral vectors stably transduce both dividing and nondividing cells. Virus pseudotyping with vesicular stomatitis virus envelope G (VSV-G) protein broadens the host range of lentiviral vector and enables vector concentration by ultracentrifugation. However, as a result of virus vector concentration, contaminating protein debris derived from vector-producing cell culture media is toxic to target cells and reduces the transduction efficiency. Here we report a new and rapid technique for purifying lentivirus vector using the strong anion exchange column that significantly improves gene transfer rates. We purified VSV-G pseudotyped self-inactivating lentivirus vector and obtained two protein elution peaks (Peak 1 and Peak 2) corresponding to transducing activity. Peak 1 viral particles were 4–8 times more effective in transducing target cells than Peak 2 or non-purified (pre-HPLC) viral particles. We used purified lentivirus vector expressing the human Fanconi anemia group A (FANCA) gene to transduce murine hematopoietic stem/progenitor cells. We observed a consistent 2- to 3-fold increase in gene transfer rates using Peak 1 purified virus compared with non-purified virus. We conclude that the purification method using the HPLC system provides the highly purified virus vector that reduces cell toxicity and significantly improves gene transfer in primary cells.*

## INTRODUCTION

Lentivirus vector based on the human immunodeficiency virus-1 (HIV-1) appears to be one of the promising vectors for gene transfer studies (1,2). The advantageous feature of lentivirus vector is the ability of gene transfer and integration into dividing and nondividing cells (3). The current method of lentivirus vector production is based on the co-transfection method with multiple plasmids expressing virus proteins (the gag, pol, rev, and envelope) and the gene of interest in 293T cells. To date, considerable effort has been made to improve the safety of lentivirus vector (4). Furthermore, the pseudotyped envelope with vesicular stomatitis virus envelope G (VSV-G) protein broadens the target cell range and can be concentrated by ultracentrifugation, enabling the higher MOI required for the effective gene transfer of primary cells (5). As a consequence, the ultracentrifugation step also concentrates cellular debris, membrane fragments, and denatured proteins derived from culture media of virus-producing cells. This unwarranted material in the crude vector preparation is toxic to target cells, especially primary cells, and may cause immunogenic reactions in experimental animal models by in vivo vector administration. Therefore, to reduce undesirable effects and increase gene transfer efficiency, the purification of virus vector is required. Lentivirus vector purification using cation exchange column chromatography has been reported (6). Generally, a low pH condition is essential to enhance cationic charge on a virus envelope for the efficient virus separation that decreases the stability of membrane proteins. More recently, the gene transfer of primary hematopoietic cells using lentivirus vector purified by a weak anion exchange column was demonstrated (7). However, no characterization of these particles was performed, including the comparison of transduction efficiency with non-purified vector.

In this study, we examined the utility of column chromatography using the HPLC system to purify VSV-G pseudotyped lentivirus vector. It is well known that a poly-cation such as polybrene and protamine sulfate enhances transduction in retrovirus and lentivirus vector systems (8,9). Based on the known electrostatic interaction between the cell surface and virus envelope membrane, we speculated that a strong anion exchange column might be useful to increase the efficiency of virus vector purification. Here we demonstrate that a strong cationic charged matrix enriches for functional lentivirus vector particles and can eliminate contaminating protein and defective particles. The overall result is the decreased cell toxicity and an improvement in gene transfer rates.

## MATERIALS AND METHODS

### Plasmid Construct

The plasmids that express virus protein (gag, pol, and rev; pMDLg/pRRE and pRSV-Rev), vector plasmid containing the sequences of human PGK internal promoter and EGFP [pRRLsin.hPGK.cPPT.CTS.GFPpre (10)], and VSV-G (pMD.G) were kindly provided by Dr. Thomas Dull (Cell Genesys, Foster City, CA, USA). Human PGK, EGFP, and woodchuck hepatitis virus post-transcriptional element (WPRE)

were removed by restriction enzyme digestion of pRRLsin.hPGK.cPPT.CTS.GFPpre using *Eco*RI. EF1-α internal promoter (excised from pBudCE4.1 plasmid; Invitrogen, Carlsbad, CA, USA) and a 4.5-kb coding region of Fanconi anemia group A (FANCA) cDNA were ligated into the backbone plasmid to make the pSIN/EF1-α/FANCA (Figure 1).

