METHODS FOR THE REMOVAL OF FREE FACTOR VIII FROM PREPARATIONS OF LENTIVIRAL VECTORS MODIFIED TO EXPRESS SAID PROTEIN

RELATED APPLICATIONS

The instant application claims the benefit of priority to US Provisional Application No. 63/043/697, filed June 24, 2020 and US Provisional Application No. 63/062,120, filed August 6, 2020. The contents of the aforementioned provisional applications are incorporated by reference herein in their entireties.

BACKGROUND

Lentiviral vectors (LVs) and other viral vectors are an attractive tool for gene therapy (Thomas et al., 2003). LVs can transduce a broad range of tissues, including non-dividing cells such as hepatocytes, neurons and hematopoietic stem cells. Moreover, LVs can integrate into target cell genomes and provide long-term transgene expression.

In view of the ever-increasing need for viral vectors for gene therapy, improved methods of purification would be highly desired. Herein, we disclose improved methods for generating viral vector compositions that are suitable for systemic administration to subjects.

SUMMARY

The present disclosure relates to the purification of enveloped viral vectors for gene therapy applications. The present disclosure is based, at least in part, on the discovery that undesirable transgene protein contaminants can co-purify with a viral vector during conventional purification processes. In certain embodiments, the transgene protein contaminant is a by-product of transgene activity or secreted protein due to a promoter-mediated transcription product (e.g., a transgene protein contaminant that a viral vector encodes) generated during the bioproduction process. For example, packaging of a lentiviral vector requires the transfection of two or more plasmids into a host cell. In some embodiments, the plasmids include: (1) an envelope plasmid encoding an envelope protein (e.g., VSV-G); (2) a transfer plasmid encoding the transgene of interest; and (3) one or more packaging plasmids (e.g., a plasmid encoding gag and pol and a plasmid encoding rev, or a plasmid encoding gag, pol, and rev). In certain embodiments, the transgene protein contaminant is a promoter-mediated transcription product of a transfer plasmid. In certain embodiments, the promoter-mediated transcription product of the transfer plasmid is produced by

the host cell (e.g., host packaging cell). In certain embodiments, the transgene protein contaminant is present in in the viral vector product sample, and may be present in detectable quantities.

The presence of a transgene protein contaminant in the viral vector drug product could result in one or more side effects when used for therapeutic treatment. This is particularly important in gene therapy approaches where the viral vector drug product is systemically administered. Systemic administration of viral vectors typically requires high viral vector doses to achieve efficient transduction of cells within a patient. In the case where a transgene protein contaminant is not sufficiently reduced and/or eliminated, the contaminating protein may be co-administered into a patient, and negatively impact the viral vector mediated production of the same protein within transduced cells of the body. Accordingly, the present invention provides methods to remove these transgene protein contaminants such that the viral vector drug substance is substantially free of the transgene protein contaminant.

In one aspect, the present invention provides a method of purifying a viral vector from a host cell, the method comprising: (i) contacting a composition comprising the viral vector and a transgene protein contaminant with a first chromatography matrix capable of selectively binding the transgene protein contaminant; and (ii) recovering the viral vector in the flow-through of the chromatography matrix, thereby separating the viral vector from the transgene protein contaminant.

In certain exemplary embodiments, the method further comprises (iii) contacting the composition comprising the viral vector and the transgene protein contaminant with a second chromatography matrix capable of selectively binding the viral vector, and (iv) eluting the viral vector from the second chromatography matrix, wherein steps (iii) and (iv) are performed prior to step (i).

In certain exemplary embodiments, the method further comprises (iii) contacting the viral vector recovered from step (ii) with a second chromatography matrix capable of selectively binding the viral vector, and (iv) eluting the viral vector from the second chromatography matrix.

In certain exemplary embodiments, steps (i) and (ii) are repeated multiple times.

In certain exemplary embodiments, step (i) is conducted in the presence of an agent that stabilizes the transgene protein contaminant. In certain exemplary embodiments, the agent is $CaCl_2$.

In certain exemplary embodiments, the method further comprises the step of (v) adjusting the concentration of a salt of the composition comprising the lentiviral vector and a FVIII transgene protein contaminant to a target salt concentration.

In certain exemplary embodiments, step (v) is performed prior to step (i).

In certain exemplary embodiments, if steps (iii) and (iv) are performed prior to step (i), then step (v) is performed between steps (iv) and (i).

In certain exemplary embodiments, the salt is NaCl.

In certain exemplary embodiments, the target salt concentration is from about 0.2M to about 0.6M. In certain exemplary embodiments, the target salt concentration is about 0.4M. In certain exemplary embodiments, the target salt concentration is from about 200 mM NaCl to about 600 mM NaCl.

In certain exemplary embodiments, step (i) is performed by loading the first chromatography matrix with the composition comprising the viral vector and the transgene protein contaminant in a loading buffer comprising Tris-HCl buffer at a pH about 7.0 to about 7.5. In certain embodiments, the loading buffer further comprises about 200 to about 600 mM NaCl and optionally about 2 mM $MgCl_2$.

In other exemplary embodiments, step (i) is performed by loading the first chromatography matrix with the composition comprising the viral vector and the transgene protein contaminant in a loading buffer comprising a phosphate buffer at a pH about 7.0 to about 7.5. In certain embodiments, the loading buffer further comprises about 200 to about 600 mM NaCl and optionally about 2 mM $MgCl_2$.

In certain exemplary embodiments, step (iv) is performed by eluting the viral vector from the second chromatography matrix with an elution buffer comprising Tris-HCl buffer at a pH about 7.0 to about 7.5. In certain embodiments, the elution buffer further comprises about 200 to about 600 mM NaCl and optionally about 2 mM $MgCl_2$.

In other exemplary embodiments, step (iv) is performed eluting the viral vector from the second chromatography matrix with an elution buffer comprising a phosphate buffer at a pH about 7.0 to about 7.5. In certain embodiments, the elution buffer further comprises about 200 to about 600 mM NaCl and optionally about 2 mM $MgCl_2$.

In certain exemplary embodiments, the method further comprises the step of (vi) combining the viral vector separated from the transgene protein contaminant with one or more pharmaceutical excipients to produce a pharmaceutical composition comprising the viral vector, wherein the pharmaceutical composition is substantially free of the transgene protein contaminant.

In certain exemplary embodiments, step (vi) is performed by ultrafiltration / diafiltration (UF/DF) of the viral vector separated from the transgene protein contaminant with a formulation buffer. In certain exemplary embodiments, the UF/DF step comprises tangential flow filtration (TFF).

In certain exemplary embodiments, the formulation buffer is a phosphate or histidine buffer comprising NaCl and Sucrose.

In certain exemplary embodiments, the pharmaceutical composition contains less than 20% of the transgene protein contaminant present in a reference viral vector composition purified with the second chromatography matrix but not the first chromatography matrix.

In certain exemplary embodiments, the viral vector is an enveloped viral vector. In certain exemplary embodiments, the enveloped viral vector is a lentiviral vector.

In certain exemplary embodiments, the viral vector encodes the transgene protein contaminant. In certain exemplary embodiments, the transgene protein contaminant is produced in the host cell by expression from a transfer plasmid.

In certain exemplary embodiments, the first chromatography matrix is selected from the group consisting of an affinity chromatography column, a cationic exchange (CEX) chromatography column, a multimodal chromatography (MMC) column and a hydrophobic interaction chromatography (HIC) column.

In certain exemplary embodiments, the first chromatography matrix is an affinity chromatography column comprising an affinity ligand that specifically binds to the transgene protein contaminant.

In certain exemplary embodiments, the second chromatography matrix is an anionic exchange (AEX) chromatography matrix. In certain exemplary embodiments, the second chromatography matrix is an anionic exchange (AEX) membrane.

In certain exemplary embodiments, the transgene protein is a clotting factor. In certain exemplary embodiments, the clotting factor is FVIII or FVIIIXTEN.

In certain exemplary embodiments, the first chromatography matrix is a VIIISelect affinity chromatography matrix.

In certain exemplary embodiments, the host cell is a mammalian cell or an insect cell. In certain exemplary embodiments, the mammalian cell is a CHO cell, a HEK293 cell, or a HeLa cell.

In certain exemplary embodiments, the composition comprising the viral vector and the transgene protein contaminant is a cell culture supernatant generated by culturing the host cell and separating and clarifying the cell culture supernatant from the host cell.

In certain exemplary embodiments, the cell culture supernatant is subjected to nuclease treatment.

In another aspect, the present invention provides a method of purifying a lentiviral vector (LVV) from a host cell, the lentiviral vector comprising a FVIII transgene, the method comprising: (i) contacting a composition comprising the lentiviral vector and a FVIII transgene protein contaminant with a first chromatography matrix capable of selectively binding FVIII transgene protein contaminant; and (ii) recovering the lentiviral vector in the flow-through of the chromatography matrix, thereby separating the lentiviral vector from the FVIII protein contaminant.

In certain exemplary embodiments, the method further comprises (iii) contacting the composition comprising the lentiviral vector and the FVIII transgene protein contaminant with a second chromatography matrix capable of selectively binding the lentiviral vector, and (iv) eluting the lentiviral vector from the second chromatography matrix, wherein steps (iii) and (iv) are performed prior to step (i), thereby separating the lentiviral vector from host cell contaminants.

In certain exemplary embodiments, the method further comprises (iii) contacting the lentiviral vector recovered from step (ii) with a second chromatography matrix capable of selectively binding the lentiviral vector, and (iv) eluting the lentiviral vector from the second chromatography matrix.

In certain exemplary embodiments, steps (i) and (ii) are repeated multiple times.

In certain exemplary embodiments, step (i) is conducted in the presence an agent that stabilizes the transgene protein contaminant. In certain exemplary embodiments, the agent is $CaCl_2$.

In certain exemplary embodiments, the method further comprises the step of (v) adjusting the concentration of a salt of the composition comprising the lentiviral vector and a FVIII transgene protein contaminant to a target salt concentration.

In certain exemplary embodiments, step (v) is performed prior to step (i).

In certain exemplary embodiments, if steps (iii) and (iv) are performed prior to step (i), then step (v) is performed between steps (iv) and (i).

In certain exemplary embodiments, the salt is NaCl.

In certain exemplary embodiments, the target salt concentration is from about 0.2M to about 0.6M. In certain exemplary embodiments, the target salt concentration is about 0.4M. In certain exemplary embodiments, the target salt concentration is from about 200 mM NaCl to about 600 mM NaCl.

In certain exemplary embodiments, step (i) is performed by loading the first chromatography matrix with the composition comprising the lentiviral vector and the FVIII transgene protein contaminant in a loading buffer comprising Tris-HCl buffer at a pH about 7.0 to about 7.5. In certain embodiments, the loading buffer further comprises about 200 to about 600 mM NaCl and optionally about 2 mM $MgCl_2$.

In other exemplary embodiments, step (i) is performed by loading the first chromatography matrix with the composition comprising the lentiviral vector and the FVIII transgene protein contaminant in a loading buffer comprising a phosphate buffer at a pH about 7.0 to about 7.5. In certain embodiments, the loading buffer further comprises about 200 to about 600 mM NaCl and optionally about 2 mM $MgCl_2$.

In certain exemplary embodiments, step (iv) is performed by eluting the lentiviral vector from the second chromatography matrix with an elution buffer comprising Tris-HCl buffer at a pH about 7.0 to about 7.5. In certain embodiments, the elution buffer further comprises about 200 to about 600 mM NaCl and optionally about 2 mM $MgCl_2$.

In other exemplary embodiments, step (iv) is performed eluting the lentiviral vector from the second chromatography matrix with an elution buffer comprising a phosphate buffer at a pH about 7.0 to about 7.5. In certain embodiments, the elution buffer further comprises about 200 to about 600 mM NaCl and optionally about 2 mM $MgCl_2$.

In certain exemplary embodiments, the method further comprises the step of (vi) combining the lentiviral vector separated from the transgene protein contaminant with one or more pharmaceutical excipients to make a pharmaceutical composition comprising the lentiviral vector, wherein the pharmaceutical composition is substantially free of the FVIII transgene protein contaminant.

In certain exemplary embodiments, step (vi) is performed by ultrafiltration / diafiltration (UF/DF) of the lentiviral vector separated from the transgene protein contaminant with a formulation buffer. In certain exemplary embodiments, the formulation buffer is a phosphate or histidine buffer comprising NaCl and Sucrose. In certain exemplary embodiments, the UF/DF step comprises tangential flow filtration (TFF).

In certain exemplary embodiments, the pharmaceutical composition contains less than 20% of the FVIII transgene protein contaminant present in a reference viral vector composition purified with the second chromatography matrix but not the first chromatography matrix.

In certain exemplary embodiments, the first chromatography matrix is selected from the group consisting of affinity chromatography column, cationic exchange (CEX) chromatography column, multimodal chromatography (MMC) column and hydrophobic interaction chromatography (HIC) column.

In certain exemplary embodiments, the first chromatography matrix is an affinity chromatography column comprising an affinity ligand that specifically binds to the recombinant FVIII protein. In certain exemplary embodiments, the affinity ligand is VIIISelect.

In certain exemplary embodiments, the FVIII transgene protein contaminant is a B-domain deleted FVIII protein. In certain exemplary embodiments, the FVIII transgene protein contaminant is a human FVIII protein comprising the amino acid sequence of SEQ ID NO:4. In certain exemplary embodiments, the FVIII transgene protein contaminant is a FVIIIXTEN molecule. In certain exemplary embodiments, the FVIIIXTEN molecule comprises the amino acid sequence of SEQ ID NO:5.

In certain exemplary embodiments, the second chromatography matrix is an anionic exchange (AEX) chromatography matrix. In certain exemplary embodiments, the second chromatography matrix is an anionic exchange (AEX) membrane.

In certain exemplary embodiments, the host cell is a mammalian cell or an insect cell. In certain exemplary embodiments, the mammalian cell is a CHO cell, a HEK293 cell, or a HeLa cell.

In certain exemplary embodiments, the composition comprising the viral vector and the FVIII transgene protein contaminant is a cell culture supernatant generated by culturing the host cell and separating and clarifying the cell culture supernatant from the host cell. \

In certain exemplary embodiments, the cell culture supernatant is subjected to nuclease treatment.

In another aspect, the present invention provides a composition comprising a viral vector produced according a method described herein.

In certain exemplary embodiments, the composition is a pharmaceutical composition comprising the viral vector and one or more pharmaceutical excipients, and wherein the composition is substantially free of transgene protein contaminant.

In certain exemplary embodiments, the transgene protein is a clotting factor. In certain exemplary embodiments, the clotting factor is FVIII, FVIIIXTEN, or FIX. In certain exemplary embodiments, the clotting factor is a B-domain deleted FVIII protein. In certain exemplary embodiments, the clotting factor is a human FVIII protein comprising the amino acid sequence of SEQ ID NO:4. In certain exemplary embodiments, the clotting factor is a FVIII-XTEN molecule. In certain exemplary embodiments, the FVIII-XTEN molecule comprises the amino acid sequence of SEQ ID NO:5.

In certain exemplary embodiments, the composition contains less than 20% of the transgene protein present in a reference viral vector composition purified with the second chromatography matrix but not the first chromatography matrix. In certain exemplary embodiments, the composition has a total FVIII activity level of less than 10% of the reference viral vector composition purified with the second chromatography matrix but not the first chromatography matrix. In one exemplary embodiment, the composition has a total FVIII activity level of about 1 IU or less.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows schematics of a conventional process work flow to manufacture an enveloped viral vector drug substance for gene therapy applications. FIG. 1A shows the conventional downstream work flow, involving a single vector capture purification chromatography step that is primarily responsible for purifying viral vector from process- and product-related impurities. FIG. 1B shows an improved downstream work flow according to one embodiment of the present disclosure, involving at least two purification chromatography steps, one of which is a transgene protein capture step of the invention.

FIG. 2 are plots showing the activity of FVIIIXTEN transgene protein per 1E9 TU of lentiviral vector during each step of the conventional purification process as shown in FIG. 1A. The contaminating FVIIIXTEN protein activity is consistently present during various stages of the production process, including in the final drug substance (DS) and drug product (DP).

