Large-scale Purification of a Lentiviral Vector by Size Exclusion Chromatography or Mustang Q Ion Exchange Capsule

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The use of virus-based vectors for gene transfer has become an important delivery method for both *in vitro* applications and *in vivo* experimental clinical therapies. In small-scale experimental applications, most vectors can easily be concentrated and purified by simple methods (for example, ultracentrifugation.) However, it is challenging to scale up centrifugation-based vector purification methods for the large-scale production required for clinical use. In particular, when considering production of vector for human use, additional steps such as final sterilization by filtration must be taken to ensure the purity and safety of the vector preparation. Because the vector aggregates when pelleted by centrifugation, sterile filtration will eliminate vector particles from the solution. An efficient vector purification process that maintains vector potency is an important step in vector production for gene therapy.

Several chromatographic methods have been published and patented for the purification of viruses and vectors. These methods primarily encompass the use of anion exchange resin alone (USP 6,261,823, and USP 5,661,023), affinity resins alone (USP 6,242,239), or a sequential combination of anion exchange followed by affinity chromatography (USP 5,837,520) or ultrafiltration followed by anion exchange (USP 6,485,958, and USP 5,661,022).[1,2]

Typically there is a precipitation or ultrafiltration step to concentrate virus and reduce volumes of the vector-containing supernatant to amounts that can be further purified on a column. Mustang Q ion exchange capsules have recently become commercially available. These capsules purify using a filter instead of a resin, which allows a larger volume of vector to be purified at a higher flow rate, thus obviating the need for initial concentration of material. The high flow rate results from a novel design utilizing functional quaternary amines, which facilitates binding through large pores that allow continual fluid movement, in contrast to the non-continuous flow rate of other ion exchange columns. Therefore, this technology is particularly useful for the purification of large biomolecules such as viruses and vectors, and is highly scalable. In an abstract presented at the 6th annual American Society of Gene Therapy (ASGT) meeting (June 4–8, 2003, Washington, D.C.), Marino *et al.* presented data using Mustang Q Acrodiscs for small-scale purification of lentiviral vectors with and without prior precipitation with polyethylene glycol (PEG).[3] This group recognized the value of Mustang Q to purify vector particles. However, they were unable to achieve good recovery of vector without precipitation prior to application onto Acrodiscs.

Efficient vector purification methods that yield pure product while preserving high titers are valuable. The vector purification process is the first step in clinical therapies that uses gene transfer, and is directly linked to safety and efficacy in terms of virus purity and titer. The quality control procedures performed at each step of our current clinical vector purification process is presented in detail in a recent report published in the *BioProcessing Journal*.[4] Presented in this report is a detailed

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description of our current vector purification process and corresponding vector purity and yield compared to a new, more scalable method for vector purification, currently in development, using Mustang Q ion exchange capsules, which obviates the need for a preliminary precipitation or concentration step.

Materials and Methods

Vector Construct and Production

The clinical vector, VRX496, and a GFP-expressing analogue to VRX496, VRX494, were made by calcium phosphate cotransfection of 293 cells with the vector construct and a single helper plasmid, VIRPAC, in a 10-layer Nunc® cell factory system (VWR, Westchester, PA). Vector was collected two or three times, at 24, 48, and/or 72 hours post-transfection. VIRPAC and VRX496 are designed with several safety features that prevent generation of a replication-competent lentivirus (RCL). VRX496 is a VSV-G pseudotyped HIV-1 based vector containing the cppt/CTS sequences and an LTR-driven antisense payload of 937 bases directed against the envelope region of HIV. This vector system is outlined in detail in a manuscript recently submitted for publication by Lu *et al.*

Vector Purification (Current Method, Using Size Exclusion)

Clarification

The bulk harvest was clarified by passage through a filter train, using Sartorius 2ft^2 capsule filters of decreasing pore size. Bulk harvest was a pool of two or three harvests totaling approximately 36–52 liters of vector-containing media. Filters were pre-wetted with cell culture medium before filtration.

Concentration and Diafiltration

Vector eluate was concentrated approximately 30- to 40-fold by ultrafiltration to about 1–2 L by passage through a hollow fiber cartridge. Buffer was then replaced by diafiltration with Benzonase buffer, pH 8.0.

Benzonase Treatment

To destroy residual nucleic acid, vector was treated with Benzonase at a concentration of 500 units per ml of material, for 30 minutes at 37° C.