### Production of Lentivirus Vector

The generation of lentivirus vectors was performed by the transfection of $2 \times 10^6$ 293T cells with each 8 µg pMDLg/pRRE, pMD.G, and pRRLsin.hPGK.cPPT.CTS.GFPpre or pSIN/EF1-α/FANCA plasmids in addition of 2.5 µg pRSV-Rev plasmid using calcium phosphate. After 48 h, the supernatant was harvested and filtered through a 0.45-µm pore size filter and then concentrated to 1/100 volume by ultracentrifugation with 50 000× *g* at 4°C for 2.5 h using a SW28 rotor (Beckman Coulter, Fullerton, CA, USA) and Sorvall Ultra 80® (Kendro Laboratory Products, Newtown, CT, USA). The virus particle pellet was resuspended in PBS and frozen at -80°C until use.

### Purification of Lentivirus Vectors by Anion Exchange Column

The strong anion exchange column (5 mL gel volume column capable of adsorbing 50 mg protein/mL column volume; HiTrap™ Q, HP, Amersham Biosciences, Piscataway, NJ, USA) was used with the Dynamax HPLC system (Rainin Instrument, Woburn, MA, USA) at 4°C. Protein elution was monitored with UV absorbency at 280 nm with Dynamax UV-C (Rainin Instrument).

Before the virus preparation was loaded onto the column, the column was washed with five column volumes (25 mL) of starting buffer (PBS adjusted pH at 7.5) at a flow rate of 5 mL/min. The column was then washed with the elution buffer (PBS containing 1 M NaCl) at 5 mL/min for 5 min and equilibrated with the starting buffer at 5 mL/min for 5 min. The 100-fold concentrated virus vector (0.5 mL) was suspended in the starting buffer adjusted to a total volume of 1–1.5 mL. The virus vector suspension was centrifuged to remove large debris at 6000× *g* for 5 min. Virus vector suspension was loaded through a 5-mL loading capacity injection loop. The protein elution program was designed to include the washing step for 5 min and salt gradient step (0–1 M NaCl) for 10 min. A 2.5 mL/min of flow rate was applied. In total, 60 fractions were collected (1 mL/tube). Consistently, two protein peaks were obtained and termed as the "Peak 1" and "Peak 2" (Figure 2a). Each fraction was analyzed for virus particle number and transduction efficiency on 293T cells. The vector expressing the FANCA gene was purified using the identical conditions. Desalting was performed with a centrifugal filter device (Centricon® Plus-20, cut-off 100 000 molecular weight; Millipore, Bedford, MA, USA). The membrane was sterilized by washing with 70% ethanol. Peak 1 and 2 virus fractions (3–5 fractions) were diluted with 10 mL PBS and then applied onto the filter device. Virus vector suspensions were centrifuged to desalt and concentrate using a swinging bucket rotor at 2000× *g* for 15–20 min at 4°C. Virus vector was then recovered from an inverted filter cup with an additional centrifugation step, according to the manufacturer's protocol. The final virus vector volume was adjusted in 200–300 µL.

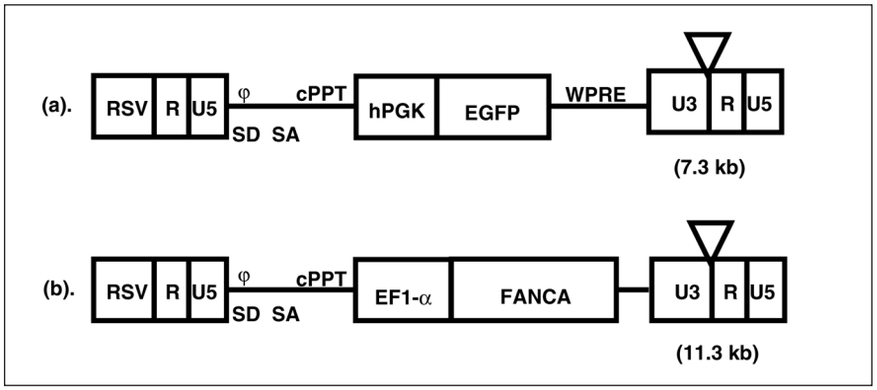


**Figure 1. Schematic diagram of vector plasmid constructs.** (a) pRRLsin.hPGK.cPPT.CTS.GFPpre. (b) pSIN/EF1-αFANCA. FANCA expression vector (b) was constructed using pRRLsin.hPGK.cPPT.CTS.GFPpre (a). *Eco*RI restriction enzyme excised the fragment containing hPGK, EGFP, and WPRE. Human elongation factor 1-α promoter and 4.5 kb FANCA coding sequence were ligated into the backbone plasmid. The triangles represent the deleted sequence in the U3 region.

