Advances in Lentivirus Purification

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Lentiviral vectors (LVs) have been increasingly used as a tool for gene and cell therapies since they can stably integrate the genome in dividing and nondividing cells. LV production and purification processes have evolved substantially over the last decades. However, the increasing demands for higher quantities with more restrictive purity requirements are stimulating the development of novel materials and strategies to supply the market with LV in a cost-effective manner. A detailed review of each downstream process unit operation is performed, limitations, strengths, and potential outcomes being covered. Currently, the majority of large-scale LV manufacturing processes are still based on adherent cell culture, although it is known that the industry is migrating fast to suspension cultures. Regarding the purification strategy, it consists of batch chromatography and membrane technology. Nevertheless, new solutions are being created to improve the current production schemes and expand its clinical use.

1. Introduction

Lentiviruses are enveloped viruses members of the Retroviridae family that have become increasingly relevant for biopharmaceuticals since they lead to stable integration of the transgene(s) to be expressed in both dividing and nondividing cells. Additionally, their integration patterns seem less risky than γ-retroviral vectors, justifying their widespread usage from functional genomics to cell engineering studies, as well as, recombinant protein production and clinical gene therapy. Over the past years, the number of gene therapy clinical trials based on lentiviral vectors (LVs) has grown to attain 10.1% of the total gene therapy trials in 2019. Their use in gene therapy for several conditions has been reported both ex vivo—involving, usually, the transduction of hematopoietic stem cells or T-cells—and in vivo—with gene delivery to the central nervous system and the retina. A comprehensive review of the clinical applications of LV has been published recently. The approval by the US Food and Drug Administration (FDA), in 2017, of two chimeric antigen receptor (CAR) T-cell therapies for the treatment of B-cells lymphoma and leukemia evidences the advantages of these viral vectors and is proof of its successful use at a clinical level. For other types of cancer, the use of LV for immunization remains a promising application.

The success of these clinical trials requires improved methods for purification of LV both quantitively, increasing process capacity, and qualitatively replacing traditional methods used at small scales that are not economically and/or technically viable for larger scales.

The current production technologies commonly result in low functional titers, ranging from 10⁶ to 10⁸ transducing units per mL (TU mL⁻¹), depending on the production system, LV pseudotype, harvest conditions, or even the titration technique. Additionally, impurities vary with the production system. For example, the serum is present when LVs are produced by adherent cell lines, and the plasmid DNA is used for transient LV production. Therefore, when developing a downstream process (DSP), the concentration of LV is pursued along with increasing LV purity, which must consider the impurities profile of the production broth. Just like any other biological product, the degree of concentration and purification needed is fundamentally connected to what the LVs are intended for. Research/small-scale purposes do not generally require a very pure product. However, when the product is intended for clinical use, the safety of the drug is a concern. The standard depends essentially on the type of administration. For ex vivo applications the purity level of the drug product is more flexible. Additionally, the multiplicity of infection (MOI) must be kept as low as possible to reduce the tumorigenic potential and cytotoxicity. For an in vivo approach doses of, at least, 10¹³–10¹⁵ per patient are needed, which will also vary according to target tissue. In this case, stricter control of the purity to reduce an immunogenic response by the patients is needed. DSP must also consider the low stability of the LV functional particles, which is affected by temperature, pH, ionic strength, shear stress, and other environmental conditions, the stability is highly dependent on the particle...
itself, mainly on the envelope protein (pseudotype) but also on the viral core.[26,35,36] Vesicular stomatitis virus G protein (VSV-G) is the most used LV pseudotype due to its broad tropism and robustness throughout the purification processes. Nevertheless, DSP schemes for other protein envelopes (e.g., baboon endogenous virus (BaEV), RD114-TR, gibbon ape leukemia virus (GaLV)) are emerging since they present higher tropism for specific target tissues per cells.[26,37,38]

The present article aims to comprehensively review the latest technical developments in the purification of LV and trace possible future trends in the field. First, the traditional methods are identified and then the more promising and easier scalable technologies are reviewed.