FIG. 3 are plots showing the evaluation of VIIISelect affinity chromatography for the purification of lentiviral vector from FVIII transgene protein encoded by the viral vector. The experiments were conducted with either FVIII (Exp 1) or FVIIIXTEN (Exp 2) transgene protein. FIG. 3A is a plot showing the level of transgene protein activity remaining in product following purification by a conventional One Column Process (e.g., as shown in FIG. 1A) or a Two Column Process of the invention that includes VIIISelect as the additional transgene protein capture step (e.g., as shown in FIG. 1B). FIG. 3B is a plot showing percent reduction of transgene protein activation for the Two Column Process as compared to the One Column Process.

FIG. 4 is a plot showing the results of using a calcium chloride in a Two Column Process of the invention. VIIISelect affinity chromatography was employed to capture FVIII transgene protein in the presence ("+CaCl2") or absence of $CaCl_2$ ("-CaCl2"). Lentiviral vector encoding FVIIIXTEN (LV-FVIIIXTEN) was purified in phosphate buffer (Exp 3), whereas lentiviral vector encoding FVIII (LV-FVIII) was purified in histidine buffer (Exp 4).

FIG. 5 is a plot showing the total FVIII activity remaining in a lentiviral vector preparation encoding FVIII (LV-FVIII) after conducting multiple transgene protein capture steps. The viral sample was recycled by passing it through a VIIISelect affinity chromatography resin multiple times to capture transgene protein encoded by the viral vector. Each transgene protein capture step

was performed in the presence of $CaCl_2$. The viral vector LV-FVIII was purified in histidine buffer.

FIG. 6 shows schematics of a downstream process work flow to manufacture an enveloped viral vector drug substance for gene therapy applications, according to one embodiment of the invention. FIG. 6A shows an improved downstream work flow according to one embodiment of the present disclosure, wherein the load sample for the second chromatography purification step is adjusted. FIG. 6B shows an improved downstream work flow according to one embodiment of the present disclosure, wherein the load sample for the first chromatography purification step is adjusted, and the load sample for the second chromatography purification step is adjusted.

FIG. 7 are plots showing the impact of salt concentration on the effectiveness of purification using an improved downstream work flow according to one embodiment of the present disclosure. FIG. 7A is a plot showing the level of FVIIIXTEN activity detected as a function of total functional vector recovery before (black bars) and after (grey bars) applying a sample to an VIIISelect affinity chromatography resin, wherein the load sample comprises 400 mM NaCl (Exp5) or 100 mM NaCl (Exp6). FIG. 7B is a plot showing the level of FVIIIXTEN activity detected as a function of total P24 capsid recovery before (black bars) and after (grey bars) applying a sample to an VIIISelect affinity chromatography resin, wherein the load sample comprises 400 mM NaCl (Exp5) or 100 mM NaCl (Exp6).

FIG. 8 is a plot showing the total FVIIIXTEN activity detected (black bars), FVIIIXTEN activity detected as a function of total functional vector recovery (dark grey bars), and FVIIIXTEN activity detected as a function of total P24 capsid recovery (light grey bars)

FIG. 9 is a plot showing the impact of various salt concentrations (200, 400 or 600 mM NaCl) on the removal of contaminating FVIII transgene protein during purification of FVIII-encoding viral vectors in a two-step purification process using AEX followed by VIIISelect with TRIS or Phosphate buffers. Total contaminant FVIII transgene activity (IU) was measured in the loading sample (solid black bar for TRIS buffer; solid grey bar for Phosphate loading buffer) following AEX elution and prior to VIIISelect purification and in the flow-through product fraction following VIIISelect purification (lined back bar for TRIS buffer; lined grey bar for Phosphate buffer).

DETAILED DESCRIPTION

The present disclosure is based on the finding that during bioproduction of viral vector, the final viral vector product could contain a transgene protein contaminant. For example, during manufacturing of lentiviral vector (LV-FVIII or LV-FVIIIXTEN) product using HEK293T cells, it was unexpectedly found that upstream CMV promoter activity in the transgene encoding plasmid (e.g., transfer plasmid) could mediate transgene mRNA production that would result in the production of contaminating FVIII or FVIIIXTEN protein. During purification, such FVIII or FVIIIXTEN transgene protein contaminant could be inadvertently co-purified with lentiviral vector via non-specific association with vector particles and end up being inadvertently co-administered to a patient. Administering a viral vector product that contains a transgene protein contaminant could interfere with the efficiency of the viral vector to transduce target cells (e.g., target cells within a patient), and/or with viral vector mediated expression of the same transgene protein. Furthermore, the existence of contaminating transgene protein (e.g., FVIII or FVIIIXTEN) in the lentiviral vector drug product (LV-FVIII or LV-FVIIIXTEN) could potentially elicit unwanted immune responses that result in the generation of antibodies against the transgene protein contaminant (e.g., anti-drug antibodies (ADAs)) and/or potential cytotoxic T-cell responses against LV-transduced cells.

Therefore, to circumvent the issue of the presence of a transgene protein contaminant in a viral vector product, the instant disclosure provides methods for the purification of a viral vector from a host cell, comprising the reduction and/or elimination of a transgene protein contaminant that the viral vector encodes. In certain embodiments, purification of a viral vector utilizing the methods provided herein results in a viral vector product that is substantially free of a transgene protein contaminant.

Generally, nomenclature used in connection with cell and tissue culture, molecular biology, biophysics, immunology, microbiology, genetics, and protein and nucleic acid chemistry described herein is well-known and commonly used in the art. The methods and techniques provided herein are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. Enzymatic reactions and purification techniques are performed according to manufacturer's specifications, as commonly accomplished in the art or as

described herein. The nomenclature used in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein is well-known and commonly used in the art. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients.

Unless otherwise defined herein, scientific and technical terms used herein have the meanings that are commonly understood by those of ordinary skill in the art. In the event of any latent ambiguity, definitions provided herein take precedence over any dictionary or extrinsic definition. Unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The use of "or" means "and/or" unless stated otherwise. The use of the term "including," as well as other forms, such as "includes" and "included," is not limiting.

So that the invention may be more readily understood, certain terms are first defined.

As used herein, the term "about," when used in reference to a particular recited numerical value, means approximately, roughly, around, or in the regions of. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the upper and lower boundaries of the numerical values set forth. In general, the term "about" is used herein to modify a numerical value above and below the stated value by a variance of 10 percent, higher or lower than the stated value. For example, as used herein, the expression "about 100" includes 90 and 110 and all values in between (e.g., 91, 92, 93, 94, etc.). In certain embodiments, the term "about" means that the value may vary from the recited value by no more than 1%. For example, as used herein, the expression "about 100" includes 99 and 101 and all values in between (e.g., 99.1, 99.2, 99.3, 99.4, etc.).

As used herein, the term "vector" refers to any vehicle for the cloning of and/or transfer of a nucleic acid into a host cell. A vector can be a replicon to which another nucleic acid segment can be attached so as to bring about the replication of the attached segment. The term "vector" includes both viral and non-viral vehicles for introducing the nucleic acid into a cell *in vitro*, *ex vivo* or *in vivo*. A large number of vectors are known and used in the art including, for example, plasmids, modified eukaryotic viruses, or modified bacterial viruses. Insertion of a polynucleotide into a suitable vector can be accomplished by ligating the appropriate polynucleotide fragments into a chosen vector that has complementary cohesive termini.

As used herein, the term "viral vector" refers to any viral vehicle for introducing a nucleic acid into a cell. A viral vector may be utilized for introducing a nucleic acid into any cell in any way, for example, *in vitro*, *ex vivo*, or *in vivo*. As known to those of skill in the art, a viral vector is packaged (produced) by a packaging cell (e.g., a packaging cell line). In some embodiments, one or more plasmids are introduced into a packaging cell in order to produce the viral vector. In some embodiments, three or more plasmids are introduced into a packaging cell in order to produce the viral vector. In the production of a lentiviral vector, typically, (1) a transfer plasmid comprising a transgene of interest; (2) an envelope plasmid comprising an envelope protein coding sequence (e.g., VSV-G); and (3) one or more packaging plasmids are co-transfected into a packaging cell. The lentiviral packaging plasmids comprise at least the gag, pol, and rev genes, on a single plasmid (e.g., in the case of second generation lentiviral vector production), or on separate plasmids (i.e., one packaging plasmid comprising the gag and pol genes, and another packaging plasmids comprising the rev gene; e.g., in the case of third generation lentiviral vector production). In the production of adeno-associated viral (AAV) vector, typically, (1) a transfer plasmid comprising a transgene of interest; (2) a rep/cap plasmid comprising the rep and cap genes required for the AAV life cycle; and (3) a helper plasmid comprising genes that mediate AAV replication are co-transfected into a packaging cell. Variations on the production of viral vector are known to those of skill in the art. For example, one of skill in the art will appreciate that one or more of the vectors can be combined into a single vector.

As used herein, the phrase "recombinant lentiviral vector" refers to a vector with sufficient lentiviral genetic information to allow packaging of an RNA genome (*e.g.,* a heterologous RNA genome), in the presence of packaging components, into a viral particle capable of infecting a target cell. Infection of the target cell may include reverse transcription and integration into the target cell genome. The recombinant lentiviral vector carries non-viral coding sequences which are to be delivered by the vector to the target cell. A recombinant lentiviral vector is incapable of independent replication to produce infectious lentiviral particles within the final target cell. Usually the recombinant lentiviral vector lacks a functional gag-pol and/or env gene and/or other genes essential for replication.

As used herein the terms "heterologous" or "exogenous" refer to such molecules that are not normally found in a given context, e.g., in a cell or in a polypeptide. For example, an exogenous or heterologous molecule can be introduced into a cell and are only present after

manipulation of the cell, e.g., by transfection or other forms of genetic engineering or a heterologous amino acid sequence can be present in a protein in which it is not naturally found. Accordingly, a recombinant lentiviral vector comprising a heterologous RNA genome refers to an RNA genome that is not naturally found in a lentivirus.

As used herein, the term "transgene protein contaminant" refers to a transgene protein that is produced in a host cell during production of the viral vector in the host cell *ex vivo*. In certain embodiments, the transgene protein is encoded by a transgene of the viral vector but is unintentionally expressed in the host cell during the viral vector production. In certain embodiments, the transgene protein product may be a by-product of undesired transgene activity in the host cell. In other embodiments, the transgene protein may be secreted by the host cell due to promoter-mediated transcription. In exemplary embodiments, the transgene protein contaminant may be produced in the host cell by spurious expression from a transfer plasmid containing the transgene of interest.

As used herein, the term "capable of selectively binding" refers to having the ability to bind (*e.g.,* reversibly bind) a certain molecule over other molecules (*e.g.,* preferentially binds a particular molecule; has a greater affinity for; binds a particular molecule to a greater degree than, or to the exclusion of, other molecules). For example, when a composition comprising a viral vector is contacted with a chromatography matrix capable of selectively binding the viral vector, the chromatography matrix will bind (e.g., retain) the viral vector over other molecules that may be present in the composition (e.g., the chromatography matrix substantially binds more of the viral vector compared to other molecules present in the composition). As such, when a chromatography matrix selectively binds a viral vector, the viral vector is retained by the chromatography matrix.

As used herein, the term "treat" refers to an amelioration or reduction of one or more symptoms of a disorder. Treating can, but need not, be a cure.

As used herein, the phrase "systemically administer" refers to prescribing or giving a pharmaceutical composition comprising a drug product to a subject, such that the drug product is introduced into the circulatory system (e.g., bloodstream) of the subject. In certain embodiments, systemic administration refers to prescribing or giving a pharmaceutical composition comprising a viral vector (e.g., a lentiviral vector) to a subject, such that the viral vector is introduced into the circulatory system (e.g., bloodstream) of the subject. Examples of routes of systemic

administration include, but are not limited to, intravenous, *e.g.,* intravenous injection and intravenous infusion, *e.g.,* via central venous access, oral administration, intramuscular administration, intradermal administration, and subcutaneous administration.

A. PURIFICATION OF VIRAL VECTORS

The present disclosure is based on the introduction of a purification step (herein the "transgene protein capture step") to a viral vector production process in order to reduce and/or eliminate a transgene protein contaminant. Introduction of the transgene protein capture step allows for the reduction and/or elimination of a transgene protein contaminant from a composition comprising a viral vector, and without limitation, could provide the following clinical benefits: (1) improve potency of viral vector drug product used to transduce cells within patient body; (2) potentially improve efficacy of gene therapy product administered systemically; (3) improve the safety profile of *in vivo* gene therapy products as a result of minimizing potential immune responses against protein antigens immediately after administration (e.g., minimize the potential development of anti-drug antibodies); (4) minimize any negative therapeutic effect or side effects due to presence of transgene protein product; and (5) improve product quality and increase product purity. In certain embodiments, the transgene protein contaminant is produced in the host cell by expression from a transfer plasmid.

In certain aspects, the transgene protein capture step comprises: (i) contacting a composition comprising the viral vector and a transgene protein contaminant with a first chromatography matrix capable of selectively binding the transgene protein contaminant; and (ii) recovering the viral vector in the flow-through of the chromatography matrix, thereby separating the viral vector from the transgene protein contaminant. As will be appreciated by one of skill in the art, the transgene capture step can be performed once or multiple times (two or more times, three or more times, *etc.*) using the same or different transgene capture steps. The resultant viral vector may then be utilized for introducing a nucleic acid into any cell in any way, for example, *in vitro*, *ex vivo*, or *in vivo*. In certain embodiments, a viral vector of the present disclosure is a lentiviral vector.

As known in the art, the safety profile of a drug product can depend on the development of any unwanted immunogenicity against the drug product (e.g., development of an anti-drug antibody against the drug product). The development of unwanted immunogenicity is affected by

the route of administration (e.g., intradermal, subcutaneous, inhalation, intramuscular, intravenous routes, and the like), dose, and frequency of administration of the drug product. Methods of viral vector purification incorporating one or more transgene capture steps described herein reduces and/or eliminates a transgene protein contaminant that may be present in an administrable viral vector drug product, and as such, reduces and/or eliminates the development of unwanted immunogenicity against the transgene protein contaminant. Those of skill in the art will appreciate that the reduction and/or elimination of the development of unwanted immunogenicity can occur however the drug product (e.g., viral vector drug product) is administered, at any dosage, and at any dosing frequency.

In certain embodiments, the transgene protein capture step (e.g., the transgene impurity capture step) employs the use of one or more chromatography separation techniques. In certain embodiments, transgene protein capture step employs the use of one or more chromatography matrices. In some embodiments, the one or more chromatography matrices are selected from the group consisting of affinity chromatography, cation exchange (CEX) chromatography, anion exchange (AEX) chromatography, multimodal chromatography (MMC) and hydrophobic interaction chromatography (HIC).

In various embodiments described herein, various buffers and solutions that are known to those of skill in the art are used. Any suitable buffers and solutions known to those of skill in the art can be used. For example, a purification step may require the use of several buffers and solutions, e.g., sanitization solutions, equilibration buffers, wash buffers, elution buffers, stripping buffers, regeneration buffers, diluent buffers, and the like.

In certain embodiments, transgene protein capture step employs affinity chromatography. The term "affinity chromatography" refers to a protein separation technique in which a protein is reversibly and specifically bound to an affinity ligand (e.g., an affinity ligand that specifically binds to a transgene protein contaminant). As used herein, the term “specifically binds” refers to the ability to mediate a binding reaction with a molecule which is highly preferential to the molecule.

Affinity chromatography utilizes specific binding interactions between molecules to separate components. In affinity chromatography, an affinity ligand is immobilized to a solid support, e.g., resin, such that when a composition is passed through the solid support, molecules having specific binding affinity for the affinity ligand become retained by the resin. In certain

embodiments, transgene protein capture step utilizes an affinity chromatography column comprising an affinity ligand that specifically binds to the transgene protein contaminant. In some embodiments, affinity ligands are immobilized or coupled directly to solid support material by formation of covalent chemical bonds between particular functional groups on the affinity ligand (e.g., primary amines, sulfhydryls, carboxylic acids, aldehydes) and reactive groups on the support. In some embodiments, affinity ligands are immobilized by indirect coupling approaches, e.g., via a glutathione S-transferase (GST)-tagged fusion protein captured to a glutathione support via the glutathione-GST affinity interaction. Affinity ligands that bind to general classes of proteins (e.g., antibodies) or commonly used fusion protein tags (e.g., Histidine tag, or His-tag) are readily available in pre-immobilized forms for use in affinity purification. In certain embodiments, more specialized affinity ligands such as specific antibodies or antigens of interest can be immobilized. For example, a peptide antigen can be immobilized to a support and used to purify antibodies that recognize the peptide. Accordingly, in certain embodiments, the present disclosure provides a method of purifying a viral vector from a host cell, comprising: subjecting a composition comprising the viral vector and a transgene protein contaminant to affinity chromatography purification capable of selectively binding the transgene protein contaminant; and recovering the viral vector in the resultant flow-through of the affinity chromatography purification, thereby separating the viral vector from the transgene protein contaminant. In certain embodiments, the method of purifying a viral vector from a host cell comprises: (i) contacting a composition comprising the viral vector and a transgene protein contaminant with an affinity chromatography matrix capable of selectively binding the transgene protein contaminant; and (ii) recovering the viral vector in the flow-through of the affinity chromatography matrix, thereby separating the viral vector from the transgene protein contaminant.

