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Description

PRIOR ART

The lentiviral vectors derived from the human immunodeficiency virus (HIV-1 in particular) are among the most used vectors for gene therapy. These vectors are usually pseudotyped with glycoproteins coming from other viruses: gibbon leukemogenous virus (GALV, GALV-TR glycoproteins), vesicular stomatitis virus (VSV-G), measles virus (MV). Methods for purifying clinical lots of lentiviral vectors pseudotyped with the VSV-G protein have been described (Schweizer and Merten, 2010). However, the viral vectors pseudotyped with other envelope proteins, and more particularly with glycoproteins derived from GALV or MV, are relatively little used because no satisfactory purification protocol is currently available. The major limiting obstacle for the purification of this type of pseudotyped vector is related to the instability and fragility of certain membrane glycoproteins. However, these vectors are particularly interesting with regard to their tropism that is less wide than that of vectors pseudotyped with the VSV-G protein. For example, vectors pseudotyped with a glycoprotein derived from GALV have a more restricted tropism and target hematopoietic stem cells more specifically. The provision of an efficient method for purifying vectors pseudotyped with GALV glycoproteins is therefore a major issue in the field of gene therapy.

The inventors have therefore proposed to develop a method for purifying retroviruses, and in particular viruses pseudotyped by the GALV envelope glycoprotein or other envelope proteins, in order to produce virus preparations for clinical use.

SUMMARY OF THE INVENTION

The present invention results from the unexpected observation made by the inventors of the influence of the pH of the solutions used during the purification of an enveloped virus, and the positive influence of certain additives on the yield of said purification.

The inventors have been able to show that the addition of a polyol in one or more of the buffers used during one or more steps of a method for purifying an enveloped virus made it possible to obtain a substantial increase in purification efficiency. In particular, the improvement of the yield of a purification comprising an ultrafiltration/diafiltration step followed by anion exchange chromatography, is observed when the buffers used during this chromatography comprise a polyol.

DETAILED DESCRIPTION OF THE INVENTION

Production of viruses and enveloped vectors

The production of enveloped viruses or vectors is well known in the prior art. Those skilled in the art can refer to their general knowledge in this field, in particular as represented by Ansorge et al. 2010; Schweizer and Merten 2010; Rodrigues et al. 2011.

The host cell may be selected from among any cells allowing the production of an enveloped virus. According to a particular embodiment, said cell is chosen from a human cell (HEK293, HEK293T, HEK293FT, Te671, HT1080, CEM), a murine cell (NIH-3T3), a mustelide cell (Mpf), a canine cell (D17) (Miller and Chen 1996, Miller 2001, Merten 2004, Rodrigues et al 2011, Stacey and Merten 2011).

In methods employing transiently transfected cells, any agent for transfection of plasmids can be used. In particular, calcium phosphate or polyethyleneimine may be used, although other agents may be considered by those skilled in the art (Ansorge et al., 2010). The conditions (in particular the amount of plasmid(s), ratio between the plasmids, ratio between the plasmid(s) and the transfection agent, the type of medium, etc.) and the transfection time may be adapted by those skilled in the art depending on the characteristics of the product virus and/or the transgene introduced into the transfer plasmid.

According to a particular embodiment, the culture medium used has a neutral pH (e.g. between 7 and 7.4, especially 7, 7.1, 7.2, 7.3 or 7.4) conventionally used in the prior art for cell culture and virus production. According to one particular embodiment, the production method used comprises the cultivation of the producer cells in a moderately acidic medium. While the prior art presents the neutrality of culture media as a necessary condition for the optimal culture of cells and the optimal production of viruses and enveloped vectors, it has been found that moderately acidic conditions on the contrary make it possible to significantly improve the production of an enveloped virus, in particular a lentivirus, in particular a pseudotyped lentivirus. (e.g. with GALV protein (or GALV-TR), VSV-G, or measles virus envelope proteins).

Purification of viruses and enveloped vectors

The buffer(s) used during the ultrafiltration/diafiltration step, more particularly TFF, may also be acidic, neutral or basic buffers.

The solution loaded on or in the ultrafiltration/diafiltration device or on the anion exchange chromatography column may correspond to the cell culture supernatant optionally pretreated with benzonase and/or low speed centrifugation and/or a clarification. It is to be understood that this culture supernatant, possibly pretreated, does not correspond to a "purification buffer". However, its pH can also be adjusted before loading, if needed. If the production has been carried out at neutral or acidic pH, the optionally pretreated culture supernatant can be loaded directly, or its pH can be decreased or increased before loading. It is also possible to envisage the addition of additives in the culture supernatant, possibly pretreated, before loading. For example, it is possible to add at this step a polyol, an antioxidant (including histidine, L-methionine, L-cysteine, glutathione or vitamin C), a metal salt, especially a magnesium salt such as $MgCl_2$ or $MgSO_4$, or any other suitable additive.

According to a particular embodiment, the culture supernatant, optionally pretreated, is loaded directly on or in the ultrafiltration/diafiltration device or on the chromatography column, without pH adjustment and without addition of additive. Acidic, basic or neutral pH buffers may be, and preferably are, previously used to equilibrate the ultrafiltration/diafiltration device and/or to perform ultrafiltration/diafiltration or anion exchange chromatography as such.

According to one variant, the ultrafiltration/diafiltration step is a TFF step. In this variant, the diafiltration is carried out with a buffer whose pH is adjusted according to the modalities described above. Thus, the buffer used may be an acid buffer, in particular a pH buffer of between 5 and 5.9 (in particular equal to 5.0, 5.1, 5.2, 5.3, 5.4, 5.5 , 5.6, 5.7, 5.8 or 5.9), in particular a buffer from pH 5.5 to pH 5.9 (in particular equal to 5.5, 5.6, 5.7, 5, 8 or 5.9), more particularly a buffer of pH 5.5. In one variant, the buffer used is a buffer of pH between 6 and 8 (in particular of pH equal to 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9 or 8.0), more particularly between 7 and 8

(in particular with a pH equal to 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9 or 8.0), comprising a polyol.

The buffers used during the ultrafiltration step and during the diafiltration step may be different or identical. In a particular embodiment, the ultrafiltration step is carried out by means of a buffer having a pH of about 7 (in particular a pH of between 6.8 and 7.2 (for example equal to 6.8, 6.9, 7.0, 7.1 or 7.2), more particularly a buffer of pH 7), while the diafiltration is carried out using a buffer having a pH of between 5 and 5.9. (in particular equal to 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8 or 5.9), more particularly a pH buffer between, 5 and 5.9 (in particular equal to 5.5, 5.6, 5.7, 5.8 or 5.9), in particular a pH buffer equal to 5.5.

In one embodiment, the purification method comprises an anion exchange chromatography step followed by an ultrafiltration/diafiltration step. In another embodiment, the purification method comprises an ultrafiltration step followed by an anion exchange chromatography step.

In a particular embodiment, the purification method comprises:
(a) clarification of the cell culture medium;
(b) an ultrafiltration/diafiltration step;
(c) anion exchange chromatography;
(d) exclusion chromatography;

wherein steps (b) and (c) can be reversed. According to a preferred embodiment, step (c) follows step (b).

For the evaluation of the fractions and the selection of those which will be submitted as a result of the purification method, the column may be equipped at the output of a chromatograph provided with a 280 nm UV absorbance reader, a conductivity meter, a plotter and a fraction collector.

In the context of the present invention, the term "polyol" defines a linear, cyclic or bicyclic carbon molecule comprising between 3 and 18 carbon atoms, in particular between 3 and

12 carbon atoms, substituted by at least 3-6 hydroxyl groups, in particular 8 hydroxyl groups. The polyol may be, for example, a monosaccharide aldose or ketose, in particular a tetrapose, a pentose or a hexose. Mention may be made, in particular, of the following monosaccharide polyols: erythrose, threose, ribose, arabinose, xylose, lyxose, allose, altrose, glucose, mannose, gulose, idose, galactose, talose, erythrulose, ribulose, xylulose, fructose, psicose, sorbose, tagatose. The polyol may also be chosen from the following disaccharides or trisaccharides which constitute a nonlimiting list of other polyols that may be used in the implementation of the invention: cellobiose, gentiobiose, inulobiose, isomaltose, isomaltulose, kojibiose, lactose, lactulose, laminaribiose, leucrosis, maltose, maltulose, melibiose, nigerosis, robinosis, rutinose, sucrose, sophorosis, trehalose, trehalulose, turanose, erlose, fucosyllactose, gentianosis, inulotriosis, 1-kestosis, 6-kestosis, maltotriosis, mannotriosis, melezitosis, neokestosis, panosis, raffinose, rhamninose. In a particular embodiment, the polyol is chosen from raffinose, isomaltotriose, sucrose, mannitol, sorbitol, trehalose, glucose and glycerol. In a particular embodiment, the polyol is sucrose.