2. Traditional LV Purification and Concentration Methods

The traditional purification and concentration techniques are mostly used if the needs of LV can be satisfied by small-scale production, for instance, scientific research. The centrifugation-based methods can be used as concentration and/or purification techniques. Viral pelleting and resuspension procedure is often used as a concentration method.[39–41] The use of density gradient centrifugation or ultracentrifugation using sucrose[42,43] or iodixanol[44] has been reported and can achieve some degree of purification along with reasonable concentration.[41,45] Another technique for small-scale purification schemes uses polyethylene glycol (PEG). This polymer sterically excludes viral particles from the solution leading to their precipitation. Viral particles are recovered by centrifugation and high yields can be achieved.[43,45]

Flaws of traditional methods include concerns with scalability and the concentration of impurities in the product.[22] Although a high degree of purification can be obtained, the use of ultracentrifugation reduces the infectiousness given the stress conditions to which LVs are subject. Thus, the field is gradually moving away to more versatile technologies.

3. Scalable Methods for LV Downstream Processing

The development and use of DSP with chromatography steps and membrane-based separation operations have been described over the past years.[46] The processes can be divided into three main phases: i) harvest and clarification; ii) intermediate purification, with one or more concentration and purification steps; and iii) polishing, formulation, and sterilization. The nucleic acid digestion is usually required and can take place in different phases of the process (see Figure 1). Currently, overall recovery yields for large scale DSP range from 20% to 40%.[22]

3.1. Harvest and Clarification

LVs are extracellular bioproducts released by budding from the producer cell. The majority of large-scale LV production processes use transient transfection of human embryonic kidney (HEK) 293 or 293T adherent cells in batch mode and the harvest occurs generally once or twice at 48–72 h post-transfection. Although, more recently, the industry has been also transitioning into suspension cultures.[47–53] The harvest quality is highly dependent on cell viability which impacts the amount of host cell (HC) derived impurities. Thus, the choice of the time of harvest must consider not only the productivity but also the quality of the supernatant.[46,54]

The clarification’s main goal is to remove cells and cell debris from the LV containing media. This step generally comprises depth filtration,[21,33] normal flow microfiltration,[26,55] low-speed centrifugation, or a combination of these operations. The use of centrifugation followed by microfiltration[36,41,43] is a common strategy to reduce filter clogging and minimize fouling at small volumes. With similar goals, but for larger scales, a membrane cascade is commonly used,[26,49,55] the final pore size being 0.45 µm.[44]

The recoveries for these early steps are commonly not reported, although, after optimization, small losses in functional titer can be achieved.[21,26,33] Valkama et al. recently reported functional recoveries close to 100% using a depth filter for clarification of large supernatant volumes containing VSV-G pseudotyped LV.[21] However, the success of this operation can be highly dependent on the pseudotype. Using a GaLV-TR pseudotyped LV,
Boudeffa et al. obtained very low recoveries in the clarification step later improved by supernatant dilution and stabilization.[26]

Nonetheless, to meet the LV's increasing demand, recent developments in upstream processing (USP) are emerging and the integration of the early steps of DSP in USP can be highly advantageous.[48,49,56] Recently, Oxford Biomedica reported the use of Tangential Flow Depth Filtration (TFDF) to harvest and clarify LV produced in suspension culture[57] resulting in high recoveries and potentiating multiple harvests. Moreover, for suspension cell cultures operating under perfusion, retention devices, like the Virus Harvest Unit (VHU), allow the harvest of clarified viral stocks.[48,58,59] Regarding adherent cell cultures, several bioreactor configurations can be used for continuous LV production.[56-63] Tangential flow microfiltration[64,65] and continuous flow disk-stack centrifugation[66] are also continuous techniques, although never reported for LV clarification.