For the purposes of reducing and/or eliminating the presence of a specific transgene protein contaminant (e.g., an FVIII protein) in a viral vector product, in certain embodiments, the affinity ligand, is covalently attached to a chromatographic solid phase material and is accessible to the contaminating protein (e.g., an FVIII protein) in solution as the solution contacts the chromatographic solid phase material. The transgene protein contaminant (e.g., FVIII protein) is retained via its specific binding affinity for the affinity ligand during the chromatographic steps, while other solutes and/or proteins in the mixture (e.g., viral vector) do not bind appreciably or specifically to the ligand. Binding of the transgene protein contaminant to the immobilized ligand

allows the desired viral vector to be passed through the chromatographic medium while the contaminating protein remains specifically bound to the immobilized ligand on the solid phase material. Any component can be used as a ligand for the purposes of reducing and/or eliminating its respective specific binding partner. In certain embodiments, the affinity ligand is an antibody, antibody fragment, antibody variant, peptidomimetic or peptide having specific binding affinity for the transgene, e.g. an antibody, antibody fragment, antibody variant, peptidomimetic or peptide having binding affinity for FVIII. In an exemplary embodiment, a ligand for the FVIII protein is VIIISelect™ from GE Healthcare/Cytiva.

In certain embodiments, affinity purification employs various buffers and solutions. Any buffers and solutions known to those of skill in the art can be used. For example, a suitable sanitization buffer can comprise 0.5 M NaOH; a suitable equilibration buffer can comprise 20 mM Tris or 20 mM phosphate, 0 to 2 mM $MgCl_2$, 10-30 mM $CaCl_2$, 10 to 70 mM NaCl, and have a pH of 7.2; a suitable stripping or cleaning buffer can comprise 0.5 M NaOH, a solution containing phosphoric acid, Benzyle alcohol, or acetic acid; and a suitable regenerating buffer can comprise 20 mM Tris, 2 mM $MgCl_2$, 150 mM NaCl, and have a pH of 7.2.

In some embodiments, the transgene protein capture step comprises subjecting a composition comprising a viral vector and a transgene protein contaminant to ion exchange chromatography purification capable of retaining the transgene protein contaminant within the chromatographic substrate (e.g., matrix, resin, column). As used herein, the term "ion exchange chromatography" refers to the separation of ionizable molecules based on their total surface charge. In certain embodiment, embodiments, the ion exchange chromatography employs a cation exchange resin. The terms "cation exchange resin," "cation exchange adsorbent," or "cation exchange matrix" refer to a solid phase which is negatively charged, and which thus has free cations for exchange with cations in an aqueous solution passed over or through the solid phase. A negatively charged ligand attached to the solid phase to form the cation exchange resin can, e.g., be a carboxylate or sulfonate. Commercially available cation exchange resins include carboxy-methyl-cellulose, sulphopropyl (SP) immobilized on agarose (e.g., SP SEPHAROSE-XL, SP-SEPHAROSE- Fast Flow, SP SEPHAROSE-High Performance, CM SEPHAROSE-Fast Flow, CM SEPHAROSE.High Performance, CAPTO-S, and CAPTO-SP ImpRes from GE Healthcare Life Sciences, or FRACTOGEL EMD SE HiCap, FRACTOGEL EMD SO3-,

FRACTOGEL EMD COO-, ESHMUNO S, and ESHMUNO. CPX from EMD Millipore, or UNOSPHERE- S and NUVIA- S from Bio-Rad).

In some embodiments, the transgene protein capture step comprises subjecting a composition comprising a viral vector and a transgene protein contaminant to hydrophobic interaction chromatography (HIC) purification capable of retaining the transgene protein contaminant within the chromatographic substrate (e.g., matrix, resin, column). The term "hydrophobic interaction chromatography (HIC)," as used herein, refers to the separation of components based on hydrophobic interactions with a stationary phase (e.g., resin). The elution order in HIC enables components to be ranked on the basis of their relative hydrophobicity. Advantages of HIC include, e.g., the use of non-denaturing conditions, it does not require the use of organic solvents or high temperatures, and separations are carried out at physiological pH, which preserves virus structure when used in virus purification processes. In some embodiments, the transgene protein capture step utilizes hydrophobic interaction chromatography. Hydrophobic interaction chromatography (HIC) is a technique for separating biomolecules based on differences in their surface hydrophobicity. HIC chromatographic media contain hydrophobic ligands such as linear chain hydrocarbons (e.g., propyl (C3), butyl (C4), hexyl (C6), or octyl (C8)) or aromatics (e.g., phenyl). In pure water, the hydrophobic effect is too weak for functional interaction between the ligand and proteins, or between the proteins themselves. However, lyotropic salts enhance hydrophobic interactions, and the addition of salt drives the capture of proteins to HIC media. For this reason, HIC resins are usually loaded under high salt concentrations and eluted at lower salt concentrations. As one of ordinary skill in the art will appreciate, ammonium sulfate [$(NH_4)_2SO_4$] is the most commonly used salt to control capture of proteins via HIC chromatography, because of the high lyotropic ranking of both ammonium and sulfate ions in the Hofmeister series, and the high solubility of the salt. As one of ordinary skill in the art can appreciate, the concentration of salt (e.g., ammonium sulfate) can be manipulated to achieve the optimal concentration for transgene protein contaminant binding. In addition, co-solvents can also affect the hydrophobic interaction. For example, ethylene or propylene glycol can reduce the interaction between protein and the immobilized ligand and thus be useful for improving elution profiles. Examples of suitable hydrophobic resins include without limitation, Capto Adhere, Tosoh Butyl 650M, Tosoh SuperButyl 650C, Tosoh Phenyl 650C, and EMD Fractogel® Phenyl (Tosoh Bioscience LLC, PA).

In some embodiments, the transgene protein capture step comprises subjecting a composition comprising a viral vector and a transgene protein contaminant to multimodal chromatography purification capable of retaining the transgene protein contaminant within the chromatographic substrate (e.g., matrix, resin, column) (e.g., matrix, resin, column). As used herein, the term "mixed mode chromatography" or "multi-modal chromatography," refers to a chromatography that employs a combination of two or more mechanisms in order to achieve separation of components in a mixture. In some embodiments, the transgene protein capture step utilizes multi-modal or mixed mode chromatography (MMC) to separate product-related impurities (e.g., a transgene protein contaminant) from viral vector. In some embodiments, MMC operates on the basis of at least size differences between the virus and the impurities, and/or chemical interaction occurring between the impurities and one or more chromatography ligands. Various types of MMC are known to those of skill in the art, including physical and chemical MMC. Chemical MMC includes combinations between principles of, without limitation, ion exchange chromatography, hydrophobic interaction chromatography, size exclusion chromatography, reverse phase liquid chromatography, and hydrophilic interaction liquid chromatography. Further examples include, without limitation, resins that exploit combinations of hydrogen bonding, pi-pi bonding, and metal affinity. An example of multi-modal chromatography employs the use of Capto® Core 700 chromatography resin (GE Healthcare Bio-Sciences) which comprises octylamine ligands designed to have both hydrophobic and positively charged properties that trap molecules under a certain size, e.g., 700 kilodaltons (kDA). In such chromatography resins, the bead exteriors are inactive, and permits the purification of viral vector by size exclusion, where the desired viral vector passes through the resin and impurities are retained therein.

The steps involved in viral vector purification may be conducted in the presence of certain agents known to those of skill in the art that improve efficiency. For example, in certain embodiments, the transgene protein capture step is conducted in the presence of an agent that stabilizes the transgene protein contaminant. In certain embodiments, the transgene protein capture step is conducted in the presence of an agent that stabilizes the transgene protein contaminant, wherein the agent is $CaCl_2$.

The transgene protein capture step as described herein may be incorporated into a viral vector production process known to one of skill in the art. FIG. 1A is a generic flow diagram

depicting a typical viral vector manufacturing process comprising the upstream steps (thawing of a cell bank vial; cell growth and passaging; transfection and/or induction of host cells to produce viral vector) followed by a series of downstream steps (harvesting of viral vector comprising cell removal (clarification) and nuclease treatment; at least one purification step to separate the vector from other product- and process-related impurities (herein the "viral vector capture step"); and an ultrafiltration/diafiltration step for final formulation of drug substance). In general, the transgene capture step can be incorporated into a viral vector production process at any point between the clarification step and the final step in the downstream process.

Upstream processing steps for viral vector production generally comprises the steps of cell expansion, transfection or infection of vector producing cells, and viral vector production. Generally, transfection or infection of vector producing cells is achieved by transient transfection, or the use of stable producer cell lines in mammalian or insect cells. Cells are transfected with viral vector encoding components and are cultured to increase virus numbers and/or virus titers. Methods of transfection and culturing cells are known to those of skill in the art, and includes at least, providing necessary nutrients to the cell, e.g., a suitable culture media. Methods for culturing cells include, e.g., growth adhering to surfaces, growth in suspension, or combinations thereof. Culturing can be performed in suitable containers, for example, culture dishes, culture flasks, or roller bottles. Large scale culturing is often performed with the use of bioreactors using a variety of systems available to those of skill in the art, including, e.g., batch, fed-batch, continuous systems, hollow fiber, etc.

Following upstream processing steps, downstream processing steps are typically as outlined in FIG. 1A, including the downstream processing steps of clarification, nuclease treatment, viral vector capture, and sterile filtration. The goal of downstream processing is to separate the viral vector from the various impurities produced during upstream processing and to get the vector into the appropriate state for formulation and administration to patients. The skilled artisan would be able to determine the optimal order of the aforementioned steps in order to obtain the most efficient process for viral vector manufacturing (e.g., the optimal order of steps in the process to yield the most viral vector).

The clarification step includes the elimination of large debris and macromolecular complexes from the initial crude suspension. In certain embodiments, clarification includes cell lysis in order to improve viral vector yield. A variety of methods are known to those of skill in

the art seeking to lyse cells, including physical methods such as with the use of microfluidizers or heat-shock treatments to promote lysis. Lysis is often achieved using detergents. Detergents are available in nonionic form, e.g., Triton X-100, Triton X-114, Tween 20, Tween 80, NP-40, octyl glucoside, and octyl thioglucoside, in anionic form, e.g., SDS, and in zwitterionic form, e.g., CHAPS and CHAPSO. In some embodiments, clarification is performed by a filtration step to remove cellular debris and other impurities. Suitable filters include, without limitation, filters comprising cellulose filters, e.g., cellulose fibers combined with inorganic filter aids (e.g., fumed silica, perlite, diatomaceous earth), cellulose fibers combined with organic resins, or any combination thereof, and polymeric filters such as those comprising nylon, polyethersulfone, or polypropylene. Any clarification approach known to those of skill in the art would be suitable for a viral vector production process of the present invention. Suitable clarification approaches include, without limitation, centrifugation, microfiltration, dead-end filtration, depth filtration, membrane filtration, or a combination thereof. Accordingly, clarification may include the use of a combination of filters with decreasing pore size, e.g., down to 0.2 um.

Culturing a host cell (e.g., a viral vector producing cell) followed by subsequent lysis and clarification steps results in a composition comprising a viral vector and a transgene protein contaminant. In certain embodiments, the composition comprising the viral vector and a transgene protein contaminant is a cell culture supernatant generated by culturing the host cell and separating and clarifying the cell culture supernatant from the host cell. The host cell may be any host cell suitable for viral vector production known to those of skill in the art. In certain embodiments, the host cell is selected from the group consisting of a CHO cell, a HEK293 cell (e.g., a HEK293T cell), a HeLa cell, and an insect cell. In certain embodiments, the host cell is a HEK293 cell. In certain embodiments, the host cell is a HEK293T cell.

During and/or after the cell lysis step, a nuclease treatment step may be performed in order to degrade nucleic acids (e.g., host cell nucleic acids, RNA and/or DNA contaminants) and break up any macromolecular complexes. The nuclease treatment step may be performed during and/or after clarification. Suitable nucleases for use in a viral vector production process of the present invention include, without limitation, Benzonase, Denarase, and any other DNase and/or RNase known to those of skill in the art. In some embodiments, instead of, or in addition to nuclease treatment, selective precipitation of nucleic acid impurities may be performed, e.g. by precipitation with a suitable precipitation agent such as, without limitation, polyethylene imine (PEI),

tetradecyltrimethyl-ammonium chloride (TTA), domiphen bromide (DB), cetylpyridinium chloride (CPC), benzethonium chloride (BTC), and cetyl trimethylammonium bromide (CTAB).

In certain embodiments, cell separation and nuclease treatment is carried out using a phosphate or Tris buffer having a pH of about 7.

After cell removal and nuclease treatment, one or more viral vector capture steps may be employed to purify the viral vector from various host cell contaminants. Whereas the transgene protein capture step of the invention comprises subjecting a composition comprising a viral vector and a transgene protein contaminant to chromatographic purification capable of selectively binding the transgene protein contaminant within the chromatographic substrate, the viral vector capture step employs a chromatographic substrate capable of selectively binding the viral vector within the chromatographic substrate (e.g., matrix, resin, column). In certain embodiments, the viral vector capture step comprises (i) contacting a composition comprising the viral vector (and one or most host cell contaminants) with a chromatography matrix capable of selectively binding the viral vector, and (ii) eluting the viral vector from the chromatography matrix, thereby separating the viral vector from host cell contaminants.

One or more of a variety of chromatographic purification techniques can be used for viral vector capture. In some embodiments, the viral vector capture step chromatography is selected from the group consisting of anion exchange chromatography, size exclusion chromatography, hydrophobic interaction chromatography (HIC), reverse-phase chromatography (RPC), and immobilized metal ion affinity chromatography (IMAC). In certain embodiments, the ion exchange chromatography employs an anionic exchange resin. The terms "anion exchange resin," "anion exchange adsorbent," or "anion exchange matrix" are used herein to refer to a solid phase which is positively charged, e.g., having one or more positively charged ligands, such as quaternary amino groups, attached thereto. Anion exchange chromatography resins have affinity for molecules having net negative surface charges and are useful in separating negatively charged molecules from a composition. Commercially available anion exchange resins include DEAE SEPHAROSE™ Fast Flow, Q SEPHAROSE™ Fast Flow, Q SEPHAROSE™ High Performance, Q SEPHAROSE™ XL, CAPTO™ DEAE, CAPTO™ Q, and CAPTO™ Q ImpRes from GE Healthcare Life Sciences, or FRACTOGEL™ EMD TMAE HiCap, FRACTOGEL™ EMD DEAE, and ESHMUNO™ Q from EMD Millipore, or UNOSPHERE™ Q and NUVIA™ Q from Bio-Rad.

In certain embodiments, the ion exchange chromatography comprises the use of membrane exchange. Membrane exchange chromatography is a type of ion exchange chromatography using a membrane absorber, for which there are various types including flat sheet, hollow fibre, and radial flow. In some embodiments, a membrane exchange column is packed with microporous membranes comprising internal pores which contain adsorptive moieties that can bind the viral vectors. Adsorptive membranes are available in a variety of shapes and chemistries which allows them to be suitable for purification purposes.