### Virus Particle Number and EGFP Fluorescence

Human 293T cells were plated into a 48-well plate at $1 \times 10^4$ cells/well and then transduced with EGFP-expressing vector in the presence of 5 µg/mL protamine sulfate. Two days later, EGFP fluorescence was measured using a flow cytometer (FACSCalibur™; BD Biosciences, San Jose, CA, USA).

For an estimation of the vector particle number in the same volume used for the transduction of 293T cells, virus genomic RNA was harvested as described previously (11). Virus RNA was subjected to dot blot and hybridized using α-dCTP$^{32}$-labeled probe generated using the full length of EGFP cDNA (0.6 kb). A standard curve was established using a serial dilution of vector plasmid. After hybridization, the phosphorimage value was captured by a Storm™ 860 phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA). Then, the particle number of each sample was calculated depending on standard curve. The ratio of virus particle number and EGFP fluorescence was determined. The particle number of vector expressing FANCA was also determined using a 1.8-kb fragment of FANCA cDNA (restricted with *Nhe*I and *Bam*HI enzymes) as a hybridization probe.

### Electron Microscopy

Virus vector particles were stained using a negative staining technique. A glow discharged, pioloform-coated, 400 mesh nickel grid was placed film-side down onto a 10-µL drop of virus vector suspension for 10 min to allow for particle adsorption. The grid was rinsed briefly by floating on two drops of deionized water and stained by transfer-

ring it to a drop of 2% aqueous uranyl acetate (pH 3.5) for 1 min, followed by air drying. The grids were examined at 80 kV in a LEO EM-910 transmission electron microscope (LEO Electron Microscopy, Thornwood, NY, USA).

### Transduction of Mouse Bone Marrow Hematopoietic Cells and Clonogenic Assay

Bone marrow was harvested from FANCA knockout mice (kindly provided by Dr. Fre Arwert, Amsterdam, Netherlands). Sca-1 antigen positive cells were selected using the Sca-1 MultiSort kit (Miltenyi Biotech, Auburn, CA, USA) according to the manufacturer's instructions. The cells were incubated with pre-HPLC, and Peak 1 and Peak 2 lentivirus vectors without cytokines were incubated in serum-free media (X-VIVO 10; CAMBREX, Walkersville, MD, USA) for 12 h. The minimal MOI (100) needed for mouse bone marrow transduction (12) was used. After transduction, the cells were subjected to clonogenic assay using methylcellulose culture media (MethoCult™ GF M3534; Stem Cell Technologies, Vancouver, Canada) with serial concentrations of mitomycin C (MMC) (Calbiochem, San Diego, CA, USA) ranging from 0 to 10 nM, and the colonies were enumerated 14 days later.

**Table 1. Transduction Efficiency of the Column-Purified Virus**

| Purification | EGFP-Positive Cells (%) | RNA Genome Particles ($\times 10^5$) | Transducing Activity (Genome Particle/ EGFP-Positive Cell) | Fold |
|---|---|---|---|---|
| Experiment 1 | | | | |
| pre-HPLC | 32.8 | 50.3 | 1487 : 1 | 1.0 |
| Peak 1 | 32.8 | 10.6 | 323 : 1 | 4.6 |
| Peak 2 | 15.7 | 12.1 | 770 : 1 | 1.9 |
| Experiment 2 | | | | |
| pre-HPLC | 34.0 | 31.2 | 918 : 1 | 1.0 |
| Peak 1 | 19.2 | 2.1 | 109 : 1 | 8.4 |
| Peak 2 | 12.9 | 4.1 | 314 : 1 | 2.9 |