Purification

Benzonase-treated vector material was purified by size exclusion chromatography using a Sephacryl S-500 packed column. The ratio of vector load volume to column volume was

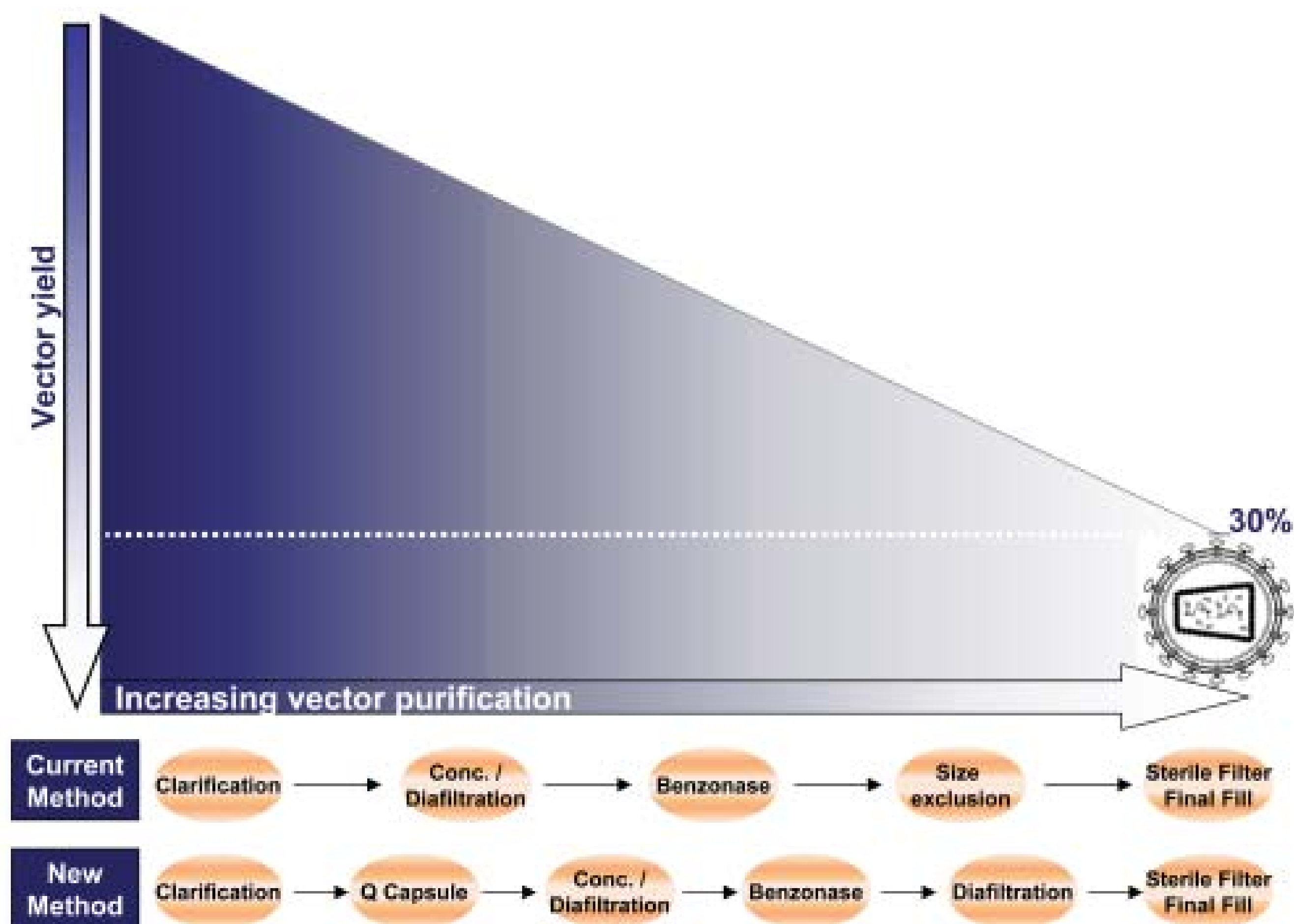

Figure 1. Overview of vector purification. Represented schematically on the y-axis is the relative level of vector recovery during the purification process, for an overall final recovery of approximately 30% of the starting yield. Each purification step is listed at the bottom for the currently used process and the new method of purification in development using the recently available Mustang Q ion exchange capsule. The vector is a VSV-G pseudotyped HIV-based lentivirus containing a cppt/CTS sequence and a 937-base antisense sequence against HIV env under the control of the LTR.

approximately 0.15. The column was equilibrated with vector storage buffer. Vector solution was loaded on the column, which was monitored for peak elution by UV spectrophotometry at 254 nm. As the peak elution begins, the vector was collected until the peak ended.

Sterile Filtration

A 0.22-µm capsule filter was pre-wetted with vector storage buffer. Vector was filtered at a rate of 30 ml per minute, and the filter was then washed/chased with about 50 ml of vector storage buffer. Vector was aliquoted into 50-ml plastic bags and stored at –80° C.