Various buffers and solutions known to those of skill in the art may be used for viral vector capture. For example, a suitable sanitization solution can comprise 0.5 M NaOH; a suitable equilibration buffer can comprise 20 mM Tris, 2 mM $MgCl_2$, 150 mM NaCl, and have a pH of 7.2; a suitable wash buffer can comprise 20 mM Tris, 2 mM $MgCl_2$, 400 mM NaCl, and have a pH of 7.2; a suitable elution buffer can comprise 20 mM Tris, 2 mM $MgCl_2$, 1200 mM NaCl, and have a pH of 7.2; a suitable stripping buffer can comprise 20 mM Tris, 2 mM $MgCl_2$, 2000 mM NaCl, and have a pH of 7.2; and a suitable regeneration buffer can comprise 20 mM Tris, 2 mM $MgCl_2$, 150 mM NaCl, and have a pH of 7.2.

In certain embodiments, as shown in FIG. 1B, the viral vector capture step can be performed before the transgene protein purification step. For example, in certain aspects, the present disclosure provides a method of purifying a viral vector from a host cell, comprising: (i) contacting a composition comprising the viral vector and a transgene protein contaminant with a first chromatography matrix capable of selectively binding the transgene protein contaminant; (ii) recovering the viral vector in the flow-through of the chromatography matrix, thereby separating the viral vector from the transgene protein contaminant; (iii) contacting the viral vector recovered from step (ii) with a second chromatography matrix capable of selectively binding the viral vector, and (iv) eluting the viral vector from the second chromatography matrix, thereby separating the viral vector from host cell contaminants.

In other embodiments, the viral vector capture step can be performed after the transgene protein capture step. Accordingly, in certain embodiments, the present disclosure provides a method of purifying a viral vector from a host cell, comprising: (i) contacting the composition comprising the viral vector and the transgene protein contaminant with a chromatography matrix capable of selectively binding the viral vector, (ii) eluting the viral vector (and any remaining transgene protein contaminant) from the second chromatography matrix, (iii) contacting the eluate

of step (ii) with a first chromatography matrix capable of selectively binding the transgene protein contaminant; and (iv) recovering the viral vector in the flow-through of the chromatography matrix, thereby separating the viral vector from the transgene protein contaminant.

In certain embodiments, after conducting the transgene protein capture step and/or viral vector capture step, the viral vector composition may be subjected to one or more steps of ultrafiltration, in order to further concentrate the viral vector. The ultrafiltration step is sometimes referred to as diafiltration when used for buffer exchange. In certain embodiments, an ultrafiltration/diafiltration step comprises concentrating the vector and/or exchanging the buffer. Any filtration process, e.g., direct flow filtration, tangential flow filtration, is suitable for an ultrafiltration/diafiltration step of a viral vector production process described herein. In general, the filtration process concentrates vector by forcing a diluent through a filter such that the diluent is removed from the composition, but the vector is unable to pass through the filter, thereby resulting in a concentrated vector composition. In certain embodiments, tangential flow filtration (TFF) is employed in the ultrafiltration/diafiltration step. TFF systems are composed of three distinct process streams: the feed solution, the permeate and the retentate (e.g., retentate containing the viral vector). Those of skill in the art will readily factor in considerations regarding fluid characteristics, sample volumes, and processing times, in order to select the optimal membrane and device for us in TFF applications for the purpose of concentration the vector and/or exchanging the buffer. For example, a membrane with molecular weight cut off that is three to six times smaller than the molecular weight of the viral vector to be retained is typically selected for use in filtration systems. Membranes can be flat sheets or hollow fibers.

In certain embodiments, wherein the viral vector capture step follows the transgene protein capture step, the eluate from the viral vector capture step is concentrated and further purified by ultrafiltration/diafilturation (UF/DF). In other embodiments, where the transgene protein capture step follows the viral vector capture step, the eluate from the transgene protein capture step is concentrated and further purified by UF/DF. In certain embodiments, the eluate may first be diluted before subjecting it to UF/DF. In certain embodiments, dilution of the eluant of the first purification step comprises the use of a diluent buffer, e.g., a diluent buffer comprising 20 mM Tris or 20 mM phosphate, 2 mM $MgCl_2$, 30mM to 120mM $CaCl_2$, and having a pH of 6.5 to 7.5. In certain embodiments, the diluent buffer is chilled prior to dilution (having a temperature < 15ºC). In certain embodiments, post dilution conductivity is 20 to 40 mS/cm.

In various embodiments described herein, prior to loading a sample to carry out the transgene protein capture step of the invention, the load sample may be adjusted to a suitable loading condition. As used herein, the term "load sample" refers to the sample that is applied to the downstream chromatography matrix. For example, an anion exchange chromatography load sample refers to the sample that is applied to the downstream anion exchange chromatography matrix. As another example, an affinity chromatography load sample refers to the sample that is applied to the downstream affinity chromatography matrix.

In an exemplary embodiment, as shown in FIG. 6A, where the downstream processing steps for viral vector production comprises a transgene protein capture step that follows a viral vector capture step, the eluate of the viral vector capture step (first chromatography purification step; the eluate of the viral vector capture step is also referred to herein as the load sample for the transgene protein capture step) is adjusted to a suitable loading condition before being applied to the transgene protein capture chromatography matrix. In such an embodiment, the load sample for the transgene protein capture step (i.e., eluate of the viral vector capture step) is obtained by eluting bound vector from the viral vector capture chromatography matrix (e.g., anion exchange media) using elution buffer that has a salt (e.g., NaCl or KCl) concentration from about 300 mM to about 1,500 mM. The load sample for the transgene protein capture step is then adjusted to suitable loading conditions as described herein.

In another embodiment, as shown in FIG. 6B, where the downstream processing steps for viral vector production comprises a viral vector capture step that follows a transgene protein capture step, the load sample for the transgene protein capture step is adjusted to a suitable loading condition before being applied to the transgene protein capture chromatography matrix. After collecting the flow-through from the transgene protein capture step (flow-through that contains the purified viral vector), the flow-through sample may be adjusted to a suitable loading condition for applying to the subsequent viral vector capture chromatography matrix. In certain embodiments, the flow-through sample of the transgene protein capture step (e.g., load sample for a viral vector capture step) is adjusted to a target salt concentration to achieve a target sample conductivity of less than 20 mS/cm.

As such, in certain embodiments, the downstream processing steps for viral vector production described in the present disclosure comprises the step of adjusting the concentration of

a salt of the composition comprising the viral vector and the transgene protein contaminant to a target salt concentration.

In certain embodiments, the adjusting of the load sample for the transgene protein capture step comprises adjusting the concentration of a salt to a target salt concentration. Adjustment of the load sample for the transgene protein capture step may comprise adjusting the concentration of salt and ionic components, including, without limitation, NaCl, KCl, $CaCl_2$, and $MgCl_2$. In some embodiments, adjusting of the load sample for the transgene protein capture step comprises diluting the load sample to a factor of 1:0, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, and the like, to decrease the concentration of salt in the load sample to a target salt concentration. In some embodiments, adjusting of the load sample for the transgene protein capture step comprises addition of salt (e.g., via bolus addition of stock salt) to increase the concentration of salt in the load sample to a target salt concentration. Those of ordinary skill in the art will readily be able to determine the amount of dilution required, or the amount of salt that is needed to be added to the load sample to achieve the target salt concentration suitable for applying to the transgene protein capture chromatography matrix. The adjusted load sample for the transgene protein capture step may have a target salt concentration of from about 200 mM (0.2 M) and about 600 mM (0.6 M). For example, in certain embodiments, the target salt concentration is about 200 mM, about 250 mM, about 300 mM, about 350 mM, about 400 mM, about 450 mM, about 500 mM, about 550 mM, about 600 mM. In certain exemplary embodiments, the target salt concentration is about 400 mM. In some embodiments, the salt in the load sample for the transgene protein capture step is NaCl or KCl. As such, in some embodiments, the adjusted load sample for the transgene protein capture step may have a target salt concentration of from about 200 mM NaCl and about 600 mM NaCl. In certain exemplary embodiments, the adjusted load sample for the transgene protein capture step has a target NaCl concentration of about 400 mM. In other embodiments, the adjusted load sample for the transgene protein capture step may have a target salt concentration of from about 200 mM KCl and about 600 mM KCl. In certain exemplary embodiments, the adjusted load sample for the transgene protein capture step has a target KCl concentration of about 400 mM. Without being bound to any theory, adjusting the salt concentration of the load sample for the transgene protein capture step of the invention may improve the efficiency of the transgene protein capture step by providing an optimal salt concentration in which the viral vector is separated from any bound contaminating transgene protein.

Addition to or dilution of the load sample for the transgene protein capture step to adjust for other ionic components and/or excipients may be performed to facilitate optimal removal of contaminating transgene protein in a transgene protein capture step of the invention. Buffer systems such as Tris, phosphate, bis-Tris, or other buffer systems known in the art suitable for a transgene protein capture step of the invention may be used. Such buffer systems may contain excipients such as sugar (e.g., sucrose), Poloxamer, polysorbate, and other suitable excipients known in the art. In certain embodiments, the buffer system also contains one or more ionic components such as $MgCl_2$, $CaCl_2$, or others known in the art to enhance the efficiency of transgene protein capture and removal in a transgene protein capture step of the invention. In certain exemplary embodiments, ionic components such as MgCl2 and CaCl2 may be adjusted to a target concentration of from 0 mM to about 20 mM. In certain exemplary embodiments, a suitable adjusted load sample for the transgene protein capture step of the invention may comprise from about 200 mM to about 600 mM of NaCl or KCl, from about 0 mM to about 20 mM MgCl2 or CaCl2, and may or may not contain excipients such as sugar (e.g., sucrose), Poloxamer, polysorbate, and/or any other excipients known in the art to be suitable.

The adjusted load sample for the transgene protein capture step of the invention may be stored at a lower temperature for temporary hold depending on processing need. In certain embodiments, the adjusted load sample is applied directly to the transgene protein capture chromatography matrix. Suitable conditions (e.g., temperature from about 4°C to about 25°C) for carrying out the transgene protein capture step of the invention are known to those of skill in the art, and may be provided by the manufacturer of the transgene protein capture chromatography matrix. The adjusted sample is loaded into the transgene protein capture chromatography matrix (e.g., transgene protein capture chromatography column) at a residence time value ranging from about 4 to about 12 minutes, allowing for sufficient time to elapse in order for the transgene protein contaminant to be retained within the transgene protein capture chromatography matrix. The unbound viral vector particles flow through the matrix, resulting in a purified viral vector product. To recover any remaining viral vector particles from the chromatography matrix (e.g., void space of a packed column), a chase of equilibration buffer is applied to the chromatography matrix and the flow-through is collected until a desired level of viral vector particles has been obtained.

At this stage in a viral production process described herein, the viral vector composition (i.e., viral vector eluate) is combined with one or more pharmaceutical excipients to make a

pharmaceutical composition, which is then optionally subjected to filter sterilization to render the composition suitable for clinical use. Various filter sterilization processes and sterilizing filters are known to those of skill in the art. Accordingly, a viral vector production process described herein comprises combining the viral vector separated from the transgene protein contaminant (e.g., viral vector eluate) with one or more pharmaceutical excipients to make a pharmaceutical composition comprising the viral vector. In certain embodiments, the combining of viral vector separated from the transgene protein contaminant (e.g., viral vector eluate) with one or more pharmaceutical excipients is achieved through UF/DF of the viral vector separated from the transgene protein contaminant (e.g., viral vector eluate) with a formulation buffer.

In certain embodiments, a viral vector production process described herein results in a composition comprising a viral vector, wherein the composition is substantially free of a transgene protein contaminant. As used herein, the term "substantially free of a transgene protein contaminant" refers to a composition comprising less than about 50% of the amount of transgene protein contaminant that is present in a reference viral vector composition purified without a transgene protein capture step of the invention (e.g., a viral vector composition purified according to a conventional purification process that employs a viral vector capture step but not a transgene protein capture step). For example, a composition is substantially free of a transgene protein contaminant when the composition comprises less than about 45%, less than about 40%, less than about 35%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, or less than about 5% of the amount of transgene protein contaminant present in the reference viral vector composition. In certain embodiments, the composition contains less than 20% of the transgene protein contaminant present in the reference viral vector composition.

Various methods of assaying the amount of a transgene protein contaminant are known to those of skill in the art and are suitable. In certain embodiments, for a transgene protein contaminant having a specific activity (e.g., an enzymatic activity), the activity of the transgene protein contaminant in the composition of the invention can be assayed and compared to the activity of the transgene protein contaminant in a reference viral vector composition. For example, the level of FVIII transgene activity (mU/ml) can be assayed by a variety of chromogenic assays known to those of skill in the art. In certain embodiments, the purified FVIII viral vector composition of the invention comprises less than 50000 mU/ml of FVIII activity (e.g., less than 40000, 30000, 20000, 10000 or 500 mU of FVIII activity). In certain exemplary embodiments,

the purified FVIII viral vector composition of the invention comprises a total FVIII activity of about 1 IU or less.

In certain embodiments, the amount of a transgene protein contaminant can be detected via various techniques known in the art to quantify the presence of actual contaminant protein. In certain embodiments, the presence of the transgene protein contaminant in the composition of the invention can be assayed and compared to the presence of the transgene protein contaminant in a reference viral vector composition. For example, an enzyme-linked immunosorbent assay (ELISA) can be performed to detect the presence of a transgene protein contaminant. Other examples that may be suitable include spectrometry methods (e.g., high-performance liquid chromatography (HPLC), and liquid chromatography-mass spectrometry (LC/MS), etc.), and antibody-based methods of protein detection (e.g., protein immunoprecipitation, immunoelectrophoresis, etc.).

B. VIRAL VECTORS

The methods and processes provided herein are suitable for the production and purification of any viral vector. In certain embodiments, the viral vector is an enveloped viral vector. As known in the art enveloped viral vectors include, without limitation, enveloped DNA viral vectors, e.g., herpesvirus and poxvirus, and enveloped RNA viral vectors, e.g., alphavirus and paramyxovirus. Enveloped viral vectors also include retroviruses, e.g., oncoretroviruses, lentiviruses, and spumaviruses. In certain embodiments, the enveloped viral vector is a lentiviral vector. Lentiviral vectors are part of a larger group of retroviral vectors (Coffin et al. (1997) "Retroviruses" Cold Spring Harbor Laboratory Press Eds: J M Coffin, S M Hughes, H E Varmus pp 758-763). Examples of primate lentiviruses include: the human immunodeficiency virus (HIV) and the simian immunodeficiency virus (SIV). The lentivirus family differs from retroviruses in that lentiviruses have the capability to infect both dividing and non-dividing cells (Lewis et al. (1992); Lewis and Emerman (1994)).

As used herein, the term "lentiviral vector" refers to a vector which comprises at least one component part derivable from a lentivirus. In some embodiments, the component part is involved in the biological mechanisms by which the vector infects cells, expresses genes or is replicated. In some embodiments, a recombinant lentiviral vector at least part of one or more protein coding regions essential for replication may be removed from the virus. Accordingly, in some

embodiments, recombinant lentiviral vectors are replication-defective. Portions of the viral genome may also be replaced by a transgene, thus rendering the vector capable of transducing a target non-dividing host cell and/or integrating its genome into a host genome.

In certain embodiments, a recombinant enveloped virus (*e.g.,* a recombinant lentivirus) is pseudotyped to alter or modify the tropism of the recombinant lentiviral vector. Pseudotyping can confer one or more advantages. For example, the env gene product of the HIV based vectors would restrict these vectors to infecting only cells that express a protein called CD4. In some embodiments, if the env gene in these vectors has been substituted with env sequences from other RNA viruses, this may confer a broader infectious spectrum (Verma and Somia (1997)). The envelope glycoprotein (G) of Vesicular stomatitis virus (VSV), a rhabdovirus, is an envelope protein that has been shown to be capable of pseudotyping certain retroviruses. Pseudotyped VSV-G vectors may be used to transduce a wide range of mammalian cells. The incorporation of a non-lentiviral pseudotyping envelope, such as VSV-G protein, gives the advantage that vector particles can be concentrated to a high titre without loss of infectivity (Akkina et al. (1996) J. Virol. 70:2581-5).

Lentiviruses include members of the bovine lentivirus group, equine lentivirus group, feline lentivirus group, ovine lentivirus group, caprine lentivirus group, and primate lentivirus group. The development of lentivirus vectors for gene therapy has been reviewed in Klimatcheva et al. (1999) Frontiers in Bioscience 4:481-496. The design and use of lentiviral vectors suitable for gene therapy is described for example in U.S. Pat. Nos. 6,207,455 and 6,615,782. Examples of lentivirus include, but are not limited to, HIV-1, HIV-2, HIV-1/HIV-2 pseudotype, HIV-1/SIV, FIV, caprine arthritis encephalitis virus (CAEV), equine infectious anemia virus, and bovine immunodeficiency virus.