The polyol concentration may vary to a large extent, and may be different, in particular, for each of the different buffers implemented during this step. The concentration of polyol may be, in particular, between 1% and 15% (w/v) especially between 1.5% and 10%, in particular between 2% and 8%, more particularly between 2% and 5%. In a particular embodiment, the polyol concentration of one or more of the buffers of the anion exchange chromatography is 5% (w/v). In a particular embodiment, all the buffers used during the purification method comprise a polyol, in particular sucrose, in particular at 5% (w/v). Thus, according to this embodiment the method may comprise a TFF step, an anion exchange chromatography step and an exclusion chromatography step in which all the buffers used comprise a polyol. In another embodiment, the buffers of the TFF and anion exchange chromatography steps comprise a polyol while the buffers used to equilibrate and elute the exclusion chromatography column do not include a polyol, these buffers corresponding to a buffer formulation whose composition will largely depend on the therapeutic purpose and the mode of administration of the finished product.

In another embodiment, one or more of the buffers used in the method of the present invention, including buffers used during ultrafiltration/diafiltration and/or anion

exchange chromatography, comprise a magnesium salt, in particular magnesium chloride or magnesium sulfate. The concentration of magnesium salt, in particular magnesium chloride or magnesium sulfate in each of the buffers can, independently for each buffer, be between 0.1 mM and 5 mM, especially between 1 and 3 mM, in particular 2 mM.

In another embodiment, one or more of the buffers used in the method of the present invention include L-His, L-Met, L-Cys, glutathione, or vitamin C for inactivating free radicals. The concentration of these components in each of the buffers may be, independently for each buffer, between 0.1 mM and 20 mM.

The purification method according to the invention may also comprise one or more steps of treatment of the sample(s) with a nuclease, in particular a benzonase. Nuclease may be used before or after each step. In one embodiment, nuclease, in particular benzonase, is used in the culture medium of the producer cells after the transfection step of the plasmids.

According to one embodiment, one or more purification step(s) are carried out at a temperature below room temperature, in particular at a temperature of between 2 and 12°C, more particularly between 4 and 10°C. According to a particular embodiment, one, more or all the steps of the purification are carried out at about 4°C.

DESCRIPTION OF FIGURES

Fig. 1. Two methods for the purification of lentiviral particles LV-GALV-TR: method (A) is a simplified method implementing a single exclusion chromatography step (gel filtration) after the tangential filtration step in flow; while method (B) is a more elaborate method for obtaining a higher purity than when using method (A) - for example for the production of vectors for clinical use.

Fig. 2. Comparison of membranes with a cutoff of 500 kDa and 750 kDa in the removal of contaminating proteins (SDS-PAGE (top) and Western Blot anti-p24 (bottom)). The band at 24/25 kDa (= p24) is clearly visible for all samples on the Western Blot: 1) size markers; 2) diafiltration (a) (test 1) at 750 kDa; 3) diafiltration (b) (test 2) at 750 kDa; 4) ultracentrifugation at 68338g for 3h (resuspension in X-vivo culture medium 20); 5)

diafiltration (a) (test 1) at 500 kDa; 6) diafiltration (b) (test 2) at 500 kDa; 7) culture supernatant containing LV -GALV-TR vectors.

Fig. 3. Effect of NaCl on the stability of LV-GALV-TR vectors encoding GFP stored at room temperature. The vectors are incubated for 4 hours at pH 7.0 (PBS) at room temperature (RT). To optimise the anion exchange chromatography step, we tested the stability of the vectors in a NaCl salt medium. For this, the vectors were incubated after the UF/DF step in pH 7.0 PBS buffers of different NaCl concentrations for 4 hours at room temperature. Then the vectors were titrated on HCT116 cells. 48 hours later, cells were switched to the FACS to measure the percentage of expression of GFP.

Fig. 4. Effect of pH and salinity of the elution buffer on the efficiency of purification of infectious lentiviral vectors after an anion exchange chromatography step. The vector preparation was produced by transfection of HEK293T cells, clarified and concentrated/diafiltered by TFF for use in the evaluation of different (weak) anion exchange media. Different supports were evaluated: Toyopearl 650C DEAE, CIM© (DEAE) and Poros D. The 100% yield is equivalent to the infectious titer after the previous step of TFF.

Fig. 5. Purification of a lentiviral vector preparation GALV-TR by exclusion chromatography (Capto Core 700). Three ml of a lentiviral preparation was concentrated/diafiltered and then passed through a column of Capto Core 700 (4.5ml). PBS buffer (pH 7.0), 5% sucrose, 2mM $MgCl_2$ was used during this step for column equilibration and formulation. Fractions of 1 ml were collected and analyzed for the vector concentration (TU): a) Chromatogram showing the titer (TU) by fraction, the cumulative amount of vector for fractions 4-9 and recovery (%) cumulative for fractions 4-9; b) Western Blot of all fractions; c) SDS-PAGE of all fractions.

Fig. 6. Transduction of CD34+SC (ambilical cord blood) cells with HIV-GALV-TR vector of MOI 20

Gross: percentage of cells expressing GFP determined by flow cytometry of CD34+ cells transduced with the raw product HIV-GALV-TR, DF/UF: percentage of cells expressing GFP determined by flow cytometry of CD34+ cells transduced with the preparation of HIV - GALV-TR vectors obtained after purification and concentration by TFF of the crude product.

EXAMPLES

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Material and methods

Cells: HEK293T and HCT116 cell lines (CCL-247 colorectal cancer cells, origin: ATCC) are cultured at 37°C, 5% CO_2 in Dulbecco's Modified Eagle's medium (Gibco) (DMEM+Glutamax) supplemented with 2-10% fetal calf serum (FCS) (Life Technologies). Culture medium: DMEM/SVF buffered to pH 6.0 by the addition of hydrochloric acid (37% HCl, Sigma-Aldrich), then filtered using a Corning® 1000mL filter (0.22µm PES (polyethersulfone)).

Production of viral vectors:

Virus vectors derived from HIV-1 pseudotyped with different glycoproteins are produced by transient calcium phosphate transient transfection in 293T cells, by 4 plasmids as described by Merten et al. (2011). 2x108 293T cells are seeded in Hyperflask 1760-cm^2 (Corning) in 550ml of DMEM 10% FCS (Kutner et al., 2009). 24 hours later, the culture medium is replaced by the transfection medium, combining in the DNA/CaCl2/HBS complex. The 4 plasmids: gagpol (pKLgagpol) 136 µg, rev (pKrev) 52.25 µg, transgene plasmid (pCCL-eGFP) 206.8 µg, envelope plasmid suitable for each pseudotype: GALV-TR: pBA.GALV-TRIAmphoKana (Gibbon ape Leukemia Virus) 223 µg to generate LV-GALV-TRs; VSV-GpMDG (Vesicular stomatitis virus-g) 68.13 µg to generate LV-VSV-g; pFΔ30 and pHCMH2 (modified envelope proteins of measles virus) 40µg and 14µg to generate LVMV; sufficient quantity for 18ml H_2O and 8.9ml TEO.1X, mix with 3ml of $CaCl_2$ (2.5M) then add 30ml of HBS2X, wait for the formation of the complex for 4 min and add the mix to the culture medium. After 16 hours the supernatant is replaced by the fresh medium 2% SVF 15U Benzonase (Merck) and 2mM $MgCl_2$ (Sigma-Aldrich). The harvesting is carried out after 48 hours post transfection and the supernatant is filtered through a 0.45 µm cellulose acetate (CA) IL filter (Corning).

The MLV-GALV retroviral vectors are produced by the PG 13 cells. They are MLV-GALV vector producing cells (Miller et al., 1991), the cells are maintained in Dulbecco's modified Eagle's medium (Gibco) (DMEM + Glutamax) supplemented with 2-10% FCS at 37°C, 5% CO_2. The harvesting of the vectors is done after 24 hours of change of culture medium.

Then the production supernatant is clarified on a 0.45 µm cellulose acetate filter (Corning).

Concentration of viral vectors by tangential flow filtration (TFF):

This step consists of concentrating the production supernatant and then replacing the culture medium with an appropriate buffer for the remainder of the method.

Ultrafiltration (UF) is performed after preparation of the UF cassette and determination of the normalised water permeability (NWP) at 0.5 bar and 20°C. The membrane is then equilibrated with Bis-Tris buffer pH 6.0 5% sucrose, 2mM $MgCl_2$ or other buffers for concentration at other pHs (e.g. PBS, pH 7.0, 5% M, sucrose, 2mM $MgCl_2$). The entire method is carried out at about 4°C and the concentrate product reservoir put in an ice bucket.