Vector Purification (New Method, Using Mustang Q Ion Exchange)

The new, scalable vector purification scheme is similar to the original described above, except that the size exclusion chromatography is replaced with purification by ion exchange chromatography at an earlier step of the purification process using the recently available Mustang Q capsule. Bulk harvest was collected and clarified as described above. A 60-ml Mustang Q capsule (Pall Trincor, Exton, PA) was used to capture vector from the harvest filtrate. The capsule was equilibrated with one liter of 0.15 M NaCl in buffer, pH 7.5. Harvest filtrate was loaded at 200 ml/min. Washing, elution, and stripping were performed at 150 ml/min. After loading, the capsule was washed with 0.4 M NaCl in buffer until the absorbance returned to baseline. Elution was performed using 1.5 M NaCl in buffer and eluate was diluted in buffer to bring the NaCl concentration to approximately 150 mM.

Eluted vector was concentrated by ultrafiltration, then the buffer was replaced with Benzonase buffer, pH 8.0, by diafiltration as described above. Vector was treated with Benzonase and, following treatment, the vector was diafiltered again as described above, then sterile filtered.

Titer Assay

HeLa-tat Titer

To determine the titer of viral vectors, HeLa-tat cells were transduced

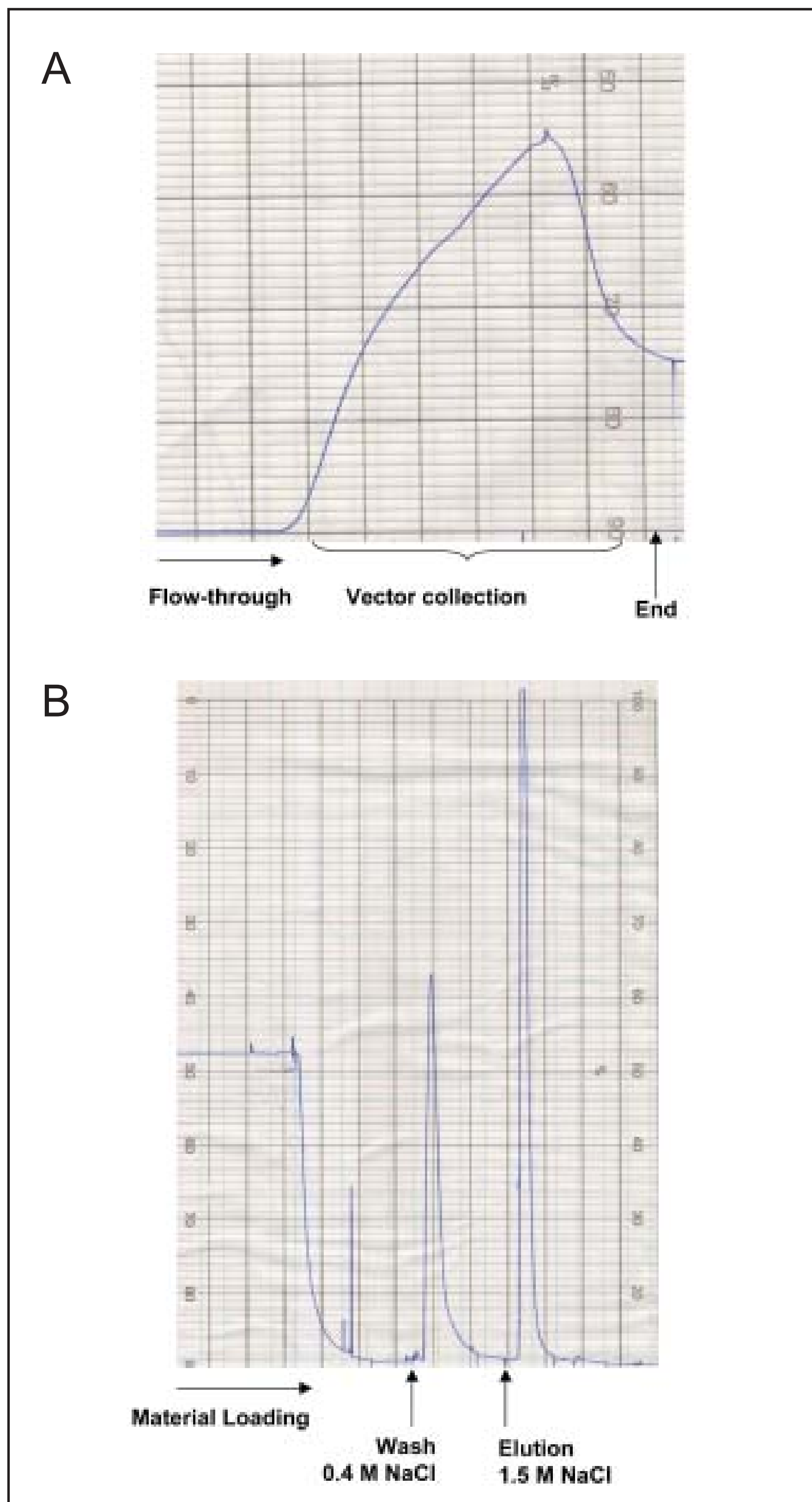

Figure 2. Chromatographic profile of virus purification by size exclusion chromatography or ion exchange chromatography. Vector is purified using either a Sephacryl-500 packed size exclusion column, or a Mustang Q ion exchange capsule. (A) Vector collection starts at the onset of the first peak during size exclusion, and collection ends when the peak ends. (B) Vector is loaded for capture by ion exchange; the column is washed, then vector is eluted with sodium chloride as indicated.

with serially diluted vector-containing supernatants or purified vector in the presence of 4 μg/ml of polybrene in DMEM containing 5% FBS overnight. The next day, medium was replaced with DMEM containing 10% FBS. Forty-eight to 72 hours after transduction, the transduced cells were analyzed for GFP expression by flow cytometry or by TaqMan PCR for vector copy number per cell depending upon whether a GFP expressing vector was used. This data was used to back calculate the titer.