### 3.2. Nucleic Acid Cleavage

To increase the overall biosafety of the final product[20] and comply with the regulatory standards[60] a nucleic acid cleavage step must occur. The use of Benzonase or Denarase reduces DNA size and decreases bulk viscosity.[41,46] These enzymes require magnesium ions as a co-factor and show optimum activity at 37 °C; however, different experimental conditions (e.g., amount of enzyme, temperature, incubation time, salt concentration, pH, and the presence of enzyme inhibitors) are reported regarding nucleic acid cleavage step. Recently, Oxford Biomedica developed a system (SecNuc) that avoids the addition of nucleases that are counterweighted by easier subsequent DNA clearance and also nuclease removal which must be accomplished if LVs are intended for clinical use.[4]

Many times, nuclease step immediately precedes[21,68] or succeeds clarification.[24,69] In other processes, it is included in the USP[26,55] or later DSP phases after some volume reduction.[70-72] The early location of this step results in higher nuclease consumption increasing the manufacturing costs which can be counterweighted by easier subsequent DNA clearance and also nuclease removal which must be accomplished if LVs are intended for clinical use.[4]

### 3.3. Intermediate Purification

Following clarification, scalable purification steps for LV use chromatography and/or Tangential Flow Filtration (TFF), both techniques achieving some degree of concentration of the bulk product.

Chromatography is a key technology in purification protocols for the main biotherapeutic products offering scalability, purity, high recovery rates, and reproducibility.[73] The classic resin media were originally designed for protein purification; thus, the large hydrodynamic radius of the LV raised some technical constraints. The use of beads often restricts access to the binding sites, resulting in small dynamic binding capacities.[74,75] Additionally, the high column backpressure also leads to limited flow rates. The mass transfer in this kind of stationary phase is predominantly limited by diffusion, whereas in membrane adsorbers and monoliths it is mostly convective.[73,76] This allows a quicker processing and larger throughput, making this supports ideal for virus capture. Anion-exchange and affinity chromatography have been used in the early purification steps of LV (Table 1).

#### 3.3.1. Anion-Exchange Chromatography

Anion-exchange chromatography (AEX) is widely used for capture in large-scale bioprocesses. Briefly, the purification of these viruses is performed in a bind-elute mode where the LV particles, having a negative surface charge at working pH, are adsorbed by the positively charged matrix.[41,34] The elution is performed with buffer ionic strengths between 0.5–1 m NaCl.[4,56] As LVs are labile, high salt concentration can lead to virus inactivation[1,77] A loss of 50% of LV biological activity after 1 h of exposure to 1 m NaCl has been reported.[78] To reduce losses during the AEX step it is often necessary to dilute the viral vector right after the elution step.[79]

Membranes and monoliths stationary phases, as with conventional chromatography resins, can be functionalized with strong or weak ion ligands.[41,80] Strong ion exchangers with quaternary amines as ligands have been used to produce high-quality virus stocks,[81] in particular with Sartobind Q and Mustang Q commercial membranes.[21,26] Conversely, weak exchangers, for example, CIM Monolith with DEAE (diethylaminoethanol) ligand, have also been reported in LV purification with high recovery yields.[13] In general, this technique results in functional particle recovery yields ranging from 30% to 80% (Table 1).

Since binding is driven by electrostatic interactions, AEX matrices are not selective for LV, and negatively charged nucleic acids and proteins bind to the chromatographic media.[54,77] The high salt concentration required to elute LV leads also to the release of bound impurities. Merten et al. achieved around 99% and 86% of protein and DNA removals.[24] However, additional purification steps are usually required to obtain clinical-grade virus stocks.[81]

Novel stationary phases are continually being developed. Ruscic et al. reported LV purification using regenerated cellulose nanofibers derivatized with a quaternary amine and produced by electrospinning. The authors claimed to achieve a volumetric concentration factor of 100, with a recovery yield of 85%.[86]

#### 3.3.2. Affinity Chromatography

The affinity chromatography (AC) is used to separate particles from the broth-based on a highly specific interaction between them and a selected immobilized ligand.[8,82] Unlike AEX, AC presents a high selectivity, which potentially leads to higher purity and concentration outcomes. Therefore, its incorporation in a DSP can lead to a reduction in the number of unit operations required and consequently greater overall recoveries.[4]