In some embodiments, a lentiviral vector is a "third-generation" lentiviral vector. As used herein, the term "third-generation" lentiviral vector refers to a lentiviral packaging system that has the characteristics of a second-generation vector system, and that further lacks a functional tat gene, such as one from which the tat gene has been deleted or inactivated. Typically, the gene encoding rev is provided on a separate expression construct. See, *e.g.*, Dull et al. (1998) J. Virol. 72: 8463-8471. As used herein, a "second-generation" lentiviral vector system refers to a lentiviral packaging system that lacks functional accessory genes, such as one from which the accessory genes vif, vpr, vpu, and nef have been deleted or inactivated. See, *e.g.*, Zufferey et al. (1997) Nat.

Biotechnol. 15:871-875. As used herein, "packaging system" refers to a set of viral constructs comprising genes that encode viral proteins involved in packaging a recombinant virus. Typically, the constructs of the packaging system will ultimately be incorporated into a packaging cell.

In certain embodiments, the lentiviral vector is a vector of a recombinant lentivirus capable of infecting non-dividing cells. In certain embodiments, the lentiviral vector is a vector of a recombinant lentivirus capable of infecting liver cells (*e.g.*, hepatocytes). The lentiviral genome and the proviral DNA typically have the three genes found in retroviruses: gag, pol and env, which are flanked by two long terminal repeat (LTR) sequences. The gag gene encodes the internal structural (matrix, capsid and nucleocapsid) proteins; the pol gene encodes the RNA-directed DNA polymerase (reverse transcriptase), a protease and an integrase; and the env gene encodes viral envelope glycoproteins. The 5' and 3' LTR's serve to promote transcription and polyadenylation of the virion RNA's. The LTR contains all other cis-acting sequences necessary for viral replication. Lentiviruses have additional genes including vif, vpr, tat, rev, vpu, nef and vpx (in HIV-l, HIV-2 and/or SIV). Adjacent to the 5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsidation of viral RNA into particles (the Psi site).

In some embodiments, a method of producing a recombinant lentivirus capable of infecting a non-dividing cell comprises transfecting a suitable host cell with two or more vectors carrying the packaging functions, namely gag, pol and env, as well as rev and tat. Thus, for example, a first vector can provide a nucleic acid encoding a viral gag and a viral pol and another vector can provide a nucleic acid encoding a viral env to produce a packaging cell. Introducing a vector providing a heterologous gene, herein identified as a transfer vector, into that packaging cell yields a producer cell which releases infectious viral particles carrying the foreign gene of interest.

According to the above-indicated configuration of vectors and foreign genes, the second vector can provide a nucleic acid encoding a viral envelope (env) gene. The env gene can be derived from nearly any suitable virus, including retroviruses. In some embodiments, the env protein is an amphotropic envelope protein which allows transduction of cells of human and other species.

Examples of retroviral-derived env genes include, but are not limited to: Moloney murine leukemia virus (MoMuLV or MMLV), Harvey murine sarcoma virus (HaMuSV or HSV), murine mammary tumor virus (MuMTV or MMTV), gibbon ape leukemia virus (GaLV or GALV), human

immunodeficiency virus (HIV) and Rous sarcoma virus (RSV). Other env genes such as Vesicular stomatitis virus (VSV) protein G (VSV-G), that of hepatitis viruses and of influenza also can be used.

In certain embodiments, a lentiviral vector of the instant disclosure comprises one or more nucleotide sequences encoding a gag protein, a Rev-response element, a central polypurine track (cPPT), or any combination thereof.

In some embodiments, the lentiviral vector expresses on its surface one or more polypeptides that improve the targeting and/or activity of the lentiviral vector or the encoded FVIII polypeptide. The one or more polypeptides can be encoded by the lentiviral vector or can be incorporated during budding of the lentiviral vector from a host cell. During lentiviral production, viral particles bud off from a producing host cell. During the budding process, the viral particle takes on a lipid coat, which is derived from the lipid membrane of the host cell. As a result, the lipid coat of the viral particle can include membrane bound polypeptides that were previously present on the surface of the host cell.

In some embodiments, the lentiviral vector expresses one or more polypeptides on its surface that inhibit an immune response to the lentiviral vector following administration to a human subject. In some embodiments, the surface of the lentiviral vector comprises one or more CD47 molecules. CD47 is a "marker of self" protein, which is ubiquitously expressed on human cells. Surface expression of CD47 inhibits macrophage-induced phagocytosis of endogenous cells through the interaction of CD47 and macrophage expressed-SIRPα. Cells expressing high levels of CD47 are less likely to be targeted and destroyed by human macrophages *in vivo*.

In some embodiments, the lentiviral vector comprises a high concentration of CD47 polypeptide molecules on its surface. In some embodiments, the lentiviral vector is produced in a cell line that has a high expression level of CD47. In certain embodiments, the lentiviral vector is produced in a $CD47^{high}$ cell, wherein the cell has high expression of CD47 on the cell membrane. In particular embodiments, the lentiviral vector is produced in a $CD47^{high}$ HEK 293T cell, wherein the HEK 293T is has high expression of CD47 on the cell membrane. In some embodiments, the HEK 293T cell is modified to have increased expression of CD47 relative to unmodified HEK 293T cells. In certain embodiments, the CD47 is human CD47.

In some embodiments, the lentiviral vector has little or no surface expression of major histocompatibility complex class I (MHC-I). Surface expressed MHC-I displays peptide fragments

of "non-self" proteins from within a cell, such as protein fragments indicative of an infection, facilitating an immune response against the cell. In some embodiments, the lentiviral vector is produced in a MHC-I^{low} cell, wherein the cell has reduced expression of MHC-I on the cell membrane. In some embodiments, the lentiviral vector is produced in an MHC-I^{-} (or "MHC-I^{free}", "MHC-1^{neg}" or "MHC-negative") cell, wherein the cell lacks expression of MHC-I.

In particular embodiments, the lentiviral vector comprises a lipid coat comprising a high concentration of CD47 polypeptides and lacking MHC-I polypeptides. In certain embodiments, the lentiviral vector is produced in a $CD47^{high}$/MHC-I^{low} cell line, *e.g.*, a $CD47^{high}$/MHC-I^{low} HEK 293T cell line. In some embodiments, the lentiviral vector is produced in a $CD47^{high}$/MHC-I^{free} cell line, *e.g.*, a $CD47^{high}$/MHC-I^{free} HEK 293T cell line.

Examples of lentiviral vectors are disclosed in U.S. Patent No. 9,050,269 and International Publication Nos. WO9931251, W09712622, W09817815, W09817816, and WO9818934, which are incorporated herein by reference in their entireties.

Expression Control Elements

In some embodiments, the nucleic acid molecule or vector of the disclosure further comprises at least one expression control sequence. An expression control sequence as used herein is any regulatory nucleotide sequence, such as a promoter sequence or promoter-enhancer combination, which facilitates the efficient transcription and translation of the coding nucleic acid to which it is operably linked. For example, the isolated nucleic acid molecule of the disclosure can be operably linked to at least one transcription control sequence.

The gene expression control sequence can, for example, be a mammalian or viral promoter, such as a constitutive or inducible promoter. Constitutive mammalian promoters include, but are not limited to, the promoters for the following genes: hypoxanthine phosphoribosyl transferase (HPRT), adenosine deaminase, pyruvate kinase, beta-actin promoter, and other constitutive promoters. Exemplary viral promoters which function constitutively in eukaryotic cells include, for example, promoters from the cytomegalovirus (CMV), simian virus (*e.g.*, SV40), papilloma virus, adenovirus, human immunodeficiency virus (HIV), Rous sarcoma virus, cytomegalovirus, the long terminal repeats (LTR) of Moloney leukemia virus, and other retroviruses, and the thymidine kinase promoter of herpes simplex virus.

Other constitutive promoters are known to those of ordinary skill in the art. The promoters useful as gene expression sequences of the disclosure also include inducible promoters. Inducible promoters are expressed in the presence of an inducing agent. For example, the metallothionein promoter is induced to promote transcription and translation in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the art.

In one embodiment, the disclosure includes expression of a transgene under the control of a tissue specific promoter and/or enhancer. In another embodiment, the promoter or other expression control sequence selectively enhances expression of the transgene in liver cells. Examples of liver specific promoters include, but are not limited to, a mouse thyretin promoter (mTTR), an endogenous human factor VIII (F8) promoter, an endogenous human factor IX (F9) promoter, human alpha-1-antitrypsin promoter (hAAT), human albumin minimal promoter, and mouse albumin promoter. In a particular embodiment, the promoter comprises a mTTR promoter. The mTTR promoter is described in R. H. Costa et al., 1986, *Mol. Cell. Biol.* 6:4697. The F8 promoter is described in Figueiredo and Brownlee, 1995, *J. Biol. Chem.* 270:11828-11838. In certain embodiments, the promoter comprises any of the mTTR promoters (*e.g.*, mTTR202 promoter, mTTR202opt promoter, mTTR482 promoter) as disclosed in U.S. patent publication no. US2019/0048362, which is incorporated by reference herein in its entirety.

Expression levels can be further enhanced to achieve therapeutic efficacy using one or more enhancers. One or more enhancers can be provided either alone or together with one or more promoter elements. Typically, the expression control sequence comprises a plurality of enhancer elements and a tissue specific promoter. In one embodiment, an enhancer comprises one or more copies of the α-1-microglobulin/bikunin enhancer (Rouet et al., 1992, *J. Biol. Chem.* 267:20765-20773; Rouet et al., 1995, *Nucleic Acids Res.* 23:395-404; Rouet et al., 1998, *Biochem. J.* 334:577-584; Ill et al., 1997, *Blood Coagulation Fibrinolysis* 8:S23-S30). In another embodiment, an enhancer is derived from liver specific transcription factor binding sites, such as EBP, DBP, HNF1, HNF3, HNF4, HNF6, with Enh1, comprising HNF1, (sense)-HNF3, (sense)-HNF4, (antisense)-HNF1, (antisense)-HNF6, (sense)-EBP, (antisense)-HNF4 (antisense).

In a particular example, a promoter useful for the disclosure is an ET promoter, which is also known as GenBank No. AY661265. *See also* Vigna et al., *Molecular Therapy 11(5)*:763 (2005). Examples of other suitable vectors and gene regulatory elements are described in WO

02/092134, EP1395293, or US Patent Nos. 6,808,905, 7,745,179, or 7,179,903, which are incorporated by reference herein in their entireties.

In general, the expression control sequences shall include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription and translation, respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribing sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined coding nucleic acid. The gene expression sequences optionally include enhancer sequences or upstream activator sequences as desired.

In certain embodiments, it will be useful to include within the lentiviral vector one or more miRNA target sequences which, for example, are operably linked to a transgene within a lentiviral vector. More than one copy of a miRNA target sequence included in the lentiviral vector can increase the effectiveness of the system.

Also included are different miRNA target sequences. For example, lentiviral vectors which express more than one transgene can have the transgene under control of more than one miRNA target sequence, which can be the same or different. The miRNA target sequences can be in tandem, but other arrangements are also included. The transgene expression cassette, containing miRNA target sequences, can also be inserted within the lentiviral vector in antisense orientation. Antisense orientation can be useful in the production of viral particles to avoid expression of gene products which can otherwise be toxic to the producer cells.

In other embodiments, the lentiviral vector comprises 1, 2, 3, 4, 5 ,6, 7 or 8 copies of the same or different miRNA target sequence. In certain embodiments, the lentiviral vector does not include any miRNA target sequence. Choice of whether or not to include an miRNA target sequence (and how many) will be guided by known parameters such as the intended tissue target, the level of expression required, etc.

In one embodiment, the target sequence is an miR-223 target which has been reported to block expression most effectively in myeloid committed progenitors and at least partially in the more primitive HSPC. miR-223 target can block expression in differentiated myeloid cells including granulocytes, monocytes, macrophages, myeloid dendritic cells. miR-223 target can also be suitable for gene therapy applications relying on robust transgene expression in the lymphoid or erythroid lineage. miR-223 target can also block expression very effectively in human HSC.

In another embodiment, the target sequence is an miR-142 target (tccataaagtaggaaacactaca (SEQ ID NO: 7)). In one embodiment, the lentiviral vector comprises 4 copies of miR-142 target sequences. In certain embodiments, the complementary sequence of hematopoietic-specific microRNAs, such as miR-142 (142T), is incorporated into the 3' untranslated region of a lentiviral vector, making the transgene-encoding transcript susceptible to miRNA-mediated down-regulation. By this method, transgene expression can be prevented in hematopoietic-lineage antigen presenting cells (APC), while being maintained in non-hematopoietic cells (Brown et al., Nat Med 2006). This strategy can impose a stringent post-transcriptional control on transgene expression and thus enables stable delivery and long-term expression of transgenes. In some embodiments, miR-142 regulation prevents immune-mediated clearance of transduced cells and/or induce antigen-specific Regulatory T cells (T regs) and mediate robust immunological tolerance to the transgene-encoded antigen.

In some embodiments, the target sequence is an miR181 target. Chen C-Z and Lodish H, Seminars in Immunology (2005) 17(2):155-165 discloses miR-181, a miRNA specifically expressed in B cells within mouse bone marrow (Chen and Lodish, 2005). It also discloses that some human miRNAs are linked to leukemias.

The target sequence can be fully or partially complementary to the miRNA. The term "fully complementary" means that the target sequence has a nucleic acid sequence which is 100 % complementary to the sequence of the miRNA which recognizes it. The term "partially complementary" means that the target sequence is only in part complementary to the sequence of the miRNA which recognizes it, whereby the partially complementary sequence is still recognized by the miRNA. In other words, a partially complementary target sequence in the context of the present disclosure is effective in recognizing the corresponding miRNA and effecting prevention or reduction of transgene expression in cells expressing that miRNA. Examples of the miRNA target sequences are described at WO2007/000668, WO2004/094642, WO2010/055413, or WO2010/125471, which are incorporated herein by reference in their entireties.

In some embodiments, a suitable third-generation lentiviral vector is a self-inactivating lentiviral vector. In some embodiments, the lentiviral vector is a VSV.G pseudo type lentiviral vector. In some embodiments, the lentiviral vector comprises a hepatocyte-specific promoter for transgene expression. In some embodiments, the hepatocyte-specific promoter is an enhanced transthyretin promoter. In some embodiments, the lentiviral vector comprises one or more target

sequences for miR-142 to reduce immune response to the transgene product. In some embodiments, incorporating one or more target sequences for miR-142 into a lentiviral vector of the present disclosure allows for a desired transgene expression profile. For example, incorporating one or more target sequences for miR-142 may suppress transgene expression in intravascular and extravascular hematopoietic lineages, whereas transgene expression is maintained in nonhematopoietic cells.

C. VIRAL VECTOR TRANSGENES

A viral vector suitable for a method described herein comprises a transgene expression cassette encoding a transgene protein, e.g., therapeutic protein, for treatment of a disease or disorder in a subject suffering therefrom. When the viral vector is administered to the subject *in vivo*, the viral vector transduces cells of the subject with the transgene expression cassette such that the protein product of the transgene in expressed in the subject as desired. However, if the transgene is expressed during the viral vector production process *ex vivo*, the transgene protein is considered a contaminant that can co-purify with the virus. Therefore, the methods of the invention may be employed to remove the transgene protein contaminant.