T Cell Titer

The titer assay was performed as described in Schonely *et al.*[4] Briefly, peripheral blood mononuclear cells (PBMCs) from normal donors were isolated and $CD4^+$ T lymphocytes were purified by positive selection. Purity was determined to be more than 95% by flow cytometric staining for CD4/CD3. $CD4^+$ T lymphocytes were cultured in X-Vivo-15 containing gentamycin, 5% human serum, and 1.6 mg/ml of N-acetyl cysteine. For transduction, T lymphocytes were plated at 1 x 10^6 cells per well in a 24-well plate. Plates precoated with Retronectin™ (Takara Bio Inc., Japan) were loaded with vector at 5%, 10%, or 20% v/v of culture medium 30 minutes prior to addition of cells. Cells were simultaneously stimulated with immobilized CD3/28 (iCD3/28) beads and IL2 (Chiron, Emeryville, CA), and cultures were carried in triplicate for three days before vector was removed. Stimulatory beads were removed at day seven. TaqMan PCR was performed on an ABI Prism 7900HT sequence detector (Applied Biosystems, Foster City, CA). DNA was isolated from 1 x 10^5 cells in duplicate using a Dneasy isolation kit (Qiagen, Valencia, CA). Forward and reverse primers specific to a 186-base tag region derived from the GFP protein (Lu *et al.*, manuscript submitted) were added at a final concentration of 300 mM, and a detection probe was added at a final concentration of 500 nM in the presence of TaqMan Universal Mix (Applied Biosystems). To measure potency of the purified vector, cultures were infected at a multiplicity of infection (MOI) of 0.001 with the NL4-3 strain of HIV (Advanced BioScience Laboratories, Inc., Kensington, MD). Non-transduced cells and cells transduced at 20% with a positive control vector were used for challenge controls. Cultures were followed for 15 days, and passaged twice, at which time supernatants were taken to assess virion production by ELISA.