One of the first reported studies used the immobilized metal affinity chromatography (IMAC). This technique explores the binding between an immobilized metal ion and a molecule capable of sharing electrons with it. For LV purification this would involve the modification of the envelope protein with the introduction of a histidine tag. Such insertion can result in lower expression or compromise the protein’s function, with loss in particle infectivity.[83–85] Furthermore, the desorption step...
Table 1. Anion exchange and affinity chromatography methods used for the purification of LV.

| Column/matrix | PT      | Load | BV   | Des. reagent | Buffer            | pH   | Rec. (%) | Ref. |
|---------------|---------|------|------|--------------|-------------------|------|----------|------|
| **Anion Exchange** |         |      |      |              |                   |      |          |      |
| Resin         |         |      |      |              |                   |      |          |      |
| HiTrap Q HP   | VSV-G   | 1 mL | 1 mL | LG: up to 1 m NaCl | 100 × 10⁻³ m Tris | 7.5  | 89.6     | [41] |
| DEAE Sepharose FF | –       | 30 mL | 16 mL | SW: 0.65 to 1 m NaCl | –     | –        | 95   | [116] |
| DEAE resin    | VSV-G   | 48 L | –    | 750 × 10⁻³ m NaCl  | PBS   | –        | –    | [24]  |
| Poros DM/50   | GaLV-TR | 45/29 mL | 8 mL | SW: elution at 0.7 m NaCl | 20 × 10⁻³ m Bis–Tris + 5% sucrose + 2 × 10⁻³ mM MgCl₂ | 6.0  | 61.5     | [26] |
| Poros D 50    | GaLV-TR | 20 mL | 4 mL | 0.65 m NaCl | 20 × 10⁻³ m Bis–Tris | 5.5  | 100      | [93] |
| **Membrane**  |         |      |      |              |                   |      |          |      |
| Sartobind Q   | VSV-G   | 11 kg | 400 mL | SW: 0.3–1.50 m NaCl | 50 × 10⁻³ m HEPES | 7.5  | 22.4     | [21] |
| Mustang Q XT5 | BaEV-R-less | 1 L | 5 mL | 2 m NaCl | 50 × 10⁻³ m Tris | 8.0  | >33     | [49] |
| Mustang Q XT5 | VSV-G   | 1 L | 5 mL | 2 m NaCl | 50 × 10⁻³ m Tris | 8.0  | >55     | [49] |
| Nanofiber (RC w/Q) | RDPro | 400 mL | 0.1 mL | LG: elution at 0.6–0.9 m NaCl | –     | –        | –    | [36] |
| Mustang Q     | VSV-G   | 6 L | 60 mL | 1.2 m NaCl | 25 × 10⁻³ m Tris–HCl | 8    | –        | [55] |
| Mustang Q XT5 | VSV-G   | 1.5 L | 5 mL | 1.5 m NaCl | PBS | 7.2      | –    | [39]  |
| Mustang Q Acrdiscs | MVG | 540 mL | 0.8 mL | LG: elution at 0.2–0.4 m NaCl | 25 × 10⁻³ m Tris–HCl | 7.4  | 64.36    | [117] |
| Mustang Q XT  | –       | 3 L | –    | SW: 0.5 and 1.2 m NaCl | 20 × 10⁻³ m Tris + 2 × 10⁻³ mM MgCl₂ | 7.6  | 50–60    | [68] |
| Sartobind Q75 | VSV-G   | –   | 75 mL | SW: elution at 1.2 m NaCl | DMEM (or TSSM) | –    | –        | [118] |
| Mustang Q capsule | VSV-G | – | – | 1.5 m NaCl | 50 × 10⁻³ m Tris–HCl | 8    | –        | [96] |
| Microcapillary film (EVOH w/Q) | VSV-G | 15 mL | 1.5 mL | 1.3 m NaCl | 25 × 10⁻³ m Tris–HCl | 8.0  | 11/10    | [119] |
| LentiSELECT 40/500/1000 | VSV-G | 40 mL | – | – | – | – | 52 | [79] |
| LentiSELECT 40/500/1000 | VSV-G | 500 mL | – | – | – | – | 38 | [79] |
| LentiSELECT 40/500/1000 | VSV-G | 1000 mL | – | – | – | – | 37 | [79] |
| Mustang Q     | VSV-G   | 36–52 L | 60 mL | 1.5 m NaCl | – | 7.5 | >60 | [72] |
| **Monolith**  |         |      |      |              |                   |      |          |      |
| CIM DEAE      | VSV-G   | –   | –    | SW: elution at 0.65 m NaCl | 10 × 10⁻³ m Tris | 8.0  | 80       | [33] |
| **Affinity**  |         |      |      |              |                   |      |          |      |
| CIMac         | RDPro   | 50 mL | 0.1 mL | 15 × 10⁻³ m biotin + BSA | X-VIVO 15 | –     | 20      | [85] |
| Pierce monomeric avidin coated column | VSV-G | 12 mL | 2 mL | 2 × 10⁻³ m biotin | PBS | –       | 68   | [86] |
| Fractogel EMD Heparin | VSV-G | 35 mL | 1 mL | 0.35 m NaCl | 20 × 10⁻³ m Tris–HCl | 7.5  | 53       | [47] |