Principle: A first concentration up to 20 ml volume followed by diafiltration with 10 volumes of ion exchange chromatography loading buffer (in this case: 10 x 20ml) are performed. These steps are followed by a second concentration to the minimum possible volume (10 ml in this case).

Membrane 750 kDa, 410 cm^2: "hollow fiber cartridge" (GE Healthcare, Ref: UFP750-E3MA) using the Kros-Flow research II TFF system (Spectrum).

After validation of the integrity of the membrane, the concentration of the vectors begins with a starting volume of 500 ml of crude supernatant and is concentrated by the membrane from 500ml to 20ml.

The concentrated product is diafiltered against 200 µl of buffer A (in order to diafilter 10 times 20 ml of concentrate). This represents a concentration factor of 25X. The final volume of the diafiltrate is 10ml. In this case the concentration factor is 50X.

Anion exchange chromatography:

In a first protocol, the anion exchange chromatography step is performed downstream of the TFF. Several chromatographic supports are tested: CIMD DEAE monolithic column,

CIM© Q (BIA Separations, Villach, Austria), column volume: 1ml; Sartobind D 75MA, volume: 2.1ml (Sartorius Stedim Biotech); Poros PI, volume of the column: 4ml; Poros D 50, column volume: 4ml, Poros HQ, column volume: 4ml (Life Technologies), Toyopearl 650C DEAE, column volume: 2ml (Tosoh).

The column to be tested is connected to a Biologic-LP (Biorad) chromatograph equipped with a 280 UV absorbance reader, a conductivity meter, a plotter (Chart recorder 1327, Bio-Rad), and a collector. Fraction (Mode 1 2110, Bio-Rad).

The column is equilibrated with 5 column volumes (5 CV) of buffer A at 2 ml/min. After loading the sample onto the column, the column is washed with 5 CV of appropriate equilibration buffer according to the desired pH, according to Table A). Two-step elution is then carried out: 0.3M NaCl, 20mM Bis-Tris, 5% sucrose, 2mM $MgCl_2$ (pH 6.0) then 650mM NaCl, 20mM Bis-Tris, 5% sucrose, 2mM $MgCl_2$ (pH 6.0) to elute the vectors. Three other pH's are tested pH 5.5, 7.0 and 8.0 using the appropriate buffers (including buffers such as Bis-propane, PBS, L-His); in the presence (5%) and in the absence of sucrose 5% and $MgCl_2$ (2 mM).

Finally the fraction is loaded immediately on the gel filtration column (exclusion chromatography) to remove the contaminants and contaminant proteins eluted with the 650 mM NaCl vectors.

In a second protocol, the clarified production supernatant is loaded onto a Poros D anion exchange chromatography column, without a prior ultrafiltration/diafiltration step to evaluate the chromatography yield under these conditions. The equilibration buffer used has a pH of 5.5, and contains 5% sucrose and 2 mM $MgCl_2$.

Exclusion chromatography:

This is the final step before sterilizing filtration for methods A and B (Fig. 1). This step consists in eliminating contaminants having a smaller size than the gel used (exp: 750kDa, or 500kDa). The Captocore 700 column was used for this step. It is a gel with dual functionality: exclusion chromatography and adsorption chromatography gel.

Before starting the loading, the column is sanitised with 1 M NaOH and equilibrated with the formulation buffer. The product UF/DF (Method A), or the fractions corresponding to the peak of chromatography of anion exchange chromatography (AXC) (Method B) is loaded on the column. 8 ml (UF/DF, or AXC fraction) are loaded at a flow rate of 0.5 ml/min. Then the formulation buffer is injected at 0.5 ml/min (5 CV formulation buffer). The fraction corresponding to the UV peak is collected (approximately 16 ml) and then filtered on a 0.22 µm filter (sterilizing filtration). Samples are stored at -80°C.

Titration of viral vectors:

The viral titer in transducing units (TU) of the vectors having the eGFP reporter gene is analyzed by transduction of HCT116 cells. 72 hours after transduction the cells were switched to FACS to determine the TU/ml titer as previously described (Pfeifer et al., 2009). For physical analysis of the virus particles, the p24 ELISA KIT (PerkinElmer) was used for the quantification of the p24 lentivirus capsid protein according to the supplier's instructions.

Transduction of umbilical cord blood cells CD34+:

CD34+ cells are isolated from umbilical cord blood by immunomagnetic selection (Miltenyi Biotec). Culture and transduction of CD34+ cells is as described (Charrier et al., 2011): first, the cells are preactivated overnight in X-Vivo (Lonza) medium and supplemented with cytokines. Preactivated cells are inoculated in a 48-well plate (5^e4 cells/100 µl). The transduction is done by the addition of 100µ1 of vectors (1^e6TU) purified in the presence of 8 µg/ml of vectofusin-1 (Fenard et al., 2013). After 6 hours of incubation, 1 ml of differentiation medium (X-VIVO-20 supplemented with 10% serum, and in the presence of cytokines (hSCF, h-11 h-3h-Flt3 h-11-6) are added as described (Charrier et al., 2011)) in each well, and after 5 days the transduction efficiency is evaluated by measuring the expression of GFP by FACS (FC500, BD Biosciences).

SDS-PAGE Western Blot:

Culture samples containing lentiviral vectors or purified samples are analyzed by SDS-PAGE and Western Blot for the presence of p24 capsid proteins. The p24 proteins are revealed according to the method developed by LI-COR, with the Odyssey device and the

Odyssey 2.1 software. The primary antibody used is an anti-p24 (Santa Cruz # SC-57823) for the detection of HIV p24 capsid proteins.

The antibody is used with a 1/200th dilution in PBS1X-Tween 0.1% + Odyssey blocker (1 : 1). The secondary goat antibody used coupled to Li-COR's "Dey 800" fluorochrome is directed against the primary antibodies.

Quantification of residual proteins and specific residual DNA:

Total proteins are quantified by Bradford method (Bio-Rad) with serum albumin as standard. The test is done according to the instructions of the supplier.

Residual DNA: The quantification of residual DNA of plasmid origin and/or from the host cell is done by quantitative PCR. The samples are treated with proteinase K (Roche) and the DNA is extracted using the MagNA Pure DNA and viral NA small volume kit system (MagNA Pure 96 Roche). Real-time quantitative PCR is then performed, with specific primers for the kanamycin gene to detect residual DNA of plasmid origin. To detect the residual DNA of the host cell, primers that target the ElA gene are used. Absolute quantification is performed with respect to a reference plasmid containing the amplified regions by quantitative PCR and whose copy number is known.

Table A: Buffers used during the method

	Buffers	pH	Sucrose (Sigma-Aldrich)	$MgCl_2$ (Sigma-Aldrich)
Buffer A	L-Histidine 20mM (Sigma-Aldrich)	5.0	5% W/V	2mM
Buffer B	Bis-Tris 20mM (Sigma-Aldrich)	5.5		
Buffer C	Bis-Tris 20mM (Sigma-Aldrich)	6		
Buffer D	PBS (GIBCO®)	7.2		
Buffer E	Bis-Propane 20mM (Sigma-Aldrich)	8		

Results

Cell culture and clarification:

These steps consist in producing retroviral and lentiviral vectors using stable cells such as PG 13 characterised by stable and continuous production of retroviral vectors in

continuous culture with regular medium exchange or cells such as HEK293 or HEK293T which should be transfected with 3 or 4 plasmids (providing the lentivirus helper functions and the recombinant vector sequence) for induction of lentiviral vector production. The transient production is limited in time and allows one or more harvests a few days after transfection. The titers in general depend on the construction (sequence) of the vector but also of the protein envelope. The following titers can be obtained with these production systems (Table 1).

Table 1. Vector concentrations obtained with the different production systems:

Pseudotyped vector-production cell	Vector concentration (TU/ml, gi/ml)	References
PG13, MLV-GaLV-TR	5*6 TU/ml	Miller et al. 1991
HEK293T, LV (HIV-1) – GaLV-TR	5*5 TU/ml	Sakuma et al. 2010
HEK293T, LV (HIV-1) –VSVg	1-5*7 TU/ml	Merten et al. 2011

Before any subsequent treatment, it is possible to remove cell debris and aggregates present in the production supernatant. Conventionally, a filter of 0.45 µm (cellulose acetate) is used. The yield of this step is 80 ±5%. However, those skilled in the art may use other membranes or membrane cascades, characterised by similar behavior and performance.