Results

Vector Purification

After clarification, vector was puri-

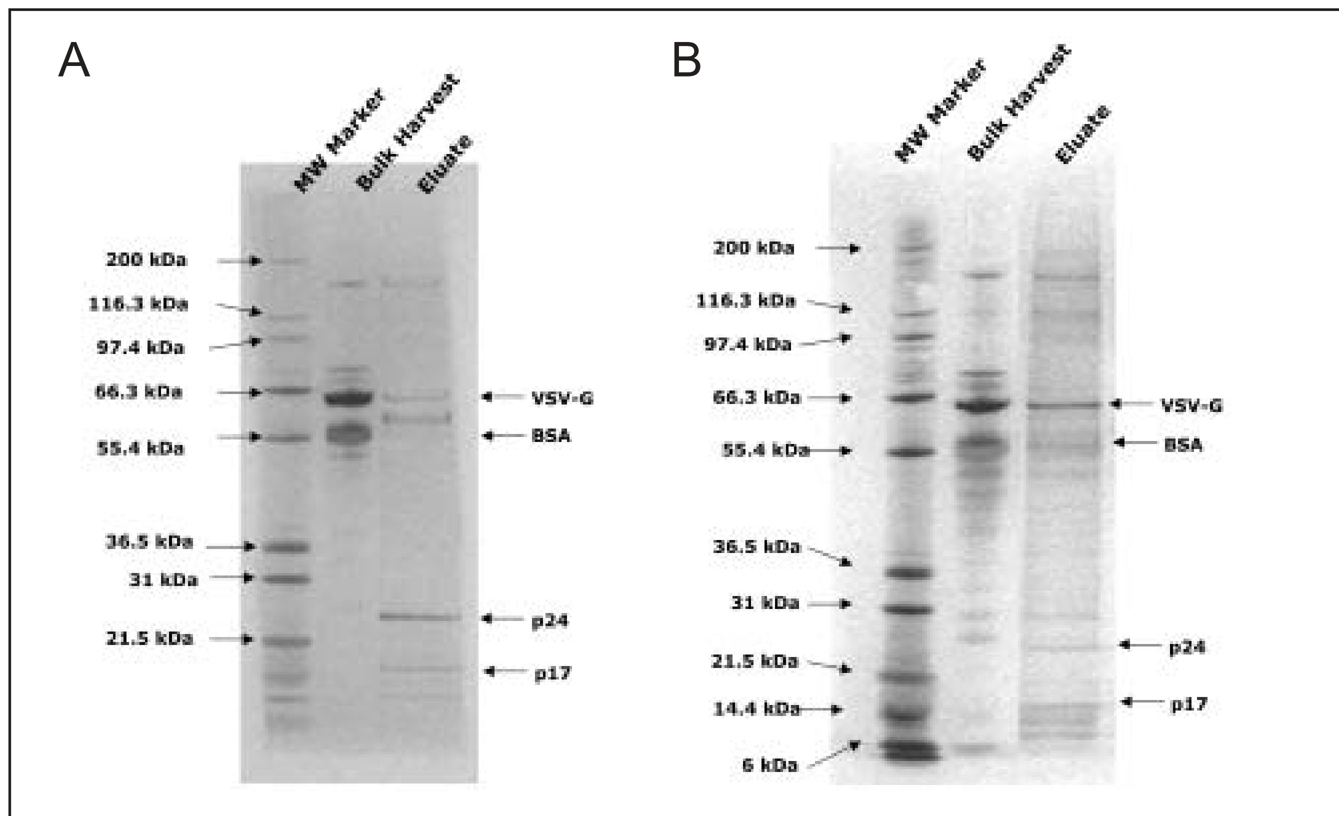

Figure 3. Electrophoretic profile of vector product. Vector was analyzed on a NuPAGE 4-12% 1 mm gradient gel, before (bulk harvest) and after (eluate) purification. Proteins were visualized by silver stain. The size of the molecular weight marker, Mark 12, is present in the left lane of each gel. The VSV-G envelope protein, p24 coat protein, and p17 matrix protein (the most abundant proteins in the vector) are indicated. BSA is also noted around 55 kDa representing a non-vector protein for evaluation of selective purification of vector particles. (A) Size exclusion: Vector purified using the current method for clinical grade vector. (B) Ion exchange: Vector purified using the new method currently in development.

fied by a series of concentration and purification steps using either the current process used to produce clinical grade vector that uses size exclusion chromatography, or a new process in development that uses Mustang Q ion exchange capsules (Fig. 1). In the current clinical process, clarified bulk was concentrated approximately 30 to 40-fold by ultrafiltration. Following treatment with Benzonase, vector was purified by size exclusion chromatography (Fig. 2A), sterile filtered, and formulated in the final fill.

Using the new method, clarified bulk harvest was purified by ion exchange chromatography using a Mustang Q capsule. Figure 2B shows the chromatographic profile of a large-scale purification run on a 60-ml Mustang Q capsule. Eluted vector was concentrated and diafiltration replaced the column elution buffer with the buffer for Benzonase treatment. Diafiltration stopped Benzonase activity by removing the enzyme and replacing the buffer with vector storage buffer without changing the volume.

Table 1. Large-scale lentivirus vector purification: comparison of purity and titer between the current purification method for clinical lentiviral vector (size exclusion) and a new, more scalable method (Mustang Q ion exchange).

	Size Exclusion		Mustang Q	
	start	finish	start	finish
Titer (TU/ml)	3.16×10^7	3.29×10^8	1.09×10^7	2.17×10^8
Total protein (µg/ml)	4,684	31.2	2,202[1]	169
p24 (µg/ml)	0.35	2.36	0.18	2.09
p24/total protein	7.47×10^{-5}	7.56×10^{-2}	8.17×10^{-5}	1.23×10^{-2}

[1] The lower initial concentration of protein here is due to the use of 5% serum (FBS) instead of 10% in the regular process.