PT, Pseudotyping; BV, Bed volume; Des. reagent, Desorbing reagent; Rec., Recovery of functional particles; SW, Stepwise elution; LG, Linear gradient elution. Calculated by the authors based on the article’s data. 

a) Recovery of more than one unit operation, including chromatography. 

b) Capture of clarified LV/capture of unclarified LV.

d) Fractogel EMD Heparin had been discontinued.

requires the use of imidazole resulting in LV inactivation. Finally, another major drawback of IMAC for LV purification is the possible adverse effects promoted by His-tag in clinical trials, thus ensuring its current rejection. 

The use of heparin AC in LV purification enables an elution under mild conditions. However, its low selectivity can result in a binding competition of host cell proteins and DNA with LV and consequently co-elution. Given the animal origin of heparin, its use will be avoided in industrial applications.

Another strategy involves LV labeling with desthiobiotin or biotin mimics that binds streptavidin columns (or magnetic beads) with low affinity. LV desorption is made with biotin. Chen et al. modified a cell line to generate desthiobiotin-labeled LV further purified in monomeric avidin-coated chromatography columns. More recently, Mekkaoui et al. reported the purification of an RDPro pseudotyped LV labeled with cTag8. This biotin mimic was genetically encoded, and the technique is envelope-protein independent.
Despite the encouraging results at a lab-scale for LV purification using AC, much has to be improved to translate this technique into an industrial setting. Particularly, modifications on the LV envelope may compromise the viral biologic activity, by vector inactivation or tropism alteration.\[21\]

### 3.3.3. Tangential Flow Filtration

Ultrafiltration/diafiltration is the gold standard for virus large-scale concentration and buffer exchange since it is a fast and robust technique, easily scaled-up and, transferred to good manufacturing practices (GMP).\[4\] Briefly, TFF is used to concentrate viral particles in the retentate, whereas small impurities such as proteins and DNA fragments are removed throughout the pores into the permeate.\[46\] Membrane fouling can be a major limitation leading to permeate flow rate decrease and transmembrane pressure (TMP) increase during the concentration process. TFF can be applied at different stages of the downstream process. First, it can be used for concentration of the feed stream, after virus clarification, reducing the feed volume for the later steps, and if diafiltration is performed, exchanging the buffer to an appropriate composition for subsequent operation units.\[27\] Second, it is also commonly employed in the downstream polishing steps for concentration and buffer exchange or formulation\[87\] (see below).

As previously mentioned, one of the major challenges in LV purification is the maintenance of their biological activity. Pressure variations, high shear forces, and foaming can lead to loss of infectivity or even envelope disruption.\[21\] Membrane chemistry and pore size are selected by experimental screening followed by optimization of the critical process parameters such as flow rate, TMP, membrane throughput, and process time to assure a smooth operation. Flat sheet cassettes\[21,88\] or hollow fibers are widely employed for TFF.