The transgene protein contaminant may correspond to any transgene of interest. In certain embodiments, the transgene may encode a protein selected from a clotting factor, a growth factor, an antibody, or a metabolic enzyme. As used herein, the term "clotting factor," refers to molecules, or analogs thereof, naturally occurring or recombinantly produced, which prevent or decrease the duration of a bleeding episode in a subject. In other words, it means molecules having pro-clotting activity, i.e., are responsible for the conversion of fibrinogen into a mesh of insoluble fibrin causing the blood to coagulate or clot. "Clotting factor" as used herein includes an activated clotting factor, its zymogen, or an activatable clotting factor. An "activatable clotting factor" is a clotting factor in an inactive form (e.g., in its zymogen form) that is capable of being converted to an active form. The term "clotting factor" includes but is not limited to factor I (FI), factor II (Fll), factor V (FV), FVII, FVIII, FIX, factor X (FX), factor XI (FXI), factor XII (FXII), factor XIII (FXIII), Von Willebrand factor (VWF), prekallikrein, high-molecular weight kininogen, fibronectin, antithrombin III, heparin cofactor II, protein C, protein S, protein Z, Protein Z-related protease inhibitor (ZPI), plasminogen, alpha 2-antiplasmin, tissue plasminogen activator(tPA), urokinase, plasminogen activator inhibitor-1 (PAI-1), plasminogen activator inhibitor-2 (PAI2), zymogens

thereof, activated forms thereof, or any combination thereof. In certain embodiments, the clotting factor comprises FVIII or a variant or fragment thereof. In other embodiments, the clotting factor comprises FVIIIXTEN or a variant or fragment thereof. In certain embodiments, the clotting factor comprises FIX or a variant or fragment thereof.

In some embodiments, the transgene encodes heterologous amino acid sequence. The heterologous amino acid sequence can be linked to the N-terminus or the C-terminus of the FVIII amino acid sequence or the FIX amino acid sequence or inserted between two amino acids in the FVIII amino acid sequence or the FIX amino acid sequence. In some embodiments, the heterologous amino acid sequence can be inserted within the FVIII polypeptide at any site disclosed in International Publication No. WO 2013/123457 A1 and WO 2015/106052 A1 or U.S. Publication No. 2015/0158929 A1, which are herein incorporated by reference in their entirety.

In some embodiments, the heterologous amino acid sequence is inserted within the B domain of FVIII or a fragment thereof. In some embodiments, the heterologous amino acid sequence is inserted within the FVIII immediately downstream of an amino acid corresponding to amino acid 745 of mature human FVIII (SEQ ID NO:4). In one particular embodiment, the FVIII comprises a deletion of amino acids 746-1646, corresponding to mature human FVIII, and the heterologous amino acid sequence is inserted immediately downstream of amino acid 745, corresponding to mature human FVIII.

In some embodiments, a heterologous moiety comprises one or more XTEN sequences, fragments, variants, or derivatives thereof. As used here "XTEN sequence" refers to extended length polypeptides with non-naturally occurring, substantially non-repetitive sequences that are composed mainly of small hydrophilic amino acids, with the sequence having a low degree or no secondary or tertiary structure under physiologic conditions. As a heterologous moiety, XTENs can serve as a half-life extension moiety. In addition, XTEN can provide desirable properties including but are not limited to enhanced pharmacokinetic parameters and solubility characteristics.

In some embodiments, the XTEN sequence useful for the disclosure is a peptide or a polypeptide having greater than about 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1200, 1400, 1600, 1800, or 2000 amino acid residues. In certain embodiments, XTEN is a peptide or a polypeptide having greater than about 20 to about 3000 amino acid residues, greater than 30 to about 2500 residues, greater

than 40 to about 2000 residues, greater than 50 to about 1500 residues, greater than 60 to about 1000 residues, greater than 70 to about 900 residues, greater than 80 to about 800 residues, greater than 90 to about 700 residues, greater than 100 to about 600 residues, greater than 110 to about 500 residues, or greater than 120 to about 400 residues. In one particular embodiment, the XTEN comprises an amino acid sequence of longer than 42 amino acids and shorter than 144 amino acids in length.

The XTEN sequence of the disclosure can comprise one or more sequence motifs of 5 to 14 (*e.g.*, 9 to 14) amino acid residues or an amino acid sequence at least 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the sequence motif, wherein the motif comprises, consists essentially of, or consists of 4 to 6 types of amino acids (*e.g.*, 5 amino acids) selected from the group consisting of glycine (G), alanine (A), serine (S), threonine (T), glutamate (E) and proline (P). Examples of XTEN sequences that can be used as heterologous moieties in chimeric proteins of the disclosure are disclosed, *e.g.*, in U.S. Patent Publication Nos. 2010/0239554 A1, 2010/0323956 A1, 2011/0046060 A1, 2011/0046061 A1, 2011/0077199 A1, or 2011/0172146 A1, or International Patent Publication Nos. WO 2010/091122 A1, WO 2010/144502 A2, WO 2010/144508 A1, WO 2011/028228 A1, WO 2011/028229 A1, or WO 2011/028344 A2, each of which is incorporated by reference herein in its entirety.

In certain embodiments, the transgene comprises a FVIII coding sequence as shown in Table 1. In certain embodiments the transgene protein encoded by the transgene comprises an amino acid sequence as shown in Table 1.

Table 1: Sequences

SEQ ID NO	Description	Sequence
1	FVIII coding sequence (non-codon optimized)	ATGCAGATTGAGCTGTCCACTTGTTTCTTCCTGTGCCTCCTGC GCTTCTGTTTCTCCGCCACTCGCCGGTACTACCTTGGAGCCGT GGAGCTTTCATGGGACTACATGCAGAGCGACCTGGGCGAAC TCCCCGTGGATGCCAGATTCCCCCCCCGCGTGCCAAAGTCCT TCCCCTTTAACACCTCCGTGGTGTACAAGAAAACCCTCTTTG TCGAGTTCACTGACCACCTGTTCAACATCGCCAAGCCGCGCC CACCTTGGATGGGCCTCCTGGGACCGACCATTCAAGCTGAAG TGTACGACACCGTGGTGATCACCCTGAAGAACATGGCGTCCC

SEQ ID NO	Description	Sequence
		ACCCCGTGTCCCTGCATGCGGTCGGAGTGTCCTACTGGAAGG CCTCCGAAGGAGCTGAGTACGACGACCAGACTAGCCAGCGG GAAAAGGAGGACGATAAAGTGTTCCCGGGCGGCTCGCATAC TTACGTGTGGCAAGTCCTGAAGGAAAACGGACCTATGGCAT CCGATCCTCTGTGCCTGACTTACTCCTACCTTTCCCATGTGGA CCTCGTGAAGGACCTGAACAGCGGGCTGATTGGTGCACTTCT CGTGTGCCGCGAAGGTTCGCTCGCTAAGGAAAAGACCCAGA CCCTCCATAAGTTCATCCTTTTGTTCGCTGTGTTCGATGAAGG AAAGTCATGGCATTCCGAAACTAAGAACTCGCTGATGCAGG ACCGGGATGCCGCCTCAGCCCGCGCCTGGCCTAAAATGCAT ACAGTCAACGGATACGTGAATCGGTCACTGCCCGGGCTCATC GGTTGTCACAGAAAGTCCGTGTACTGGCACGTCATCGGCATG GGCACTACGCCTGAAGTGCACTCCATCTTCCTGGAAGGGCAC ACCTTCCTCGTGCGCAACCACCGCCAGGCCTCTCTGGAAATC TCCCCGATTACCTTTCTGACCGCCCAGACTCTGCTCATGGAC CTGGGGCAGTTCCTTCTCTTCTGCCACATCTCCAGCCATCAG CACGACGGAATGGAGGCCTACGTGAAGGTGGACTCATGCCC GGAAGAACCTCAGTTGCGGATGAAGAACAACGAGGAGGCCG AGGACTATGACGACGATTTGACTGACTCCGAGATGGACGTC GTGCGGTTCGATGACGACAACAGCCCCAGCTTCATCCAGATT CGCAGCGTGGCCAAGAAGCACCCCAAAACCTGGGTGCACTA CATCGCGGCCGAGGAAGAAGATTGGGACTACGCCCCGTTGG TGCTGGCACCCGATGACCGGTCGTACAAGTCCCAGTATCTGA ACAATGGTCCGCAGCGGATTGGCAGAAAGTACAAGAAAGTG CGGTTCATGGCGTACACTGACGAAACGTTTAAGACCCGGGA GGCCATTCAACATGAGAGCGGCATTCTGGGACCACTGCTGTA CGGAGAGGTCGGCGATACCCTGCTCATCATCTTCAAAAACCA GGCCTCCCGGCCTTACAACATCTACCCTCACGGAATCACCGA CGTGCGGCCACTCTACTCGCGGCGCCTGCCGAAGGGCGTCA AGCACCTGAAAGACTTCCCTATCCTGCCGGGCGAAATCTTCA AGTATAAGTGGACCGTCACCGTGGAGGACGGGCCCACCAAG AGCGATCCTAGGTGTCTGACTCGGTACTACTCCAGCTTCGTG AACATGGAACGGGACCTGGCATCGGGACTCATTGGACCGCT GCTGATCTGCTACAAAGAGTCGGTGGATCAACGCGGCAACC AGATCATGTCCGACAAGCGCAACGTGATCCTGTTCTCCGTGT TTGATGAAAACAGATCCTGGTACCTCACTGAAAACATCCAG AGGTTCCTCCCAAACCCCGCAGGAGTGCAACTGGAGGACCC TGAGTTTCAGGCCTCGAATATCATGCACTCGATTAACGGTTA CGTGTTCGACTCGCTGCAGCTGAGCGTGTGCCTCCATGAAGT CGCTTACTGGTACATTCTGTCCATCGGCGCCCAGACTGACTT CCTGAGCGTGTTCTTTTCCGGTTACACCTTTAAGCACAAGAT

SEQ ID NO	Description	Sequence
		GGTGTACGAAGATACCCTGACCCTGTTCCCTTTCTCCGGCGA AACGGTGTTCATGTCGATGGAGAACCCGGGTCTGTGGATTCT GGGATGCCACAACAGCGACTTTCGGAACCGCGGAATGACTG CCCTGCTGAAGGTGTCCTCATGCGACAAGAACACCGGAGAC TACTACGAGGACTCCTACGAGGATATCTCAGCCTACCTCCTG TCCAAGAACAACGCGATCGAGCCGCGCAGCTTCAGCCAGAA CCCGCCTGTGCTGAAGAGGCACCAGCGAGAAATTACCCGGA CCACCCTCCAATCGGATCAGGAGGAAATCGACTACGACGAC ACCATCTCGGTGGAAATGAAGAAGGAAGATTTCGATATCTA CGACGAGGACGAAAATCAGTCCCCTCGCTCATTCCAAAAGA AAACTAGACACTACTTTATCGCCGCGGTGGAAAGACTGTGG GACTATGGAATGTCATCCAGCCCTCACGTCCTTCGGAACCGG GCCCAGAGCGGATCGGTGCCTCAGTTCAAGAAAGTGGTGTT CCAGGAGTTCACCGACGGCAGCTTCACCCAGCCGCTGTACC GGGGAGAACTGAACGAACACCTGGGCCTGCTCGGTCCCTAC ATCCGCGCGGAAGTGGAGGATAACATCATGGTGACCTTCCG TAACCAAGCATCCAGACCTTACTCCTTCTATTCCTCCCTGATC TCATACGAGGAGGACCAGCGCCAAGGCGCCGAGCCCCGCAA GAACTTCGTCAAGCCCAACGAGACTAAGACCTACTTCTGGA AGGTCCAACACCATATGGCCCCGACCAAGGATGAGTTTGAC TGCAAGGCCTGGGCCTACTTCTCCGACGTGGACCTTGAGAAG GATGTCCATTCCGGCCTGATCGGGCCGCTGCTCGTGTGTCAC ACCAACACCCTGAACCCAGCGCATGGACGCCAGGTCACCGT CCAGGAGTTTGCTCTGTTCTTCACCATTTTTGACGAAACTAA GTCCTGGTACTTCACCGAGAATATGGAGCGAAACTGTAGAG CGCCCTGCAATATCCAGATGGAAGATCCGACTTTCAAGGAG AACTATAGATTCCACGCCATCAACGGGTACATCATGGATACT CTGCCGGGGCTGGTCATGGCCCAGGATCAGAGGATTCGGTG GTACTTGCTGTCAATGGGATCGAACGAAAACATTCACTCCAT TCACTTCTCCGGTCACGTGTTCACTGTGCGCAAGAAGGAGGA GTACAAGATGGCGCTGTACAATCTGTACCCCGGGGTGTTCGA AACTGTGGAGATGCTGCCGTCCAAGGCCGGCATCTGGAGAG TGGAGTGCCTGATCGGAGAGCACCTCCACGCGGGGATGTCC ACCCTCTTCCTGGTGTACTCGAATAAGTGCCAGACCCCGCTG GGCATGGCCTCGGGCCACATCAGAGACTTCCAGATCACAGC AAGCGGACAATACGGCCAATGGGCGCCGAAGCTGGCCCGCT TGCACTACTCCGGATCGATCAACGCATGGTCCACCAAGGAA CCGTTCTCGTGGATTAAGGTGGACCTCCTGGCCCCTATGATT ATCCACGGAATTAAGACCCAGGGCGCCAGGCAGAAGTTCTC CTCCCTGTACATCTCGCAATTCATCATCATGTACAGCCTGGA CGGGAAGAAGTGGCAGACTTACAGGGGAAACTCCACCGGCA

SEQ ID NO	Description	Sequence
		CCCTGATGGTCTTTTTCGGCAACGTGGATTCCTCCGGCATTA AGCACAACATCTTCAACCCACCGATCATAGCCAGATATATTA GGCTCCACCCCACTCACTACTCAATCCGCTCAACTCTTCGGA TGGAACTCATGGGGTGCGACCTGAACTCCTGCTCCATGCCGT TGGGGATGGAATCAAAGGCTATTAGCGACGCCCAGATCACC GCGAGCTCCTACTTCACTAACATGTTCGCCACCTGGAGCCCC TCCAAGGCCAGGCTGCACTTGCAGGGACGGTCAAATGCCTG GCGGCCGCAAGTGAACAATCCGAAGGAATGGCTTCAAGTGG ATTTCCAAAAGACCATGAAAGTGACCGGAGTCACCACCCAG GGAGTGAAGTCCCTTCTGACCTCGATGTATGTGAAGGAGTTC CTGATTAGCAGCAGCCAGGACGGGCACCAGTGGACCCTGTT CTTCCAAAACGGAAAGGTCAAGGTGTTCCAGGGGAACCAGG ACTCGTTCACACCCGTGGTGAACTCCCTGGACCCCCCACTGC TGACGCGGTACTTGAGGATTCATCCTCAGTCCTGGGTCCATC AGATTGCATTGCGAATGGAAGTCCTGGGCTGCGAGGCCCAG GACCTGTAC
2	FVIII coding sequence comprising **XTEN** (XTEN in bold and underline) (non-codon optimized)	ATGCAGATTGAGCTGTCCACTTGTTTCTTCCTGTGCCTCCTGC GCTTCTGTTTCTCCGCCACTCGCCGGTACTACCTTGGAGCCGT GGAGCTTTCATGGGACTACATGCAGAGCGACCTGGGCGAAC TCCCCGTGGATGCCAGATTCCCCCCCCGCGTGCCAAAGTCCT TCCCCTTTAACACCTCCGTGGTGTACAAGAAAACCCTCTTTG TCGAGTTCACTGACCACCTGTTCAACATCGCCAAGCCGCGCC CACCTTGGATGGGCCTCCTGGGACCGACCATTCAAGCTGAAG TGTACGACACCGTGGTGATCACCCTGAAGAACATGGCGTCCC ACCCCGTGTCCCTGCATGCGGTCGGAGTGTCCTACTGGAAGG CCTCCGAAGGAGCTGAGTACGACGACCAGACTAGCCAGCGG GAAAAGGAGGACGATAAAGTGTTCCCGGGCGGCTCGCATAC TTACGTGTGGCAAGTCCTGAAGGAAAACGGACCTATGGCAT CCGATCCTCTGTGCCTGACTTACTCCTACCTTTCCCATGTGGA CCTCGTGAAGGACCTGAACAGCGGGCTGATTGGTGCACTTCT CGTGTGCCGCGAAGGTTCGCTCGCTAAGGAAAAGACCCAGA CCCTCCATAAGTTCATCCTTTTGTTCGCTGTGTTCGATGAAGG AAAGTCATGGCATTCCGAAACTAAGAACTCGCTGATGCAGG ACCGGGATGCCGCCTCAGCCCGCGCCTGGCCTAAAATGCAT ACAGTCAACGGATACGTGAATCGGTCACTGCCCGGGCTCATC GGTTGTCACAGAAAGTCCGTGTACTGGCACGTCATCGGCATG GGCACTACGCCTGAAGTGCACTCCATCTTCCTGGAAGGGCAC ACCTTCCTCGTGCGCAACCACCGCCAGGCCTCTCTGGAAATC TCCCCGATTACCTTTCTGACCGCCCAGACTCTGCTCATGGAC CTGGGGCAGTTCCTTCTCTTCTGCCACATCTCCAGCCATCAG CACGACGGAATGGAGGCCTACGTGAAGGTGGACTCATGCCC