Tangential flow filtration:

Tangential flow filtration comprises two successive steps of ultrafiltration and diafiltration (UF/DF). These two steps eliminate a large part of contaminants whose size is smaller than the pore exclusion size of the membrane used. This UF/DF step also makes it possible to concentrate the viral particles and to reduce the volume of the product to be purified. A 110 cm^2 membrane with a pore exclusion size of 750 kDa (GE HealthCare) was used. Before starting the UF, different concentrations of sucrose (especially 5% sucrose (weight/volume)), and different concentrations of $MgCl_2$ (especially 2mM $MgCl_2$ (final concentration)) are added to the clarified product. Then the concentration step by UF is at a flow of 80ml/min, 7psig. The reservoir of the TFF is placed in an ice bucket to ensure a low temperature during the UF/DF. The diafiltration step begins after reducing the volume from 500ml to 20ml during the UF. For the DF is used 200ml (10 volumes of the

concentrated product) diafiltration buffer: PBS, 5% sucrose, 2mM $MgCl_2$. At the end of this step 20ml of UF/DF product is recovered in a 50ml Corning tube. The choice of buffer depends on the use of the preparation or the optimum conditions of the post-filtration step (e.g. in this case other buffers may be used such as Bis-Tris (pH 6.0) 5% sucrose 2mM $MgCl_2$) - cf. Table A. Samples are titrated on HCT16 cells as described by Fenard et al. (2013).

Optimization studies of the concentration and filtration conditions:

1. The lentiviral particles have a diameter ranging from 80 to 120 nm, meaning that the membrane pore size that can be used for the filtration concentration can go up to a maximum of about 50 nm (or 750 kDa). Within the scope of this invention, the 500 kDa and 750 kDa cutoff sizes have been evaluated. Yields (in UT) were as follows: 64% for the 750 kDa membrane versus 34% TU yield for the 500 kDa membrane.

Fig. 2 shows the electrophoresis gels (SDS-PAGE and Western Blot) for vector preparations after tangential filtration using membranes with a cutoff of 500 kDa and 750 kDa. In addition to the higher yields obtained when using 750 kDa membranes, it is clear that a 750 kDa cutoff had a positive effect (Fig. 2, columns 2, 3) compared with the use of the 500 kDa membrane at the level of contaminant removal (Fig. 2, columns 5, 6). In addition, the concentrate generated with the 750 kDa membrane contains much less intense protein bands than observed for the crude supernatant.

2. Since the tangential filtration step is characterised by the generation of shear fields leading to the inactivation of the retroviral/lentiviral particles, it was necessary to optimise this step in order to maintain the functionality of these vectors. The addition of a polyol at different concentrations was evaluated in order to protect the lentiviral vector from the adverse conditions of the tangential filtration.

Table 2. Concentration yield of LV-GALV-TR filtration using different concentrations of sucrose.

	Yield % (TU)
0% sucrose	50.83
2% sucrose	80.31
5% sucrose	80.40
10% sucrose	52.37
15% sucrose	68.49

Note: 190 ml of crude supernatant was concentrated to 17 ml and several times diafiltered with PBS (pH 7) + different% sucrose and 2mM $MgCl_2$.

These results clearly show the advantage of carrying out the concentration of supernatant filtration containing LV-GALV-TR vectors in the presence of sucrose and $MgCl_2$. The best yields are obtained at concentrations of 2% to 5% sucrose (Table 2).

In addition, the use of a moderate concentration of sucrose has the advantage that the sample to be concentrated is less viscous because high concentrations of sucrose (10% - 15%) lead to an increase in viscosity.

3. Evaluation of the pH and its effect on tangential filtration and functional vector yield:
In the application FR 13 58909 filed by the present applicant, it has been shown that the production of enveloped vectors pseudotyped with different envelope proteins is increased when using a pH 6.0 (up to 2x). It was decided to evaluate the impact of the choice of the pH of the supernatant containing the lentiviral vectors on the efficiency of the tangential filtration. In this context, two different pH values were evaluated (pH 6 and pH 7) during the concentration of filtration of GALV-TR pseudotyped lentiviral vectors (Table 3). The reduction in pH from 7.0 to 6.0 resulted in a reduction in yield of about 10% (from 73.6% to 64%). However, this yield remains acceptable and it is therefore possible to envisage a concentration of filtration at acidic pH.

Table 3. Impact of the pH of the supernatant to be concentrated/diafiltration buffer on the concentration and filtration yields of pseudotyped lentiviral vectors GALV-TR and VSV–g.

Tangential filtration condition	Vector LV	Yield (%, TU)
PBS, 5% sucrose 2mM $MgCl_2$, pH 7.0	LV-GaLV-TR	73.64
BISTRIS 20Mm, 5% sucrose, 2mM $MgCl_2$, pH 6.0	LV-GaLV-TR	63.99

4. Identification of the best condition for the concentration of lentiviral vectors GALV-TR:
With regard to the GALV-TR lentiviruses, the best condition for concentration and diafiltration (tangential filtration) was as follows: the LV-GALV-TR (IL) vectors are clarified through a 0.45 µm membrane in cellulose acetate, in the presence of 5% sucrose and 2mM $MgCl_2$, followed by the TFF step (750 kDa cartridge, 410 cm^2) with volume reduction to reach 20ml (50X). A diafiltration step is then performed against a volume of 200ml of appropriate buffer (for example: 20mM Bis-Tris pH 6.0, 5% sucrose and 2mM $MgCl_2$ or PBS pH 7.0, 5% sucrose and 2mM $MgCl_2$).

The yield of this step for LV-GALV-TR vectors is 86% ± 5%, for a starting volume of 550 ml of the crude product. The volume of the concentrated product is 15ml with a concentration factor of 36.6X and the elimination of contaminants reaches more than 90%.

5. Evaluation of established tangential filtration conditions for concentration and filtration of other retroviral and lentiviral vectors pseudotyped with different envelope proteins:
In the scientific literature, different envelope proteins have been evaluated for studying and improving the tropism of retroviral and lentiviral vectors. In this context, the conditions established for the concentration/diafiltration of lentiviral vectors GALV-TR were evaluated for the concentration of filtration of retroviral and lentiviral vectors pseudotyped with different envelope proteins (Table 4). The results obtained with pseudotyped lentiviral vectors GALV-TR are indicated as reference.

Table 4. Concentration of the filtration of retroviral vectors pseudotyped with different envelope proteins:

Tangential filtration condition	Vector LV	Yield (%, TU)
BISTRIS, 5% sucrose, 2mM $MgCl_2$, pH 6.0	MLV-GaLV (PG13)	94.2
PBS, 5% sucrose, 2mM $MgCl_2$, pH 7.0	LV-GaLV-TR	73.64
BISTRIS, 5% sucrose, 2mM $MgCl_2$, pH 6.0	LV-GaLV-TR	63.99
Bis-Tris 5% sucrose 2mM MgCl2 pH 6.0	LV-MV-CMHII	61.22
PBS, 5% sucrose, 2mM $MgCl_2$, pH 7.0	LV-MV-CMHII	65.67
PBS 5% sucrose, 2mM $MgCl_2$, pH 7.0	LV-VSV-g	107
BISTRIS, 5% sucrose, 2mM $MgCl_2$, pH 6.0	LV-VSV-g	104

Note: MLV-GALV: pseudotyped murine retrovirus GALV; LV-GALV-TR: pseudotyped lentivirus GALV-TR; LV-MV: pseudotyped lentivirus with the env of the measles virus (modified CMHII); LV-VSV-g: pseudotyped lentivirus VSV-g

The results presented in Table 4 show that all retroviral or lentiviral vectors pseudotyped with different envelope proteins can be concentrated in the presence of sucrose and $MgCl_2$ at pH 7.0 leading to yields of about 74% for LV-GALV-TR at about 100% for VSV-g. With respect to the use of a pH of 6.0, no difference was observed for pseudotyped VSV-g vectors.

For the GALV-TR pseudotyped lentiviral vectors, these vectors were more stable at pH 7.0 during tangential filtration. The concentration of the filtration was higher than 90%, while the yield was around 74% for the lentiviral vectors GALV-TR.

Anion exchange chromatography:

The tangential flow filtration step considerably reduced the protein and DNA loading (see above), meaning that a significant portion of contaminants that could be competitors of the vectors to be purified for access to the ligands of the chromatography is decreased.

In principle, depending on the subsequent use, one can imagine two different ways of considering purification. They are shown in Fig. 1: a simplified method implementing a single exclusion chromatography step (A in Fig. 1) and a more elaborate method implementing an additional step of anion exchange chromatography for the preparation of lentiviral vectors for clinical use (B in Fig. 1).