Table 2. Tangential-flow filtration for concentration and/or diafiltration of LV.

| Cassette | PT | MWCO | Pressure data | Area | Feed | VCF | Rec. (%) | Ref. |
|----------|----|------|---------------|------|------|-----|----------|-----|
| 5 × Sartocon Hydrosart (Sartorius) | Cellulose | VSV-G | 100 kDa | TMP ≤ 0.1 bar | 3.0 m² | 126.8 kg | 13 | 70–80 | \[21\] |
| 2 × Sartocon Hydrosart + | Cellulose | VSV-G | 100 kDa | TMP ≤ 0.2 bar | 4.2 m² | 178.4 kg | 18 | – | \[21\] |
| 1 × Sartocube Hydrosart (Sartorius) | Cellulose | VSV-G | 100 kDa | TMP ≤ 0.2 bar | 4.2 m² | 178.4 kg | 18 | – | \[21\] |
| Sartocon Slice Hydrosart (Sartorius) | Cellulose | VSV-G | 100 kDa | TMP ≤ 0.2 bar | 0.1 m² | 835 mL\[5] | 1.5 | ≈60 | \[21\] |
| Vivaflow 50 (Sartorius) | – | VSV-G | 100 kDa | BaEV R-less | – | – | – | >55 | \[49\] |

**Hollow fiber**

| Cassette | PT | MWCO | Pressure data | Area | Feed | VCF | Rec. (%) | Ref. |
|----------|----|------|---------------|------|------|-----|----------|-----|
| (Spectrum Labs) | mPES | RDPro | 500 kDa | TMP = 0.075 bar | – | – | – | ≈20 | \[16\] |
| (Spectrum Labs) | – | VSV-G | 500 kDa | TMP = 0.35 bar | 190 cm² | 1.2 L | 8 | – | \[51\] |
| (Spectrum Labs) | mPES | GaIV-TR | 500 kDa | TMP = 0.5 bar | 115 cm² | – | – | 40\[6,8\] | \[28,91\] |
| (GE Healthcare) | PES | – | 750 kDa | – | 110 cm² | 190 | 11.2 | 64–74\[9\] | \[68\] |
| (GE Healthcare) | PES | VSV-G | 750 kDa | – | 110 cm² | 190 | 11.2 | 100\[10\] | \[68\] |
| FlexStand (GE Healthcare) | PS | VSV-G | 100 kDa | TMP = 0.5 bar | – | – | – | – | \[71\] |
| (GE Healthcare) | – | VSV-G | 500 kDa | – | – | – | 100 | \[70\] |
| (GE Healthcare) | – | VSV-G | 500 kDa | – | – | – | 20 | \[69\] |
| (GE Healthcare) | – | VSV-G | 750 kDa | – | – | – | 20 L | \[24\] |
| Tandem TFF\[5] (Spectrum Labs) | – | VSV-G | 500 kDa | $P_{\text{feed}}$ = 1 bar | 615 + 40 cm² | 5.5 L | 2000 | 94–100 | \[120\] |

PT, Pseudotyping; MWCO, Molecular weight cut-off; VCF, Volumetric Concentration Factor, Recovery of functional particles. \[5\] Calculated by the authors based on the article’s data. \[6\] The authors consistently reported a recovery increase when operating at higher feed and retentate pressures, maintaining the differential pressure constant. \[7\] pH of the DF buffer has a considerable impact on vector recovery. \[8\] Before optimization. \[9\] Uses a system with two sequential and integrated UF filters, FP1, and FP2.
As mentioned above, TFF is frequently used to concentrate and formulate LV. A proper virus formulation is crucial to provide maximum stability and guarantee therapeutic success. The LV must remain biologically active after freeze-thaw or lyophilization and during its clinical use. For ex vivo applications, there is some flexibility in the formulation that leads to the use of protein-containing media such as Lonza’s X-VIVO and CellGenix’s CellGro media. Traditionally, the stabilization of LV has been accomplished with the use of different buffers systems—PIPES, HEPES, HIS-HCl, Tris-HCl, or PBS—coupled with stabilizer adjuvants. The more common excipients include sugars (sucrose or trehalose), or proteins such as recombinant human albumin and gelatin.