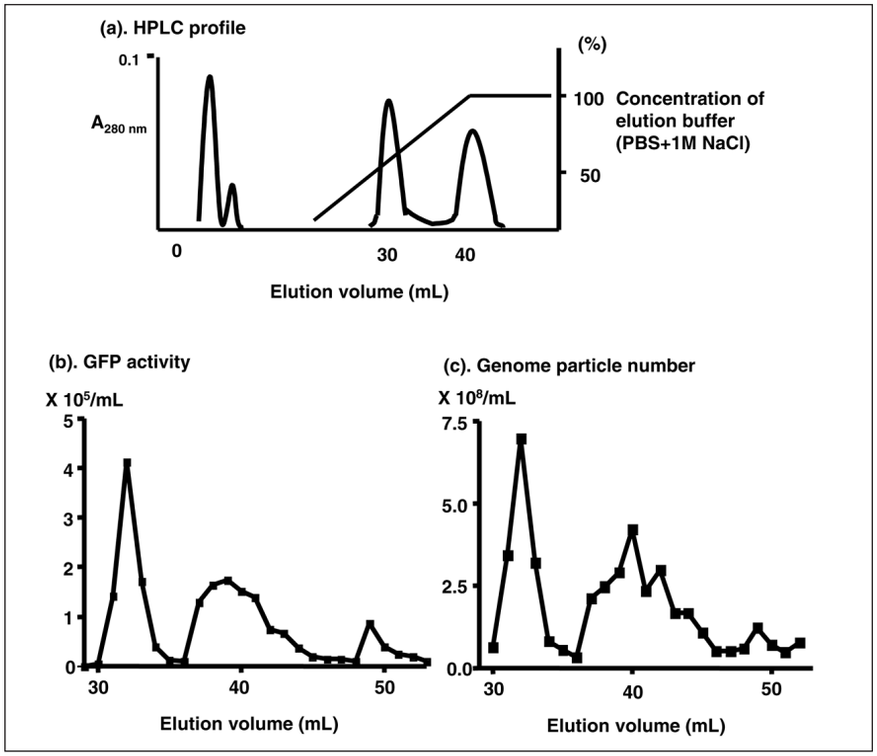


**Figure 2. Strong anion exchange column chromatograpy of lentivirus vector.** (a) Elution pattern of crude concentrated lentivirus. Virus was applied to the HiTrap Q anion exchange column, washed with PBS, and eluted with PBS containing 1 M NaCl. Fractions of 1 mL were collected. (b) EGFP activity in cells transduced with eluate. 293T cells ($1 \times 10^4$) were transduced with of HPLC elution containing lentivirus vector. Two days later, EGFP fluorescence was measured with flow cytometry. Data were expressed as the number of EGFP-positive cells. (c) Genome particle number in cells transduced with eluate. The eluted fractions were treated with DNase I. Virus RNA was extracted and subjected to dot blot analysis using an EGFP probe. The virus particle number was calculated using the standard curve generated with plasmid DNA.

### Bone Marrow Transplantation with Transduced Stem/Progenitor Cells

Lineage$^-$c-kit$^+$Sca-1$^+$ cells were isolated from FANCA knockout mice bone marrow following the published method (17) and then transduced with FANCA cDNA expressing vector using the same conditions described above. Twelve hours later after transduction, 3000 cells were injected through orbital vein into sublethally irradiated (4Gy) FANCA knockout mouse. Twenty-four hours after injection, 0.3 mg/kg MMC was administered intraperitoneally to eliminate nontransduced cells and enrich transgene-expressing cells. After bone marrow transplantation, the blood cell number was monitored weekly. One month later, all blood indices reached normal range (data not shown).

### Real-Time PCR

Peripheral blood (50 µL) was collected from recipient mice, and DNA was isolated using the DNeasy® tissue kit (Qiagen, Valencia, CA, USA). Real-time PCR was performed using the ABI PRISM® 7700 and GeneAmp® 5700 Sequence Detector and software version 1.6.3 (Applied Biosystems, Foster City, CA, USA). The primers and fluorescence-labeled probes specific for the sequence of lentivirus vector (13) and neomycin resistance gene utilized as an internal control were generated using the TaqMan® system (Applied Biosystems). The sequence of the primers and

probe for neomycin resistance gene are: forward primer 5′-CCATTCGACCACCAAGCG-3′, reverse primer 5′-CCGGCTCTCCATCCGA-3′, and probe; 5′-TET (tetrachloro-6-carboxyfluorescein)-AACATCGCATCGAGCGAGCACG-TAMRA-3′. A standard curve for the transgene copy number was established by the amplification of a series of genomic DNA mixtures derived from a vector-transduced cell line that has one transgene copy per cell and mouse peripheral blood (data not shown). The quantity of the transgene in the sample DNA was normalized by neomycin resistance gene amplified simultaneously in a separate tube.