The different possibilities of chromatography are developed later: Just after the TFF UF/DF step and in order to decrease the contaminants and to separate the viral particles, an anion exchange chromatography step is added. This technique makes it possible to separate the biomolecules according to their isoelectric points as a function of the pH and the concentration of salts. Thus, at a given pH value, a certain concentration of salts (often NaCl) is required in order to remove the biomolecules retained and this concentration must be chosen according to the interaction force between the biomolecules and the ligands: the stronger this interaction, the higher is the concentration of salts (salinity). In addition, the higher the pH of the chromatography buffer is near the isoelectric point of the biomolecule species to be purified, the less salt is needed to unhook the biomolecules from the chromatographic ligands. However, it is known that retroviral and lentiviral vectors rapidly lose their infectivity as a function of salt concentration (reviewed by Segura et al., 2006). Thus, at first, the stability of the lentiviral vectors towards different concentrations of NaCl was evaluated.

1. Impact of salinity on the stability of lentiviral vectors GALV-TR:

As indicated above, the elution of the biomolecules retained by a chromatography column is very often done with salt gradients (buffers containing NaCl) or a step of increasing the salt concentration (NaCl). Therefore, in order to evaluate the effect of the NaCl concentration, incubation tests of post-TFF lentiviral vectors were carried out in different NaCl concentrations ranging from 50mM to 1500mM, at room temperature for 4h. Fig. 3 represents the infectivity of the lentiviral vectors at room temperature as a function of the NaCl concentration relative to the conditions without added NaCl or the same vector preparation incubated at 4°C without added NaCl. This test clearly shows that a concentration of NaCl of between 50mM and 1M has a moderately detrimental effect on the stability of lentiviral vectors GALV-TR with a loss of infectivity ranging from 29.52%

(50mM NaCl) to 43.86%. % (1M NaCl) (percentage relative to the preparation stored at 4°C). In contrast, the concentration of 1 MS NaCl leads to a 63.8% loss of infectivity when the vector preparation is stored at room temperature for 4 hours. It should be noted that storage at 20°C (room temperature) for 4 hours without added NaCl also leads to some loss of vector infectivity of about 23% compared to storage at 4°C.

These results mean that it is essential to elute lentiviral vectors GALV-TR chromatographic media with the lowest salinity possible, so ideally below 1M NaCl in order to maintain maximum infectivity. In addition, it is also preferable to perform all purification (all steps) at a reduced temperature (ideally between 4°C and 10°C).

2. Evaluation of different anion exchange chromatography (AEX) media:

We used weak anion exchange chromatography media (DEAD (D)) to determine whether it was possible to limit the inactivation of vectors with this type of support, particularly by attempting to reduce the concentration of salt. necessary to pick up said vectors from the chromatography column. In preliminary tests using a concentrated supernatant from PG 13 cell cultures (MLV-GALV) it has been possible to show that the use of DEAE based chromatographic support (Tosoh TSK gel DEAE 5PW) leads to an efficiency in infectious vector of 71% approximately higher yield than when using a strong exchanger (Q Sepharose FF from GE HealthCare) whose yield was only 16%, due to the too strong interaction leading to In this example, the salt concentration required to pick up the retroviral vectors was 655 mM and 915 mM, respectively.

Based on these results, weak anion exchangers were chosen for further development: several chromatography supports were evaluated: CIM© monolite (DEAE), Poros D50 (Life Technologies), Sartobind© (Sartorius) (Bandeira et al 2012). Toyopearl650C DEAE (Merten et al., 2011).

As preliminary tests, three supports were evaluated for the purification of lentiviral GALV-TR vectors at pH 5.5 or 6.0 and 7.0. For all media tested at pH 5.5 or 6.0 and 7.0, the choice of low pH (5.5 or 6.0) was beneficial for the level of infectious vector yield: in terms of CIM© DEAE support the yield was increased from 23% (pH 7.0) to 64% (pH 6.0) when

reducing the pH of buffers used for chromatography from 7.0 to 6.0 (Fig. 4). Similar results were observed for Sartobind 75D media (yield increase of 5.8% to 15.6%) and Poros D (yield increase of 32% (pH 7.0) at 80.2% (pH 6.0 and about 100% (pH 5.5), and for Toyopearl 650C gel (23% increase in yield (pH 7.0) to 89% (pH 5.5)) (Fig. 4). In addition, in order to unhook the vectors from the supports, the salinity of the elution buffer could be lower during chromatography at pH 6.0 (thus, softer for the lentiviral vectors). As for the Poros D support used at pH 6.0, the elution of the vectors is at 650 mM NaCl (see below). In terms of overall efficiency, 'modern' media (developed more recently, generating reduced shear forces (mainly due to greater porosity than other media) during chromatography and characterised by the incompressibility of the support during modification of the buffer flow rate, such as the CIM© DEAE monolith or Poros D) have shown higher yields than the yields of membrane supports (Sartobind 75D) or compressible gel based supports (Toyopearl650C).

Finally, the choice fell on recent media because their efficiency of separation and recovery in vectors was higher compared to more conventional media. These two supports have therefore been more widely evaluated and their use has been optimised for the purification of lentiviral vectors. The two supports, CIM© DEAE and Poros D, have an interesting yield greater than 60%. The elution is carried out at 650 mM NaCl, Bis-Tris 5% 2mM sucrose $MgCl_2$ pH 6.0. Increasing the pH of the elution buffer to 7.0 (PBS) results in a fall in yield of less than 7%, but the addition of 5% sucrose to PBS results in a significant increase of about 7% to 40%. However, it is necessary in this case to use a concentration of NaCl greater than 1M. In fact, it has been found that at pH 7.0 for the elution of the vectors (buffer without added sucrose) it will take about 1 M NaCl in PBS, which is probably the explanation of the low yield. The negative effect of the concentration of salts on the stability of viral particles is known (Segura et al., 2005).

3. Evaluation of different pH values on the chromatography efficiency using the Poros D support:

The pH was varied in the range of 5.5 to 8.0 in the presence and absence of 5% sucrose. The presence of 5% sucrose has a positive effect on the yield during the anion exchange chromatography step when the pH is above 5.5 (e.g. Poros D) (Table 5). The positive effect

of the presence of sucrose on the yield is no longer observed at pH 5.5. On the other hand, the presence of sucrose is essential at pH 8.0 to recover about 58% of infectious vectors. While using pH ranging from 6.0 to 7.0, the yield is between 52 and 65%, the best yield (about 100%) is obtained at a pH of 5.5.

In general, the presence of 5% sucrose leads to a reduction of the salinity necessary to initiate the elution of the lentiviral vector with a decrease in the NaCl concentration required by about 25 mM.

Table 5. Comparison of the yields of LV -GALV-TR by chromatography on Poros D using buffers of different pHs (5.5 - 8.0) in the presence or absence of sucrose.

	With/without sucrose	Yield in TU%
pH 5.5 (Bis-Tris)	5% sucrose	185.98
	0%sucrose	101.33
pH 6.0 (Bis-Tris)	5% sucrose	52.32
	0%sucrose	29.29
pH 7.0 (PBS)	5% sucrose	65.52
	0%sucrose	10
pH 8.0 (Bis-Tris-propane)	5% sucrose	57.76
	0%sucrose	0

4. Evaluation of an alternative protocol including anion exchange chromatography as a first step:

We evaluated the yield obtained during the application of an anion exchange chromatography step immediately after the clarification step. Under these conditions, the yield observed is lower than when an ultrafiltration/diafiltration step is carried out between the clarification and the anion exchange chromatography. This latter protocol was therefore selected for further purification.

Exclusion chromatography:

Exclusion chromatography is the method of choice for the separation of biomolecules by their molecular size, thus separating particles from contaminants.

Capto Core 700 filtration gel (GE HealthCare) has been used, but other media may be considered. This step allows us to replace the buffer of the previous step with the desired formulation buffer, eliminate contaminating molecules smaller than 750 kDa and avoid dilution of the sample to be loaded. This chromatography step can be directly used after tangential flow filtration (concentration diafiltration - method A) or after an ion exchange chromatography step - method B (Fig. 1). The sample resulting from the tangential flow filtration or the sample from the fractions of the anion exchange chromatography containing the lentiviral vectors is loaded on the exclusion chromatography column. In both cases the yield of this step is 86% ± 4, depending on the fractions retained for later use.

Fig. 5 shows the purification of lentiviral vectors (concentrated and diafiltered by tangential flow filtration) by gel filtration (Capto Core 700). The elution peak of the vector is at the buffer passage front and exits the column at fractions 4-9, covering about 70% of the amount of initially loaded vectors on the column (Fig. 5a). Fig. 5B and 5C represent the analysis of each fraction by electrophoresis (Western Blot, SDS-PAGE) clearly indicating the absence of contaminating bands (Fig. Sc) and the presence of the 24-25 kDa band corresponding to the p24 protein of the capsid of the lentiviral vector.