Finally, sterilization of the drug substance is achieved by filtration through a 0.22 µm filter, before vialing. This is a standard regulatory requirement and a critical step regarding vector losses. Valkama et al. reported losses between 30% and 50% in the last step of their large-scale DSP. The recoveries on this step are dependent on the membrane material and the LV titer. Oxford BioMedica verified that sterilization after the final concentration step resulted in a low LV functional recovery. Therefore, in their DSP protocol, the sterile filtration is placed before the final TFF which is done under aseptic conditions. Sterilization can be skipped if the process is performed aseptically in a semi-closed system. The final product is then stored at −80 °C to protect the virus from thermal inactivation or lyophilized.

4. Analytical Approaches and Regulatory Requirements

From the impure LV containing supernatant to a final product, an increase of the vector titer and a decrease of the total amount of impurities is expected. Those impurities are commonly distinguished between process-related and product-related that were extensively reviewed by Segura et al. Analytical methods play a key role in process development and operation, as well as in the characterization of the produced lots. This characterization is required in GMP manufacturing and to enter clinical trials. However, like other advanced therapeutic medicinal products, there are still no strict guidelines for LV manufacturing. Efforts in standardization have been made resulting in a standard for LV analytics and an LV reference material that will be released soon.
Table 3. Assays for quality assessment of LV preparations.

| Target | Assay(s)                                                                                     | Ref. |
|--------|---------------------------------------------------------------------------------------------|------|
| Identity | Western Blot, vector sequencing, restriction enzyme mapping, reverse phase HPLC, SDS-PAGE | [100]|
| Purity | Total protein, Bradford, BCA protein assay                                                   | [24,68]|
|         | Host cell protein, ELISA                                                                   | [24]  |
|         | Residual endonuclease, ELISA                                                                | [24]  |
|         | Residual BSA, ELISA                                                                        | [24]  |
|         | Total DNA, Fluorimetry (PicoGreen)                                                         | [24]  |
|         | Host cell DNA, qPCR, fluorimetry                                                           | [36,121]|
|         | DNA size distribution, Agarose gel electrophoresis, capillary electrophoresis               | [69,122]|
|         | EIA and SV40 LTA sequences in the vector, qPCR, Southern blot                              | [24]  |
|         | Transfer of plasmid (vsv-g and gag-pol), and host-cell (EIA and SV40 LTA) sequences to target cells | [4,24]|
| Potency | Total viral particles, p24 ELISA, PERT, RT qPCR                                              | [24,69,100]|
|         | Functional viral particles (analysis of transduced cells), Flow cytometry, qPCR, ddPCR      | [24,69,100]|
| Safety  | Recombinant competent lentiviruses (RCL), RCL amplification in suitable cell line by serial passages followed by quantitation assay (e.g., PCR, p24 ELISA) | [4,24]|
|         | Adventitious agents, In vitro testing, PCR-based methods                                    | [69]  |
|         | Mycoplasma, Culture-based methods, PCR-based methods                                        | [4]   |
|         | Endotoxins/pyrogens, LAL test, rabbit pyrogen test                                           | [4]   |
|         | Sterility, Bacterial and fungal sterility                                                    | [4]   |

Ref., reference; BCA, bicinchoninic acid; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; PERT, product-enhanced reverse transcriptase; RT qPCR, quantitative reverse transcription-polymerase chain reaction; ddPCR, droplet digital polymerase chain reaction; LAL, Limulus amebocyte lysate.

must be tested for identity, purity, potency, and safety.1,4 Some examples of assays used for LV release are depicted in Table 3. The identity can be confirmed using Western Blot for gag proteins, vector sequencing, restriction enzyme mapping, reverse phase high performance liquid chromatography (HPLC), or sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).100

Regarding purity, both process-related and product-related impurities should be well characterized in the final product. The quantification of total proteins can be acquired using colorimetric assays, like Bradford or BCA.4,13,24 Other impurities such as HC proteins, nuclease, and bovine serum albumin can be quantified by ELISA.4 The DNA must be present at a concentration below 10 ng per dose and the DNA size under 200 base pairs.100 Their quantification can involve spectrofluorimetry and agarose gel electrophoresis.1,4,24,69 Since HEK293T has a tumorigenic phenotype, the LV produced with these cell lines must be analyzed to quantify E1A and SV40 LTA DNA sequences. If the production process involves transient transfection, the number of copies of plasmid sequences (vsv-g and gag-pol) must also be assessed, using qPCR or Southern Blot.4,24,69,101