## RESULTS AND DISCUSSION

### The Purification of Lentivirus Vector by Anion Exchange Column

The VSV-G pseudotyped virus pellet was resuspended in PBS and loaded onto a HiTrap Q column and eluted with a linear salt gradient (see Materials and Methods section). Consistently, two peaks were obtained after virus elution. The first peak of virus elution appeared when the salt concentration was 0.5 M in PBS (Peak 1, three fractions in total), and the second peak emerges at near 1 M salt (Peak 2, 3–5 fractions). The transduction efficiency of the lentivirus in the eluted fractions was evaluated in the 293T cell line using EGFP fluorescence. The total recovery rate of virus estimated using EGFP fluorescence was 50% of initial amount of virus loaded onto the column. Two-thirds of the functional particles collected were eluted into Peak 1. The peak fractions of EGFP fluorescence and virus particle number corresponded with the protein elution peak (Figure 2). As demonstrated in Table 1, transducing activity taken as the ratio of EGFP-positive cell number to virus particle number in the same volume of each sample was significantly different between pre-HPLC, Peak 1, and Peak 2 virus. Compared to pre-HPLC virus, column-purified virus based on the transducing activity was 4- to 8-fold higher in Peak 1 and 2- to 3-fold higher in Peak 2, in the two representative experiments. In other experiments ($n = 5$), we consistently found the similar difference of transduction efficiency between Peak 1 and 2. With electron microscopy, we observed no virus particles aggregating in both Peak 1 and 2 (Figure 3). Because of the high negative charge caused by protein aggregate, a higher salt concentration was required to elute the virus particles into Peak 2. Consequently, the stability of virus envelope may have decreased, resulting in lower transduction efficiency on 293T cells. In another experiment, empty viral particles were prepared by transfection without a transgene plasmid, concentrated by ultracentrifugation, and loaded onto the column. A similar elution pattern, including two peaks, was observed. The content of virus genome RNA did not affect the envelope charge, and the empty particles could not be eliminated from the virus preparation (data not shown).

### Electron Microscopy

The pre-HPLC, Peak 1, and Peak 2 virus fractions were examined using an electron microscope after staining with 2% uranyl acetate. The appearance and size of Peak 1 and Peak 2 virus were not different. The virus size ranged

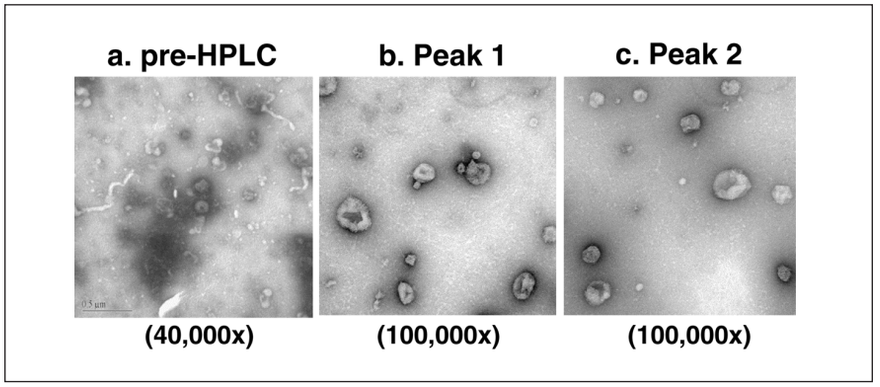


**Figure 3. Electron microscopic images of lentivirus particles.** Lentivirus preparation was negatively stained with uranyl acetate. (a) Low-magnification (40 000×) electron micrograph of pre-HPLC virus. A significant amount of contaminating protein debris (membrane fragment and proteins) is observed in the concentrated virus preparation. Some virus particles appear as aggregates. (b) High-magnification (100 000×) picture of Peak 1 virus. (c) Peak 2 virus. The removal of contaminating protein seen in panel a is notable.