Yields and purities:

The most important parameters concern the overall yield as well as the purity of the lentiviral vector preparation at the level of the charge reduction of contaminating proteins and contaminating DNA.

For protocol B (including TFF, anion exchange chromatography (AEX) and exclusion chromatography (SEC)) (Fig. 1) for the purification of lentiviral vectors for clinical use, the yield is about 50% and this protocol allows 99.9% removal of contaminating proteins and 99.9% of contaminating DNA.

Protocol A (comprising a TFF and exclusion chromatography (SEC)) (Fig. 1) intended for the purification of lentiviral vectors for use in research is simpler because it lacks the ion exchange chromatography step. The overall yield is higher due to the reduction in the number of purification steps and reaches 60.2%, the removal of residual DNA

contaminants from this simplified protocol is of the order of 96.17% and there is a reduction of contaminating proteins by 99.63%.

Practical examples of target cell transduction:

Transduction of CD34+ cells:

To determine the quality of the purified vectors, CD34+ cord blood cells are transduced. The cells are thawed after 18 hours of pre-stimulation with cytokines. The transduction is for 6 hours. Then, the cells are put in a differentiation medium for 5 days. The cells are then switched to FACS FC500 (BD Biosciences) to measure the percent expression of GFP. The following results are typically obtained (Fig. 6): Concentration filtration of lentiviral vectors (GALV-TR) leads to an increase in the transduction efficiency of CD34+ cells (expressed as a percentage of cells expressing GFP) ranging from 9% when using a 70% raw supernatant for use of a concentrated/diafiltered LV vector preparation.

Purification of a pseudotyped lentiviral vector using a modified envelope of the measles virus

A method of purifying a pseudotyped lentiviral vector using the modified measles virus envelope glycoprotein (MV pseudotyping) is described. The LV-MV-CMHII (CMHII = anti-CMHII) lentiviral vectors produced according to the procedure indicated above are purified according to the following steps:

1) concentrationldiafiltration using a TFF step

- membrane used: GE # UFP-750-E-3MA 110cm^2, for the purification of a liter of product
- diafiltration buffer: PBS ((pH 7.0), 2mM MgCl, 5% sucrose)
- volume reduction: from 500rnl/1000ml to 20ml, the buffer being replaced by the diafiltration buffer
- yield of infectious vectors: 64-70%

2) exclusion chromatography (gel filtration):

- column used: CaptoCore 700 4.7rnl

- formulation buffer: PBS, 5% sucrose, 2 mm $MgCl_2$, or else X-vivo or HANKS, containing 5% sucrose and 2mM $MgCl_2$

- equilibration of the column with 10 CV of a formulation buffer

- loading of the TFF concentrate on the CaptoCore700 column at a speed of 0.5mL/min

- washing the column with 20 CV formulation buffer

- collection of samples corresponding to the peak of OD (volume 21 mL to 50X)

- yield of infectious vectors:> 90% in TU

The overall yield of this purification is 60 to 63% in infectious vectors, which represents a major advance for the purification, and therefore the exploitation, of lentiviral vectors pseudotyped by means of the modified MV glycoprotein.

BIBLIOGRAPHIC REFERENCES

Patentkrav

1. Fremgangsmåde til oprensning af en lentivirus pseudotypiseret med kappe-glycoproteinet GaLV-TR, VSV-g eller MV, omfattende et trin til anionbytnings-kromatografi, idet bufferne anvendt under kromatografien har en pH på mellem 5 og 5,9; hvor anionbytningskromatografien er en svag anionbytningskromatografi.

2. Fremgangsmåde ifølge krav 1, idet buffernes pH er lig med 5,5.

3. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, idet et trin til ultrafiltrering/diafiltrering, især en tangentiel flowfiltrering, går forud for trinnet til anionbytningskromatografi.

4. Fremgangsmåde ifølge krav 3, idet trinnet til ultrafiltrering/diafiltrering omfatter anvendelsen af en eller flere buffere, der har en pH på mellem 5,5 og 7,5 eventuelt omfattende en polyol.

5. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, til oprensning af en lentivirus, fra dyrkningsmediet af en cellekultur af celler, der producerer lentivirussen, hvilken fremgangsmåde omfatter:

(a) klaring af celledyrkningsmediet, især ved filtrering af dyrkningsmediet gennem et retentionsfilter, hvor retentionstærsklen er på mellem 0,2 og 0,45 µm;

(b) e trin til ultrafiltrering/diafiltrering af de klarede virusser, især ved hjælp af en tangentiel flowfiltrering;

(c) en anionbytningskromatografi;

(d) en eksklusionskromatografi; især en eksklusionsresin med en eksklusionsstørrelse på mellem 300 og 1000 kDa.

6. Fremgangsmåde ifølge krav 5, idet resinet anvendt til eksklusionskromatografi er multimodalt, med en dobbelt funktionalitet til eksklusion og adsorption.

7. Fremgangsmåde ifølge et hvilket som helst af kravene 4 til 6, idet polyolen er valgt fra sukrose, mannitol, sorbitol og trehalose.

8. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 7, idet de oprensede virusser fremstilles i et neutralt medium eller i et moderat surt medium, især ved en pH på mellem pH 6 og pH 7.

9. Fremgangsmåde ifølge et hvilket som helst af kravene 4 til 8, idet polyolen er til stede i en buffer i en koncentration på mellem 1,5 og 15 vægtprocent i bufferen, især mellem 2% og 5%, i særdeleshed på 5%.

10. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, idet bufferne anvendt under fremgangsmåden også omfatter et magnesiumsalt, især magnesiumchlorid, især i en koncentration på mellem 0,1 mM og 5 mM, især mellem 1 og 3 mM, i særdeleshed på 2 mM.

11. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor anionbytningskromatografien er en anionbytningsøjlekromatografi.