A good indication of vector quality is the ratio between total and active particles, correlated with its potency. If this parameter is too high, the lot must be rejected.1,4 No reference value is defined by current guidelines but ratio values in the 10^3 order of magnitude are reported.24,69 The number of total particles is commonly inferred by p24 titration using ELISA kits.4,56,100 Other analytical methods for quantification of total particles may include the assessment of reverse transcriptase activity and determination of genomic RNA.14,102 Physical methods are commercially available and enable a quicker quantification of total particles and characterization of size distribution. Those methods include field-flow fractionation multiple-angle laser light scattering (FFF-MALLS), nanoparticle tracking analysis (NTA), flow cytometry, and tunable resistive pulse sensing (TRPS).56,103,104 The number of active particles is estimated by a gene transfer or gene expression assay in a suitable cell line.4 For research purposes, when using GFP as the transgene, the functional titer is often estimated by flow cytometry of the fluorescent cells.56,102 However, for clinical purposes, it should be quantified by the number of reverse-transcribed viral genomes integrated into target cells using droplet digital PCR (ddPCR) or quantitative PCR (qPCR) via classical TaqMan of genomic DNA100 or by another method suitable for the used transgene.61

Also, each vector batch must be evaluated regarding several safety parameters, mainly analyzing for recombinant competent lentiviruses (RCL), endotoxins, mycoplasma, adventitious agents, and sterility.4,105 Physicochemical parameters like pH, osmolarity, and appearance complement product characterization.4
5. Concluding Remarks

Over the past decade, lentiviral vectors have become a fundamental tool for gene and cell therapy, explaining the increasing number of clinical trials that use these viruses. The approval of Kymriah and Yescarta cell therapeutic products have reinforced the urgent need for simple, fast, and robust manufacturing processes of viral vectors. In recent years, this field has witnessed a tremendous effort to develop and optimize large-scale production and purification processes capable to meet the demanding number of viral doses necessary for clinical use.

In this article, a detailed review of each downstream operation unit was performed, and significant strengths and limitations were highlighted. For chromatography, the use of convective driven devices is effective in LV purification schemes and new stationary phases are emerging with very promising results. Additionally, affinity chromatography using tailor-made ligands might prove valuable due to its high selectivity allowing to decrease the number of purification steps and increasing recovery yields.

LVs purification processes have evolved significantly, however, recovery yields remain far from their maximal potential, and the amount of virus required cannot be efficiently obtained using these with current technologies. The transition to new production systems may overcome these hurdles but can introduce different challenges into the downstream processing of LV. The increase of the production titer will of course benefit the purification train, but the shift for a suspension system will increase the cell mass during the clarification step. The impurity profile will also change and an increase in process-related impurities will be observed (e.g., genomic DNA, HC proteins). Therefore, the DSP has to engage with those challenges to achieve the same high-quality viral stocks.

The biopharma industry is now moving to new operation modes trying to reach fully continuous and integrated bioprocesses. These improvements are very appealing because they allow high purity and productivity, reducing the overall costs. Much has been done to improve these continuous processes, more specifically in continuous chromatography for other viral particles such as adenovirus, influenza, and extracellular vesicles. However, the production of LV using continuous processes may be hampered due to LV cytotoxicity, particularly for VSV-G pseudotyped vectors. Until a robust LV manufacturing process is established, the transition to continuous production and purification will be difficult to apply to a larger scale but research at a lab-scale is already committed to these new processing operation modes.

In this review, some future directions were identified, and the progress made in the manufacturing of LV was described. Some success stories have already been reported and novel applications and technological developments are thriving. The trend is likely to continue, as several groups and companies are committed to investing more funding and time to push Lentiviral vectors into broader clinical use.

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Conflict of Interest

The authors declare no conflict of interest.

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