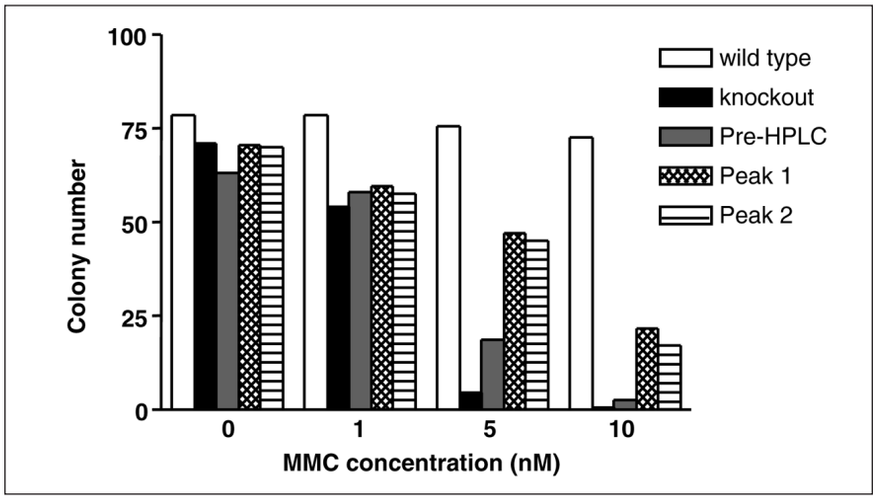


**Figure 4. MMC resistance assay of transduced Sca-1 cells.** Sca-1 cells were harvested from FANCA wild-type and knockout mouse bone marrow. Knockout Sca-1 cells were transduced with non-purified and column-purified lentivirus vector. After transduction, 1000 cells were subjected to the clonogenic assay in the presence of an increasing concentration of MMC. The colony number designated is the average of duplicate samples.

from 70 to 130 nm. Because the uranyl acetate also stains the background, in the pre-HPLC sample, we observed significant aggregation of virus particles with protein and cell debris. In Peak 1 and Peak 2 samples, virus particles were observed separately, and the removal of contaminating protein from the background was notable (Figure 3).

### The Transduction Efficiency of Mouse Bone Marrow

Fanconi anemia is an autosomal recessive disease characterized by hypersensitivity to alkylating agents such as MMC, which leads to cell death. Cells derived from FANCA knockout mice demonstrate similar sensitivity to MMC. We utilized the human FANCA gene as a biological marker for gene transfer to FANCA knockout mouse bone marrow. First, we harvested Sca-1 positive cells that produce multilineage hematopoietic cells (14,15) from FANCA knockout and wild-type mice bone marrow. After the incubation of Sca-1 cells with each virus preparation, a large amount of protein debris was seen in the serum-free media with pre-HPLC virus, and 90% of the cells were not viable. On the other hand, much less protein aggregate was observed in the culture with Peak 1 and 2 virus preparations. Transduced cells were subjected to clonogenic assay in the presence of increasing MMC concentrations. As shown in Figure 4, the number of colonies transduced with Peak 1 and Peak 2 virus was 2-fold higher at 5 nM MMC than that of pre-HPLC virus. Unlike EGFP transduction in 293T cells, we did not observe the difference in MMC resistance in bone marrow cells transduced with Peak 1 and Peak 2 virus.

Next, we transduced stem cells from FANCA knockout mice, performed bone marrow transplantation, and then determined proviral integration by real-time PCR. The proviral copy number in the peripheral blood of animals receiving the cells transduced with pre-HPLC, Peak 1, and Peak 2 virus was $0.34 \pm 0.05$, $0.73 \pm 0.07$* ($P < 0.01$*), $0.53 \pm 0.10$ ($P < 0.03^{\#}$), respectively (*, $^{\#}$; compared to pre-HPLC, by paired *t*-test). Peak 1 virus produced a 2-fold increase of transgene integration in lineage$^-$c-kit$^+$Sca-1$^+$ cells compared with the non-purified virus.

The HPLC lentivirus purification method is a rapid (total processing time is within 30 min), simple, and reproducible method for VSV-G pseudotyped virus. It is well known that only less than 1% of lentivirus vector particles prepared by ultracentrifugation is infectious (13). The RNA genome particle number of vector preparation does not represent the vector infectivity; however, the transducing activity estimated with the ratio of genome particle number and EGFP fluorescence is a better index to determine the functional transgene expression. According to this index, the HPLC virus vector purification technique effectively enriches for functional vector. This is particularly relevant for any potential human clinical gene therapy trials. As indicated in this study, methods to further enrich for functional virus should facilitate better gene transfer rates.

## ACKNOWLEDGMENTS

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