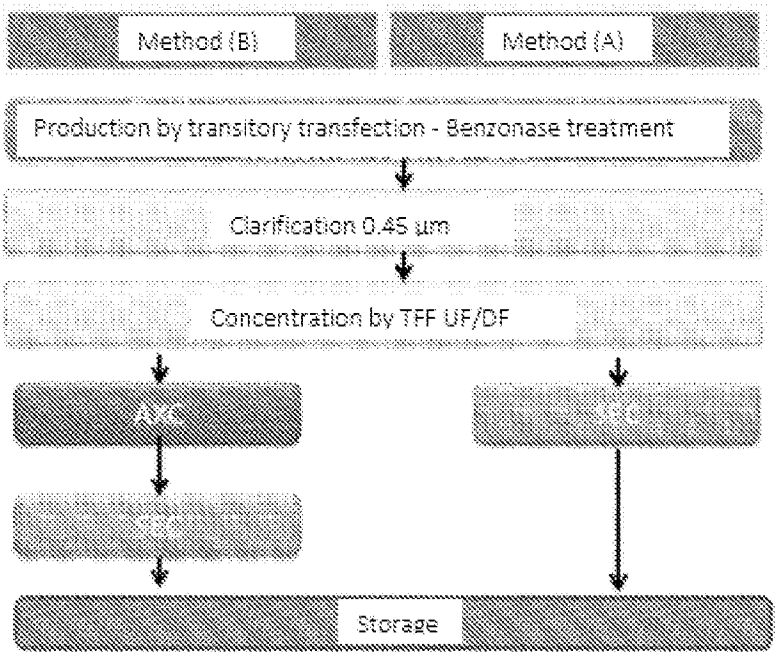

Figure 1

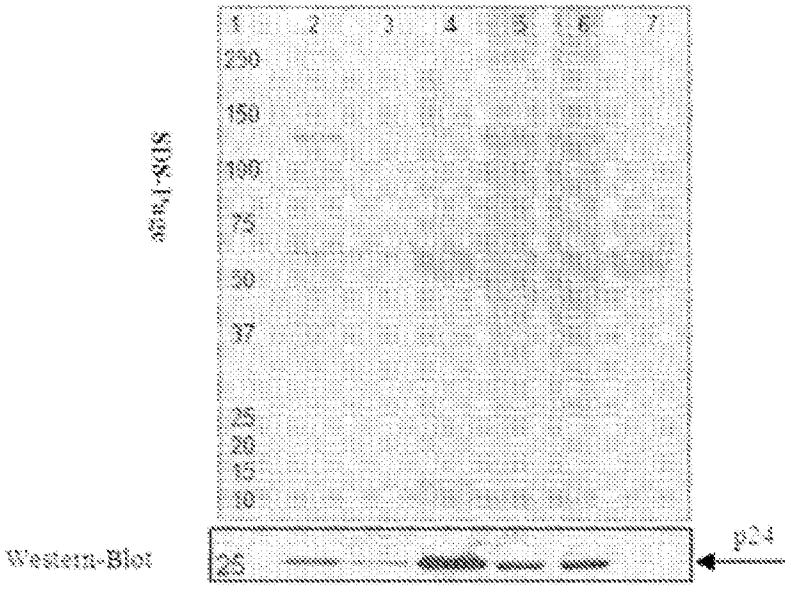

Figure 2

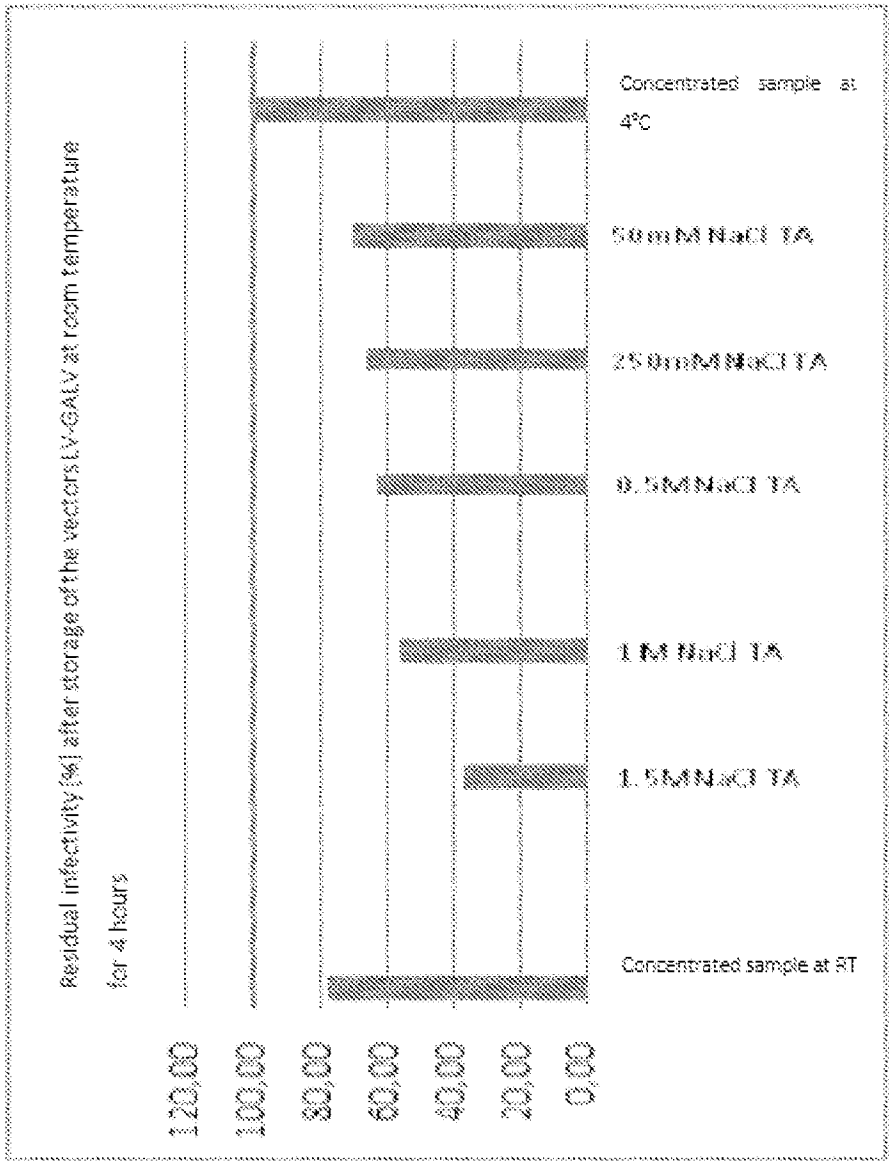
Residual infectivity [%] after storage of the vectors LV-GALV at room temperature
for 4 hours
120,00
100,00
80,00
60,00
40,00
20,00
0,00
Concentrated sample at 4°C
50 mM NaCl TA
250 mM NaCl TA
0,5M NaCl TA
1 M NaCl TA
1,5M NaCl TA
Concentrated sample at RT

Figure 3

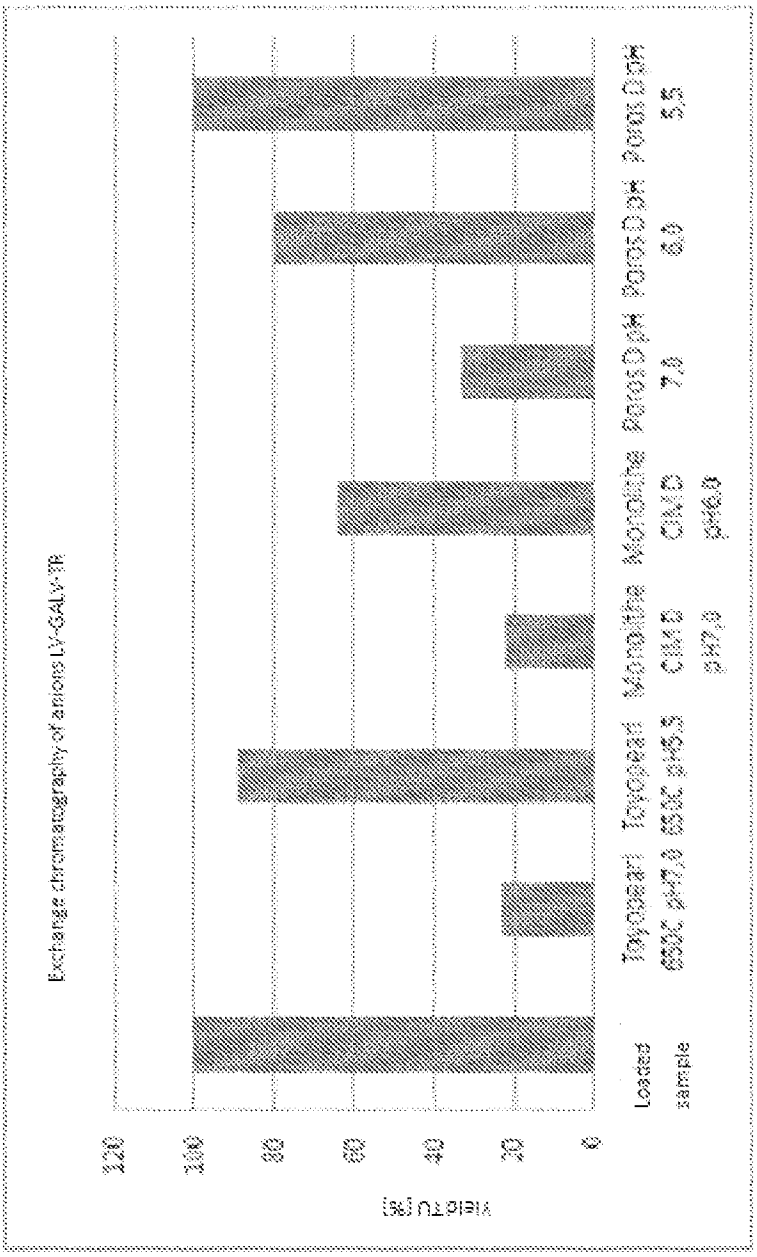
Exchange chromatography of amins LV-GALV-TR
Yield TU [%]
120
100
80
60
40
20
0
Loaded sample
Toyopearl 650C pH7,0
Toyopearl 650C pH5,5
Monolithe CIM D pH7,0
Monolithe CIM D pH4,8
Poros D pH 7,0
Poros D pH 6,0
Poros D pH 5,5
Test of supports, conditions