Vector Purity

An SDS-PAGE examining the protein electrophoretic profile after size exclusion was performed to evaluate the purity of the preparation (Fig. 3A). The gel shows the dominant bands of the VSV-G envelope protein, the p24 coat protein, and the p17 matrix protein. Little background protein contamination is observed, including residual bovine serum albumin (BSA) at approximately 55 kDa. This is not of concern because the vector product is used to transduce cells *ex vivo*. Transduced cellular product is tested for BSA by ELISA prior to patient dosing. Levels must be ≤ 1 µg/ml in the supernatant of cellular product as reported in Schonely *et al.*[4]

To examine the purification process before and after Mustang Q ion exchange purification, bulk harvest and eluted vector were run in parallel (Fig. 3B). The last column of the SDS-PAGE shows the eluted vector product, which has fewer bands of contaminating protein, as demonstrated in particular by the reduction of BSA. This gel demonstrates the specific purification of VSV-G and p24 containing particles.

A Benzonase ELISA kit II (EMD Chemicals Inc., Gibbstown, NJ), was used to ensure the enzyme was removed by diafiltration. No Benzonase was detected above the limit of assay sensitivity. Purity was also measured by the increase in the p24/total protein ratio pre- and post-purification (Table 1). This ratio increased by more than three logs (1000-fold) after purification by either method, indicating the selective purification of p24-containing particles. In this particular experiment, size exclusion purification removed approximately 2.5 times more total protein than the ion exchange method, which was negligible in terms of the purity of the final vector product.

Vector Yield

Relative percent recoveries of vector after the steps of either purification process were examined by titration and p24 values (Fig. 4 A and B). Percent recovery was normalized between the two methods after clarification (i.e. Figure 4 graphs begin with "filtration" and not "bulk harvest") since this initial step is the same for both purification schemes. Both methods resulted in a final physical particle (p24) and infectious particle (as measured by HeLa-tat titer) recovery of approximately 30% of the starting material, which is considered in the field to be a very good final recovery percentage. Small differences in recovery as measured by titration may reflect variation in the detection assay.

Most of the vector purified using the current clinical purification process (size exclusion) was lost during the concentration and diafiltration step (30%) and nearly the same amount was lost during the column purification (approximately 20% and 30% according to titration and p24 respectively). In contrast, approximately 35% of vector was lost during Mustang Q ion exchange chromatography with incremental loss at each subsequent step.

Transduction Efficiency and Potency of Purified Vector

The HeLa-tat titers of vector purified by size exclusion using Mustang Q were similar at 3.29×10^8 TU/ml and 2.17×10^8 TU/ml, respectively. Purified vector by size exclusion was also titered on primary $CD4^+$ T lymphocytes on which a titer of 2.19×10^8 TU/ml was measured. Copy numbers achieved per cell were 13.9, 22.7, and 30.1 for cultures transduced at 5%, 10%, or 20% v/v of vector.

Purified vector titer, as measured on lymphocytes, was 2.19×10^8. Lower target copy numbers can be achieved in clinical cell products by reducing the amount of vector added to the culture, as suggested by the dose-dependent level of copy numbers per cell. High copy numbers per cell are not allowed in the final cellular product for patient dosing. Cellular product is monitored for copy number per cell prior to dosing as described in Schonely *et al.* (the specification is 0.5–5 copies per cell).[4]

To test the anti-HIV potency of VRX496, which expresses antisense to the HIV envelope protein, we challenged triplicate cultures of primary $CD4^+$ T lymphocytes that had been transduced at 20% v/v with HIV at an MOI of 0.001 and subsequently monitored those cells for production of HIV virions by testing supernatants for p24 every 3–4 days for two weeks. A 3-log suppression over mock-transduced cultures lasted throughout the culture period (Fig. 5). These results demonstrate that the clinical-grade vector purified by size exclusion produces a vector of high purity and potency.