SEQ ID NO	Description	Sequence
		GGAAGAACCTCAGTTGCGGATGAAGAACAACGAGGAGGCCG AGGACTATGACGACGATTTGACTGACTCCGAGATGGACGTC GTGCGGTTCGATGACGACAACAGCCCCAGCTTCATCCAGATT CGCAGCGTGGCCAAGAAGCACCCCAAAACCTGGGTGCACTA CATCGCGGCCGAGGAAGAAGATTGGGACTACGCCCCGTTGG TGCTGGCACCCGATGACCGGTCGTACAAGTCCCAGTATCTGA ACAATGGTCCGCAGCGGATTGGCAGAAAGTACAAGAAAGTG CGGTTCATGGCGTACACTGACGAAACGTTTAAGACCCGGGA GGCCATTCAACATGAGAGCGGCATTCTGGGACCACTGCTGTA CGGAGAGGTCGGCGATACCCTGCTCATCATCTTCAAAAACCA GGCCTCCCGGCCTTACAACATCTACCCTCACGGAATCACCGA CGTGCGGCCACTCTACTCGCGGCGCCTGCCGAAGGGCGTCA AGCACCTGAAAGACTTCCCTATCCTGCCGGGCGAAATCTTCA AGTATAAGTGGACCGTCACCGTGGAGGACGGGCCCACCAAG AGCGATCCTAGGTGTCTGACTCGGTACTACTCCAGCTTCGTG AACATGGAACGGGACCTGGCATCGGGACTCATTGGACCGCT GCTGATCTGCTACAAAGAGTCGGTGGATCAACGCGGCAACC AGATCATGTCCGACAAGCGCAACGTGATCCTGTTCTCCGTGT TTGATGAAAACAGATCCTGGTACCTCACTGAAAACATCCAG AGGTTCCTCCCAAACCCCGCAGGAGTGCAACTGGAGGACCC TGAGTTTCAGGCCTCGAATATCATGCACTCGATTAACGGTTA CGTGTTCGACTCGCTGCAGCTGAGCGTGTGCCTCCATGAAGT CGCTTACTGGTACATTCTGTCCATCGGCGCCCAGACTGACTT CCTGAGCGTGTTCTTTTCCGGTTACACCTTTAAGCACAAGAT GGTGTACGAAGATACCCTGACCCTGTTCCCTTTCTCCGGCGA AACGGTGTTCATGTCGATGGAGAACCCGGGTCTGTGGATTCT GGGATGCCACAACAGCGACTTTCGGAACCGCGGAATGACTG CCCTGCTGAAGGTGTCCTCATGCGACAAGAACACCGGAGAC TACTACGAGGACTCCTACGAGGATATCTCAGCCTACCTCCTG TCCAAGAACAACGCGATCGAGCCGCGCAGCTTCAGCCAGAA C**ACATCAGAGAGCGCCACCCCTGAAAGTGGTCCCGGGAG CGAGCCAGCCACATCTGGGTCGGAAACGCCAGGCACAAG TGAGTCTGCAACTCCCGAGTCCGGACCTGGCTCCGAGCC TGCCACTAGCGGCTCCGAGACTCCGGGAACTTCCGAGAG CGCTACACCAGAAAGCGGACCCGGAACCAGTACCGAACC TAGCGAGGGCTCTGCTCCGGGCAGCCCAGCCGGCTCTCC TACATCCACGGAGGAGGGCACTTCCGAATCCGCCACCCC GGAGTCAGGGCCAGGATCTGAACCCGCTACCTCAGGCAG TGAGACGCCAGGAACGAGCGAGTCCGCTACACCGGAGA GTGGGCCAGGGAGCCCTGCTGGATCTCCTACGTCCACTG AGGAAGGGTCACCAGCGGGCTCGCCCACCAGCACTGAAG**

SEQ ID NO	Description	Sequence
		AAGGTGCCTCGAGCCCGCCTGTGCTGAAGAGGCACCAGCG AGAAATTACCCGGACCACCCTCCAATCGGATCAGGAGGAAA TCGACTACGACGACACCATCTCGGTGGAAATGAAGAAGGAA GATTTCGATATCTACGACGAGGACGAAAATCAGTCCCCTCGC TCATTCCAAAAGAAAACTAGACACTACTTTATCGCCGCGGTG GAAAGACTGTGGGACTATGGAATGTCATCCAGCCCTCACGTC CTTCGGAACCGGGCCCAGAGCGGATCGGTGCCTCAGTTCAA GAAAGTGGTGTTCCAGGAGTTCACCGACGGCAGCTTCACCC AGCCGCTGTACCGGGGAGAACTGAACGAACACCTGGGCCTG CTCGGTCCCTACATCCGCGCGGAAGTGGAGGATAACATCAT GGTGACCTTCCGTAACCAAGCATCCAGACCTTACTCCTTCTA TTCCTCCCTGATCTCATACGAGGAGGACCAGCGCCAAGGCGC CGAGCCCCGCAAGAACTTCGTCAAGCCCAACGAGACTAAGA CCTACTTCTGGAAGGTCCAACACCATATGGCCCCGACCAAGG ATGAGTTTGACTGCAAGGCCTGGGCCTACTTCTCCGACGTGG ACCTTGAGAAGGATGTCCATTCCGGCCTGATCGGGCCGCTGC TCGTGTGTCACACCAACACCCTGAACCCAGCGCATGGACGC CAGGTCACCGTCCAGGAGTTTGCTCTGTTCTTCACCATTTTTG ACGAAACTAAGTCCTGGTACTTCACCGAGAATATGGAGCGA AACTGTAGAGCGCCCTGCAATATCCAGATGGAAGATCCGAC TTTCAAGGAGAACTATAGATTCCACGCCATCAACGGGTACAT CATGGATACTCTGCCGGGGCTGGTCATGGCCCAGGATCAGA GGATTCGGTGGTACTTGCTGTCAATGGGATCGAACGAAAAC ATTCACTCCATTCACTTCTCCGGTCACGTGTTCACTGTGCGCA AGAAGGAGGAGTACAAGATGGCGCTGTACAATCTGTACCCC GGGGTGTTCGAAACTGTGGAGATGCTGCCGTCCAAGGCCGG CATCTGGAGAGTGGAGTGCCTGATCGGAGAGCACCTCCACG CGGGGATGTCCACCCTCTTCCTGGTGTACTCGAATAAGTGCC AGACCCCGCTGGGCATGGCCTCGGGCCACATCAGAGACTTC CAGATCACAGCAAGCGGACAATACGGCCAATGGGCGCCGAA GCTGGCCCGCTTGCACTACTCCGGATCGATCAACGCATGGTC CACCAAGGAACCGTTCTCGTGGATTAAGGTGGACCTCCTGGC CCCTATGATTATCCACGGAATTAAGACCCAGGGCGCCAGGC AGAAGTTCTCCTCCCTGTACATCTCGCAATTCATCATCATGT ACAGCCTGGACGGGAAGAAGTGGCAGACTTACAGGGGAAAC TCCACCGGCACCCTGATGGTCTTTTTCGGCAACGTGGATTCC TCCGGCATTAAGCACAACATCTTCAACCCACCGATCATAGCC AGATATATTAGGCTCCACCCCACTCACTACTCAATCCGCTCA ACTCTTCGGATGGAACTCATGGGGTGCGACCTGAACTCCTGC TCCATGCCGTTGGGGATGGAATCAAAGGCTATTAGCGACGC CCAGATCACCGCGAGCTCCTACTTCACTAACATGTTCGCCAC

SEQ ID NO	Description	Sequence
		CTGGAGCCCCTCCAAGGCCAGGCTGCACTTGCAGGGACGGT CAAATGCCTGGCGGCCGCAAGTGAACAATCCGAAGGAATGG CTTCAAGTGGATTTCCAAAAGACCATGAAAGTGACCGGAGT CACCACCCAGGGAGTGAAGTCCCTTCTGACCTCGATGTATGT GAAGGAGTTCCTGATTAGCAGCAGCCAGGACGGGCACCAGT GGACCCTGTTCTTCCAAAACGGAAAGGTCAAGGTGTTCCAG GGGAACCAGGACTCGTTCACACCCGTGGTGAACTCCCTGGA CCCCCCACTGCTGACGCGGTACTTGAGGATTCATCCTCAGTC CTGGGTCCATCAGATTGCATTGCGAATGGAAGTCCTGGGCTG CGAGGCCCAGGACCTGTAC
3	FIX coding sequence comprising R338L mutation (**signal peptide** in bold and underline) (Non-codon optimized)	**ATGCAGAGAGTCAACATGATTATGGCTGAGTCACCTGGG CTGATTACTATTTGCCTGCTGGGCTACCTGCTGTCCGCC GAGTGTACCGTGTTCCTGGACCATGAGAACGCAAATAAG ATCCTGAACAGGCCCAAAAGA**TACAATAGTGGGAAGCTGG AGGAATTTGTGCAGGGCAACCTGGAGAGAGAATGCATGGAG GAAAAGTGTAGCTTCGAGGAAGCCCGCGAGGTGTTTGAAAA TACAGAGCGAACCACAGAGTTCTGGAAGCAGTATGTGGACG GCGATCAGTGCGAGAGCAACCCCTGTCTGAATGGCGGAAGT TGCAAAGACGATATCAACTCATACGAATGCTGGTGTCCTTTC GGGTTTGAAGGCAAAAATTGCGAGCTGGACGTGACATGTAA CATTAAGAATGGACGGTGCGAGCAGTTTTGTAAAAACTCTGC CGATAATAAGGTGGTGTGCAGCTGTACTGAAGGATATCGCCT GGCTGAGAACCAGAAGTCCTGCGAACCAGCAGTGCCCTTCC CTTGTGGGAGGGTGAGCGTCTCCCAGACTTCAAAACTGACCA GAGCAGAGACAGTGTTTCCCGACGTGGATTACGTCAACAGC ACTGAGGCCGAAACCATCCTGGACAACATTACTCAGTCTACC CAGAGTTTCAATGACTTTACTCGGGTGGTCGGGGGCGAGGAT GCTAAACCAGGCCAGTTCCCCTGGCAGGTGGTCCTGAACGG AAAGGTGGATGCATTTTGCGGAGGGTCTATCGTGAATGAGA AATGGATTGTCACCGCCGCTCACTGCGTGGAAACCGGAGTC AAGATCACAGTGGTCGCTGGGGAGCACAACATTGAGGAAAC AGAACATACTGAGCAGAAGCGGAATGTGATCCGCATCATTC CTCACCATAACTACAATGCAGCCATCAACAAATACAATCATG ACATTGCCCTGCTGGAACTGGATGAGCCTCTGGTGCTGAACA GCTACGTCACTCCAATCTGCATTGCTGACAAAGAGTATACCA ATATCTTCCTGAAGTTTGGATCAGGGTACGTGAGCGGCTGGG GAAGAGTCTTCCACAAGGGCAGGAGCGCCCTGGTGCTCCAG TATCTGCGAGTGCCTCTGGTCGATCGAGCTACCTGTCTGCTC TCTACCAAGTTTACAATCTACAACAACATGTTCTGCGCTGGG TTTCACGAGGGAGGACGAGACTCCTGTCAGGGCGATTCTGG GGGCCCACATGTGACAGAGGTCGAAGGCACCAGCTTCCTGA

SEQ ID NO	Description	Sequence
		CTGGCATCATTTCCTGGGGAGAGGAATGTGCAATGAAGGGA AAATACGGGATCTACACCAAAGTGAGCCGCTATGTGAACTG GATCAAGGAAAAAACCAAACTGACC
4	Mature FVIII polypeptide	ATRRYYLGAVELSWDYMQSDLGELPVDARFPPRVPKSFPFNTS VVYKKTLFVEFTDHLFNIAKPRPPWMGLLGPTIQAEVYDTVVIT LKNMASHPVSLHAVGVSYWKASEGAEYDDQTSQREKEDDKVF PGGSHTYVWQVLKENGPMASDPLCLTYSYLSHVDLVKDLNSG LIGALLVCREGSLAKEKTQTLHKFILLFAVFDEGKSWHSETKNS LMQDRDAASARAWPKMHTVNGYVNRSLPGLIGCHRKSVYWH VIGMGTTPEVHSIFLEGHTFLVRNHRQASLEISPITFLTAQTLLM DLGQFLLFCHISSHQHDGMEAYVKVDSCPEEPQLRMKNNEEAE DYDDDLTDSEMDVVRFDDDNSPSFIQIRSVAKKHPKTWVHYIA AEEEDWDYAPLVLAPDDRSYKSQYLNNGPQRIGRKYKKVRFM AYTDETFKTREAIQHESGILGPLLYGEVGDTLLIIFKNQASRPYNI YPHGITDVRPLYSRRLPKGVKHLKDFPILPGEIFKYKWTVTVED GPTKSDPRCLTRYYSSFVNMERDLASGLIGPLLICYKESVDQRG NQIMSDKRNVILFSVFDENRSWYLTENIQRFLPNPAGVQLEDPE FQASNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLSV FFSGYTFKHKMVYEDTLTLFPFSGETVFMSMENPGLWILGCHN SDFRNRGMTALLKVSSCDKNTGDYYEDSYEDISAYLLSKNNAI EPRSFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTPMPKI QNVSSSDLLMLLRQSPTPHGLSLSDLQEAKYETFSDDPSPGAIDS NNSLSEMTHFRPQLHHSGDMVFTPESGLQLRLNEKLGTTAATE LKKLDFKVSSTSNNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDS QLDTTLFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESS WGKNVSSTESGRLFKGKRAHGPALLTKDNALFKVSISLLKTNK TSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTPLIH DRMLMDKNATALRLNHMSNKTTSSKNMEMVQQKKEGPIPPD AQNPDMSFFKMLFLPESARWIQRTHGKNSLNSGQGPSPKQLVS LGPEKSVEGQNFLSEKNKVVVGKGEFTKDVGLKEMVFPSSRNL FLTNLDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTG TKNFMKNLFLLSTRQNVEGSYDGAYAPVLQDFRSLNDSTNRTK KHTAHFSKKGEEENLEGLGNQTKQIVEKYACTTRISPNTSQQNF VTQRSKRALKQFRLPLEETELEKRIIVDDTSTQWSKNMKHLTPS TLTQIDYNEKEKGAITQSPLSDCLTRSHSIPQANRSPLPIAKVSSF PSIRPIYLTRVLFQDNSSHLPAASYRKKDSGVQESSHFLQGAKK NNLSLAILTLEMTGDQREVGSLGTSATNSVTYKKVENTVLPKP DLPKTSGKVELLPKVHIYQKDLFPTETSNGSPGHLDLVEGSLLQ GTEGAIKWNEANRPGKVPFLRVATESSAKTPSKLLDPLAWDNH YGTQIPKEEWKSQEKSPEKTAFKKKDTILSLNACESNHAIAAINE GQNKPEIEVTWAKQGRTERLCSQNPPVLKRHQREITRTTLQSDQ