Figure 4

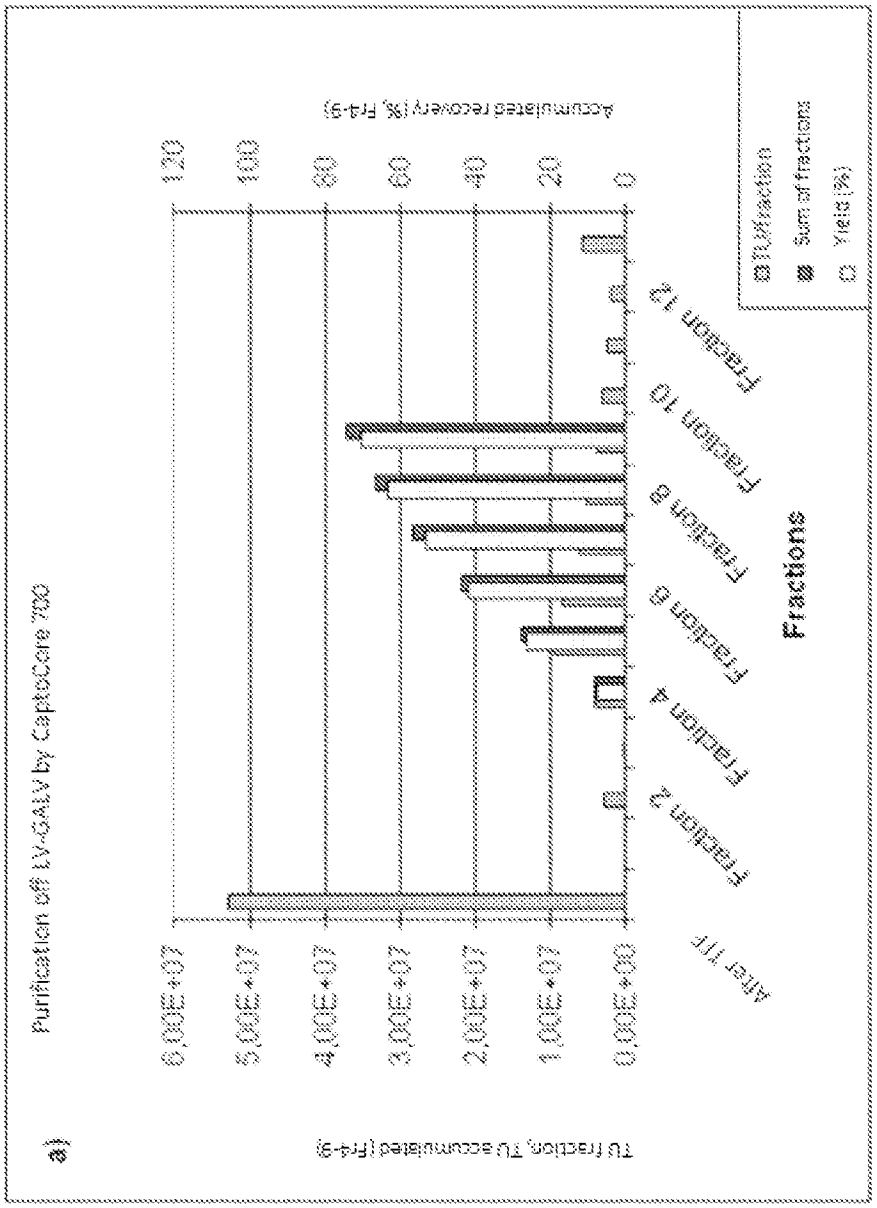
a)
Purification off UV-cAUV by CaptoCore 700
TU fraction, TU accumulated (Fr4-9)
Accumulated recovery (%, Fr4-9)
6,00E+07
5,00E+07
4,00E+07
3,00E+07
2,00E+07
1,00E+07
0,00E+00
120
100
80
60
40
20
0
After TFF
Fraction 2
Fraction 4
Fraction 6
Fraction 8
Fraction 10
Fraction 12
Fractions
TU/Fraction
Sum of fractions
Yield (%)

Figure 5

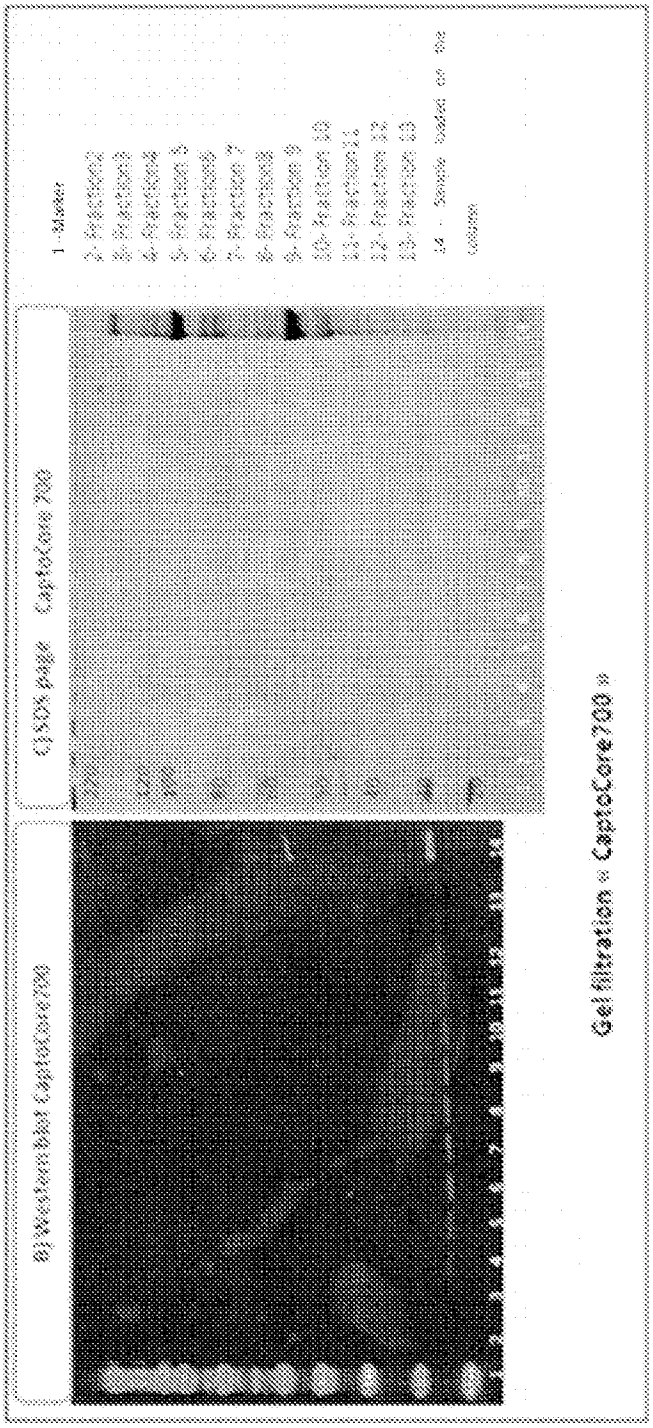

Figure 5 (continued)

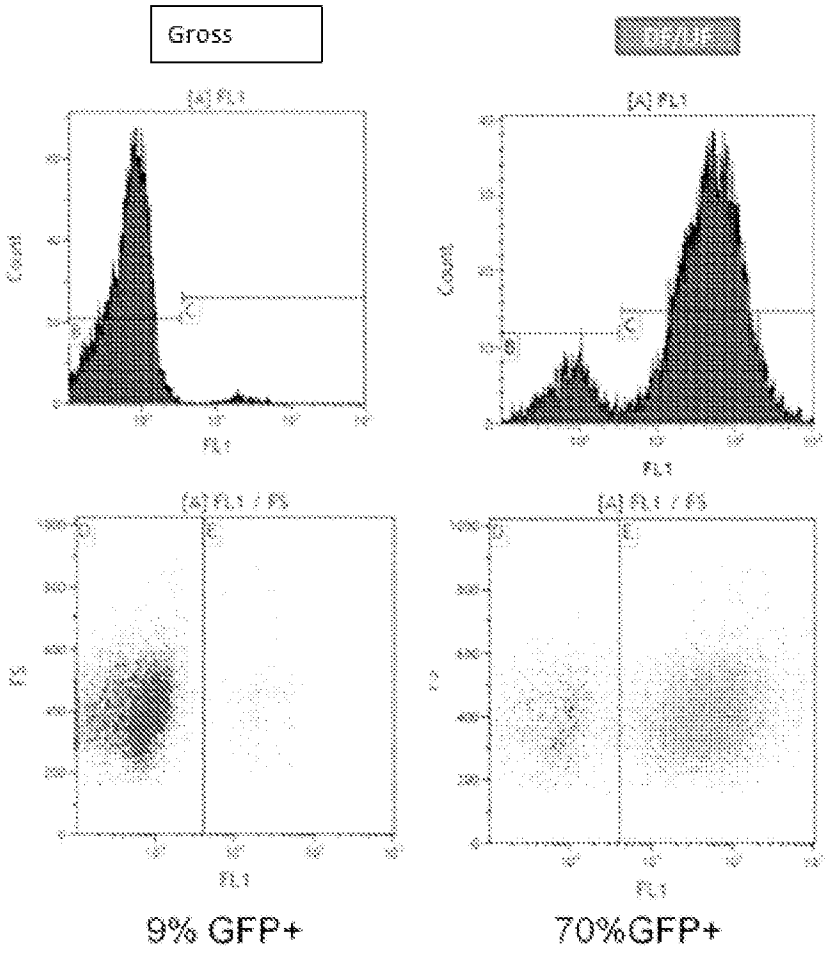

Figure 6