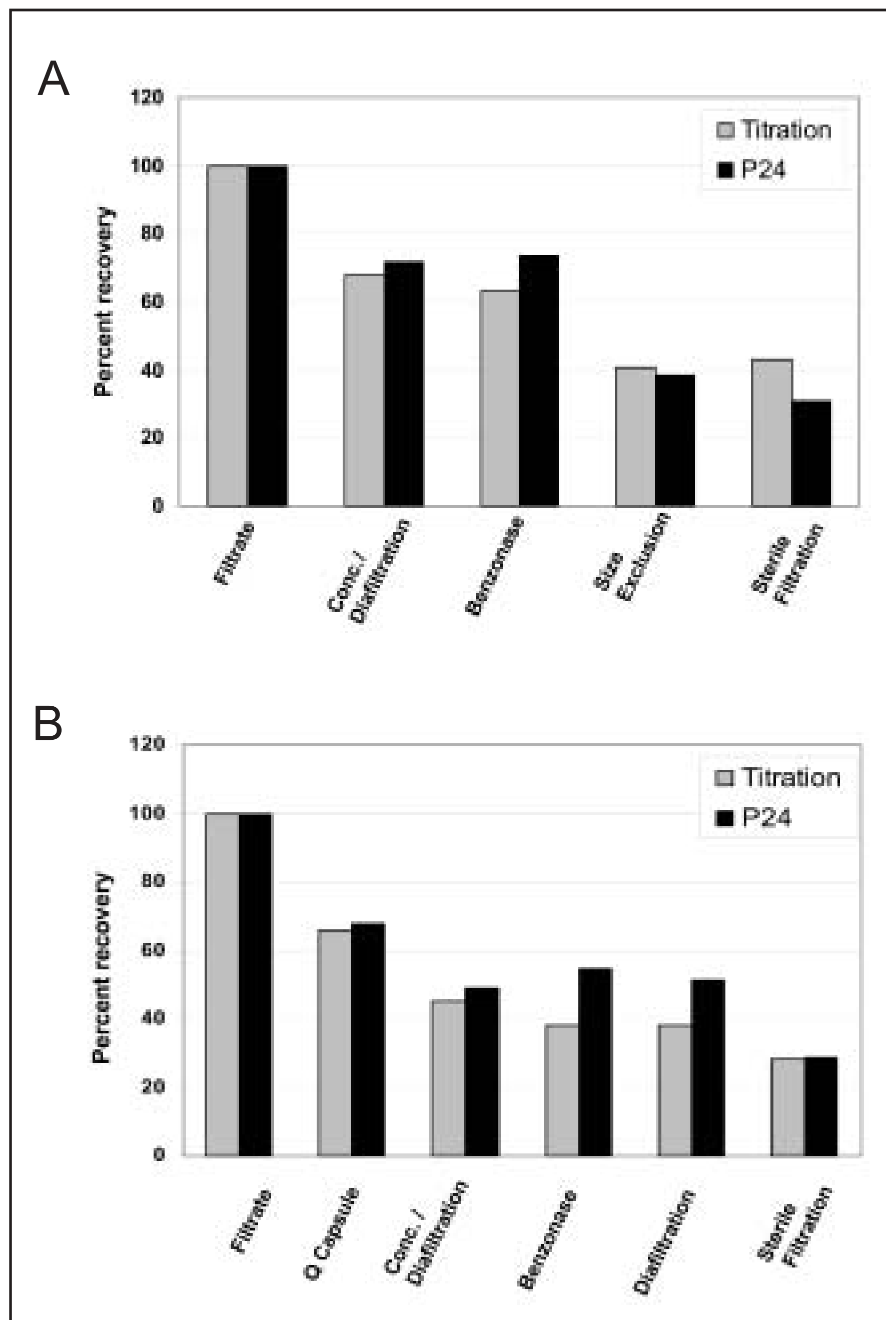

Figure 4. Overall levels of vector yield for each purification method were measured by virion production and titration. After each purification step, vector recovery was measured by virion production by ELISA for p24, and vector titer was measured by titration in HeLa-tat cells. (A) Sephacryl size exclusion chromatography, the current clinical purification process, and (B) Mustang Q capsule ion exchange chromatography.

Final fill from the ion exchange method of purification was also titered on primary $CD4^+$ T lymphocytes on which a titer of 3.7×10^7 TU/ml was measured. This demonstrates the ability of vector purified by Mustang Q to transduce primary cells. However, at this stage of development, titers in primary cells are lower than those of vector purified by our current method using size exclusion, even though titers in HeLa-tat cells are very similar. Further titer and potency experiments are underway.

Discussion

This report presents two methods for large-scale lentiviral vector purification. The first method uses an initial concentration step followed by purification by size exclusion chromatography. This is the method that was used to purify the vector for the first lentivirus vector clinical trial currently ongoing.[5] The second method is more scalable and utilizes a new ion exchange filter technology, called Mustang Q capsules, which represents a new developmental direction for large-scale vector purification. Because the concentration step for the size exclusion is not easily scalable without requiring a change in the media used in vector production (as described below) the Mustang Q capsules are a good alternative approach to purification as the industry looks toward the future of large-scale lentiviral vector manufacture.

A common approach to retroviral vector purification is a series of steps usually involving clarification, concentration and diafiltration, ion exchange, diafiltration, and a final sterile filtration step (USP 5,661,022). However, a greater yield of VRX496 vector was

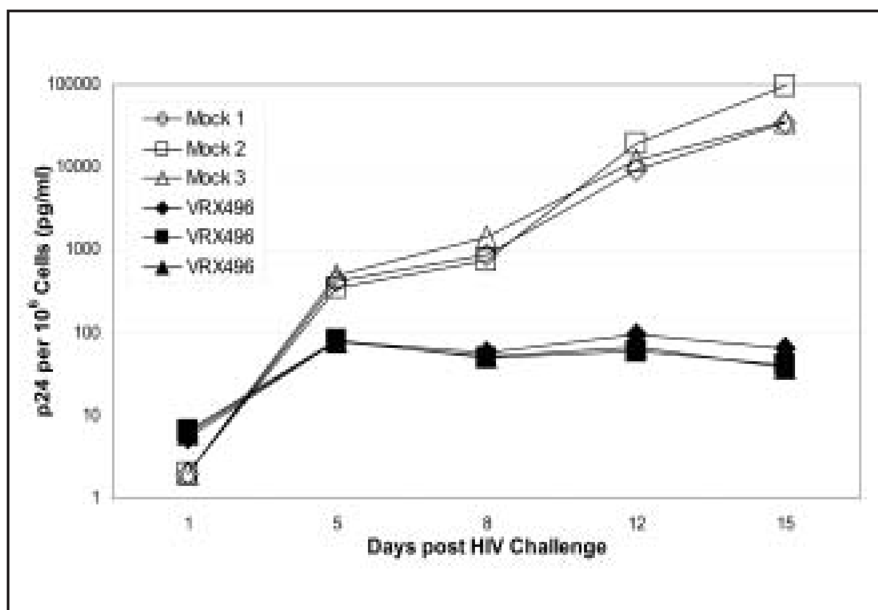

Figure 5. Determination of the potency of clinical-grade vector purified by size exclusion chromatography. Triplicate cultures of primary $CD4^+$ T lymphocytes were either transduced with clinical vector (closed symbols), or mock transduced (open symbols), and then were challenged at an MOI of 0.001 with HIV_{NL4-3}. HIV replication was measured at each cell passage by p24 ELISA.

originally obtained when size exclusion was used in place of ion exchange chromatography, and therefore we chose this method for VRX496 purification. VRX496 is a VSV-G pseudotyped vector, and the sticky nature of the VSV-G envelope protein makes it difficult to elute the particles without using a buffer with a high salt molarity. Furthermore, size exclusion chromatography is simpler to perform at a small company because it requires no special equipment and is good for small lot volumes like those required for a Phase I clinical trial.

The size exclusion-based purification of clinical-grade vector presented in this paper is estimated to be scalable to approximately 100 L of bulk harvest, given the current facility size. Size exclusion requires a high concentration of vector preparation to effectively produce purified vector at useable titers. This initial concentration is the limiting step in the scalability of this process, primarily because the polarized layer of the ultrafiltration cartridge becomes saturated with protein and limits the efficacy of concentration of large volumes of vector. Therefore, one way to increase the scalability of the ultrafiltration step is to reduce or remove the serum during vector production to reduce proteins not associated with vector, thus increasing the amount of bulk harvest vector that can be concentrated.

An alternative, highly scalable, method of purification is the use of Mustang Q ion exchange filter capsules. These capsules represent a significant advance in the field because they abrogate the need for a preliminary concentration step due to their high flow rate. This will allow culture conditions for high titer vector production that will likely require a high protein content media. The capsules range from 0.35 ml to 900 ml and, therefore, could purify an estimated 1500 L of vector product per day. The concept of using these capsules as a scalable vector purification method was put forth by Marino *et al.*[3] However, this group purified vector on a small scale using a Mustang Q "coin device" which is a flat filter. The work presented here demonstrates the feasibility of using a larger "capsule", which is self-contained and analogous to that used for large-scale purification. This method was found to be comparable to the current process of clinical-grade purification by size exclusion in terms of: 1) purity, as assessed by removal of Benzonase and p24/total protein ratio; 2) yield, as determined by virion production and titration on HeLa-Tat cells; and to some extent, by 3) final vector titers in primary T cells.

T cell titers were lower after purification by Mustang Q ion exchange chromatography when compared with purification by size exclusion. It is possible that ion exchange chromatography may be a harsher method of purification since the high density of charged amine groups result in tight binding of the vector to the matrix. This strong interaction requires a high salt elution step, which may partially damage or strip envelope proteins from the vector. Therefore, subtle differences in vector preparations that are not detectable on highly permissive cell lines, such as HeLa-tat, may become apparent on primary target cells. We are currently investigating whether this phenomenon is occurring during Mustang Q purification.

The data supports the feasibility of using a Mustang Q capsule for ion exchange chromatographic purification during large-scale manufacture of clinical-grade lentivirus vectors. This method results in vector yields and purity that are comparable with the process currently used to purify the first lentivirus vector to be tested in clinical trials.

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