SEQ ID NO	Description	Sequence
		EEIDYDDTISVEMKKEDFDIYDEDENQSPRSFQKKTRHYFIAAV ERLWDYGMSSSPHVLRNRAQSGSVPQFKKVVFQEFTDGSFTQP LYRGELNEHLGLLGPYIRAEVEDNIMVTFRNQASRPYSFYSSLIS YEEDQRQGAEPRKNFVKPNETKTYFWKVQHHMAPTKDEFDCK AWAYFSDVDLEKDVHSGLIGPLLVCHTNTLNPAHGRQVTVQEF ALFFTIFDETKSWYFTENMERNCRAPCNIQMEDPTFKENYRFHA INGYIMDTLPGLVMAQDQRIRWYLLSMGSNENIHSIHFSGHVFT VRKKEEYKMALYNLYPGVFETVEMLPSKAGIWRVECLIGEHLH AGMSTLFLVYSNKCQTPLGMASGHIRDFQITASGQYGQWAPKL ARLHYSGSINAWSTKEPFSWIKVDLLAPMIIHGIKTQGARQKFSS LYISQFIIMYSLDGKKWQTYRGNSTGTLMVFFGNVDSSGIKHNI FNPPIIARYIRLHPTHYSIRSTLRMELMGCDLNSCSMPLGMESKA ISDAQITASSYFTNMFATWSPSKARLHLQGRSNAWRPQVNNPK EWLQVDFQKTMKVTGVTTQGVKSLLTSMYVKEFLISSSQDGH QWTLFFQNGKVKVFQGNQDSFTPVVNSLDPPLLTRYLRIHPQS WVHQIALRMEVLGCEAQDLY
5	FVIII amino acid sequence comprising **XTEN** (XTEN in bold and underline)	MQIELSTCFFLCLLRFCFSATRRYYLGAVELSWDYMQSDLGELP VDARFPPRVPKSFPFNTSVVYKKTLFVEFTDHLFNIAKPRPPWM GLLGPTIQAEVYDTVVITLKNMASHPVSLHAVGVSYWKASEGA EYDDQTSQREKEDDKVFPGGSHTYVWQVLKENGPMASDPLCL TYSYLSHVDLVKDLNSGLIGALLVCREGSLAKEKTQTLHKFILL FAVFDEGKSWHSETKNSLMQDRDAASARAWPKMHTVNGYVN RSLPGLIGCHRKSVYWHVIGMGTTPEVHSIFLEGHTFLVRNHRQ ASLEISPITFLTAQTLLMDLGQFLLFCHISSHQHDGMEAYVKVD SCPEEPQLRMKNNEEAEDYDDDLTDSEMDVVRFDDDNSPSFIQI RSVAKKHPKTWVHYIAAEEEDWDYAPLVLAPDDRSYKSQYLN NGPQRIGRKYKKVRFMAYTDETFKTREAIQHESGILGPLLYGEV GDTLLIIFKNQASRPYNIYPHGITDVRPLYSRRLPKGVKHLKDFP ILPGEIFKYKWTVTVEDGPTKSDPRCLTRYYSSFVNMERDLASG LIGPLLICYKESVDQRGNQIMSDKRNVILFSVFDENRSWYLTENI QRFLPNPAGVQLEDPEFQASNIMHSINGYVFDSLQLSVCLHEVA YWYILSIGAQTDFLSVFFSGYTFKHKMVYEDTLTLFPFSGETVF MSMENPGLWILGCHNSDFRNRGMTALLKVSSCDKNTGDYYED SYEDISAYLLSKNNAIEPRSFSQN**TSESATPESGPGSEPATSGSE TPGTSESATPESGPGSEPATSGSETPGTSESATPESGPGTSTE PSEGSAPGSPAGSPTSTEEGTSESATPESGPGSEPATSGSETP GTSESATPESGPGSPAGSPTSTEEGSPAGSPTSTEEGASS**PPVL KRHQREITRTTLQSDQEEIDYDDTISVEMKKEDFDIYDEDENQS PRSFQKKTRHYFIAAVERLWDYGMSSSPHVLRNRAQSGSVPQF KKVVFQEFTDGSFTQPLYRGELNEHLGLLGPYIRAEVEDNIMVT FRNQASRPYSFYSSLISYEEDQRQGAEPRKNFVKPNETKTYFWK

SEQ ID NO	Description	Sequence
		VQHHMAPTKDEFDCKAWAYFSDVDLEKDVHSGLIGPLLVCHT NTLNPAHGRQVTVQEFALFFTIFDETKSWYFTENMERNCRAPC NIQMEDPTFKENYRFHAINGYIMDTLPGLVMAQDQRIRWYLLS MGSNENIHSIHFSGHVFTVRKKEEYKMALYNLYPGVFETVEML PSKAGIWRVECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHI RDFQITASGQYGQWAPKLARLHYSGSINAWSTKEPFSWIKVDL LAPMIIHGIKTQGARQKFSSLYISQFIIMYSLDGKKWQTYRGNST GTLMVFFGNVDSSGIKHNIFNPPIIARYIRLHPTHYSIRSTLRMEL MGCDLNSCSMPLGMESKAISDAQITASSYFTNMFATWSPSKAR LHLQGRSNAWRPQVNNPKEWLQVDFQKTMKVTGVTTQGVKS LLTSMYVKEFLISSSQDGHQWTLFFQNGKVKVFQGNQDSFTPV VNSLDPPLLTRYLRIHPQSWVHQIALRMEVLGCEAQDLY
6	FIX-R338L amino acid sequence (**signal peptide** in bold and underline)	**MQRVNMIMAESPGLITICLLGYLLSAECTVFLDHENANKIL NRPKR**YNSGKLEEFVQGNLERECMEEKCSFEEAREVFENTERT TEFWKQYVDGDQCESNPCLNGGSCKDDINSYECWCPFGFEGK NCELDVTCNIKNGRCEQFCKNSADNKVVCSCTEGYRLAENQKS CEPAVPFPCGRVSVSQTSKLTRAETVFPDVDYVNSTEAETILDNI TQSTQSFNDFTRVVGGEDAKPGQFPWQVVLNGKVDAFCGGSIV NEKWIVTAAHCVETGVKITVVAGEHNIEETEHTEQKRNVIRIIPH HNYNAAINKYNHDIALLELDEPLVLNSYVTPICIADKEYTNIFLK FGSGYVSGWGRVFHKGRSALVLQYLRVPLVDRATCLLSTKFTI YNNMFCAGFHEGGRDSCQGDSGGPHVTEVEGTSFLTGIISWGE ECAMKGKYGIYTKVSRYVNWIKEKTKLT
7	miR-142 target	tccataaagtaggaaacactaca

D. EXCIPIENTS, CARRIERS, AND OTHER CONSTITUENTS OF FORMULATIONS

In certain embodiments, viral vector compositions produced by a process or method provided herein are combined with one or more pharmaceutical excipients to make a pharmaceutical composition comprising the viral vector.

In certain embodiments, the pharmaceutical composition comprises an effective dose of a viral vector (e.g., a recombinant lentiviral vector). In certain embodiments, the pharmaceutical composition comprises an effective dose of a recombinant lentiviral vector. In certain embodiments, the pharmaceutical composition comprises a formulation buffer. In certain embodiments, the formulation buffer is a phosphate or histidine buffer comprising NaCl and sucrose. In certain embodiments, the pharmaceutical composition comprises: (a) an effective dose of a recombinant lentiviral vector; (b) a TRIS-free buffer system; (c) a salt; (d) a surfactant; and

(e) a carbohydrate, wherein the pharmaceutical composition is suitable for administration to a human subject. In certain embodiments, the pH of the buffer system is between 6.0 and 8.0. In certain embodiments, the buffer system is a phosphate buffer or a histidine buffer. In certain embodiments, the concentration of the phosphate or histidine buffer is between 5 mM and 30 mM. In certain embodiments, the concentration of the phosphate buffer is about 10 to about 20 mM, about 10 to about 15 mM, about 20 to about 30 mM, about 20 to about 25mM, or about 15 to about 20 mM. In certain embodiments, the salt is a chloride salt. In certain embodiments, the concentration of the chloride salt is between 80 mM and 150 mM. In certain embodiments, the concentration of the salt is about 100 mM, about 110 mM, about 130 mM, or about 150 mM. In certain embodiments, the surfactant is a poloxamer or a polysorbate. In certain embodiments, the concentration of the poloxamer or polysorbate is between 0.01% and 0.1%. In certain embodiments, the carbohydrate is sucrose. In certain embodiments, the concentration of the carbohydrate is between 0.5% and 5%. In certain embodiments, the chloride salt is NaCl. In certain embodiments, the poloxamer is selected from the group consisting of poloxamer 101 (P101), poloxamer 105 (P105), poloxamer 108 (P108), poloxamer 122 (P122), poloxamer 123 (P123), poloxamer 124 (P124), poloxamer 181 (P181), poloxamer 182 (P182), poloxamer 183 (P183), poloxamer 184 (P184), poloxamer 185 (P185), poloxamer 188 (P188), poloxamer 212 (P212), poloxamer 215 (P215), poloxamer 217 (P217), poloxamer 231 (P231), poloxamer 234 (P234), poloxamer 235 (P235), poloxamer 237 (P237), poloxamer 238 (P238), poloxamer 282 (P282), poloxamer 284 (P284), poloxamer 288 (P288), poloxamer 331 (P331), poloxamer 333 (P333), poloxamer 334 (P334), poloxamer 335 (P335), poloxamer 338 (P338), poloxamer 401 (P401), poloxamer 402 (P402), poloxamer 403 (P403), poloxamer 407 (P407), and a combination thereof. In certain embodiments, the polysorbate is selected from the group consisting of polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 80, and a combination thereof. In certain embodiments, the pH of the phosphate or histidine buffer is 6.1, 6.3, 6.5, 6.7, 6.9, 7.1, 7.3, 7.5, 7.7, or 7.9. In certain embodiments, the concentration of the phosphate or histidine buffer is 10 mM, 15 mM, 20 mM, or 25 mM. In certain embodiments, the chloride salt is 100 mM, 110 mM, 130 mM, or 150 mM. In certain embodiments, the concentration of the poloxamer or polysorbate is 0.03%, 0.05%, 0.07%, or 0.09%. In certain embodiments, the concentration of the carbohydrate is 1%, 2%, 3%, or 4%. In certain embodiments, the poloxamer is poloxamer 188 (P188). In certain embodiments, the poloxamer is poloxamer 407 (P407).

The viral vector of the disclosure can be administered intravenously, subcutaneously, intramuscularly, or via any mucosal surface, *e.g.,* orally, sublingually, buccally, sublingually, nasally, rectally, vaginally or via pulmonary route. The viral vector can be implanted within or linked to a biopolymer solid support that allows for the slow release of the vector to the desired site.

In one embodiment, the route of administration of the formulated drug substance comprising a viral vector is parenteral. The term parenteral as used herein includes intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, rectal or vaginal administration. The intravenous form of parenteral administration is preferred. While all these forms of administration are clearly contemplated as being within the scope of the disclosure, a form for administration would be a solution for injection, in particular for intravenous or intraarterial injection or drip. Usually, a suitable pharmaceutical composition for injection can comprise a buffer (*e.g.* acetate, phosphate or citrate buffer), a surfactant (*e.g.* polysorbate), optionally a stabilizer agent (*e.g.* human albumin), etc. However, in other methods compatible with the teachings herein, the viral vector can be delivered directly to the site of the adverse cellular population thereby increasing the exposure of the diseased tissue to the therapeutic agent.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. In the subject disclosure, pharmaceutically acceptable carriers include, but are not limited to, 0.01-0.1M and preferably 0.05M phosphate buffer or 0.8% saline. Other common parenteral vehicles include sodium phosphate solutions, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer's dextrose, and the like. Preservatives and other additives can also be present such as for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like.

More particularly, pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In such cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the

conditions of manufacture and storage and will preferably be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols, such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

In any case, sterile injectable solutions can be prepared by incorporating an active compound (*e.g.*, a polypeptide by itself or in combination with other active agents) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated herein, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle, which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying, which yields a powder of an active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The preparations for injections are processed, filled into containers such as ampoules, bags, bottles, syringes or vials, and sealed under aseptic conditions according to methods known in the art. Further, the preparations can be packaged and sold in the form of a kit. Such articles of manufacture will preferably have labels or package inserts indicating that the associated compositions are useful for treating a subject suffering from, or predisposed to clotting disorders.

The pharmaceutical composition can also be formulated for rectal administration as a suppository or retention enema, *e.g.,* containing conventional suppository bases such as cocoa butter or other glycerides.

Effective doses of the compositions of the present disclosure, for the treatment of conditions vary depending upon many different factors, including means of administration, target

site, physiological state of the patient, whether the patient is human or an animal, other medications administered, and whether treatment is prophylactic or therapeutic. Usually, the patient is a human, but non-human mammals including transgenic mammals can also be treated. Treatment dosages can be titrated using routine methods known to those of skill in the art to optimize safety and efficacy.

A pharmaceutical composition comprising a viral vector can be administered as a single dose or as multiple doses, wherein the multiple doses can be administered continuously or at specific timed intervals. *In vitro* assays can be employed to determine optimal dose ranges and/or schedules for administration. *In vitro* assays that measure clotting factor activity are known in the art. Additionally, effective doses can be extrapolated from dose-response curves obtained from animal models, *e.g.,* a hemophiliac dog (Mount et al. 2002, Blood 99 (8): 2670).

Doses intermediate in the above ranges are also intended to be within the scope of the disclosure. Subjects can be administered such doses daily, on alternative days, weekly or according to any other schedule determined by empirical analysis. An exemplary treatment entails administration in multiple dosages over a prolonged period, for example, of at least six months.

A pharmaceutical composition comprising a viral vector can be administered on multiple occasions. Intervals between single dosages can be daily, weekly, monthly or yearly. Intervals can also be irregular as indicated by measuring blood levels of modified polypeptide or antigen in the patient. Dosage and frequency of a viral vector may vary depending on the half-life of the transgene protein product in the patient.

The dosage and frequency of administration of a pharmaceutical composition comprising a viral vector can vary depending on whether the treatment is prophylactic or therapeutic. In prophylactic applications, compositions containing a viral vector are administered to a patient not already in the disease state to enhance the patient's resistance or minimize effects of disease. Such an amount is defined to be a "prophylactic effective dose." A relatively low dosage is administered at relatively infrequent intervals over a long period of time. Some patients continue to receive treatment for the rest of their lives.

A pharmaceutical composition comprising a viral vector can optionally be administered in combination with other agents that are effective in treating the disorder or condition in need of treatment (*e.g.,* prophylactic or therapeutic).

As used herein, the administration of a pharmaceutical composition comprising a viral vector in conjunction or combination with an adjunct therapy means the sequential, simultaneous, coextensive, concurrent, concomitant or contemporaneous administration or application of the therapy and the disclosed polypeptides. Those skilled in the art will appreciate that the administration or application of the various components of the combined therapeutic regimen can be timed to enhance the overall effectiveness of the treatment. A skilled artisan (*e.g.*, a physician) would be readily be able to discern effective combined therapeutic regimens without undue experimentation based on the selected adjunct therapy and the teachings of the instant specification.

It will further be appreciated that a pharmaceutical composition comprising a viral vector can be used in conjunction or combination with an agent or agents (*e.g.*, to provide a combined therapeutic regimen). Exemplary agents with which a pharmaceutical composition comprising a viral vector can be combined include agents that represent the current standard of care for a particular disorder being treated. Such agents can be chemical or biologic in nature. The term "biologic" or "biologic agent" refers to any pharmaceutically active agent made from living organisms and/or their products which is intended for use as a therapeutic.

The amount of agent to be used in combination with a pharmaceutical composition comprising a viral vector can vary by subject or can be administered according to what is known in the art. *See, e.g.*, Bruce A Chabner *et al.*, *Antineoplastic Agents, in* GOODMAN & GILMAN'S THE PHARMACOLOGICAL BASIS OF THERAPEUTICS 1233-1287 ((Joel G. Hardman *et al.*, eds., 9th ed. 1996). In another embodiment, an amount of such an agent consistent with the standard of care is administered.

In certain embodiments, a pharmaceutical composition comprising a viral vector is administered in conjunction with an immunosuppressive, anti-allergic, or anti-inflammatory agent. These agents generally refer to substances that act to suppress or mask the immune system of the subject being treated herein. These agents include substances that suppress cytokine production, downregulate or suppress self-antigen expression, or mask the MHC antigens. Examples of such agents include 2-amino-6-aryl-5-substituted pyrimidines; azathioprine; cyclophosphamide; bromocryptine; danazol; dapsone; glutaraldehyde; anti-idiotypic antibodies for MHC antigens and MHC fragments; cyclosporin A; steroids such as glucocorticosteroids, *e.g.*, prednisone, methylprednisolone, and dexamethasone; cytokine or cytokine receptor antagonists including anti-

interferon-γ, -β, or -α antibodies, anti-tumor necrosis factor-α antibodies, anti-tumor necrosis factor-β antibodies, anti-interleukin-2 antibodies and anti-IL-2 receptor antibodies; anti-LFA-1 antibodies, including anti-CD11a and anti-CD18 antibodies; anti-L3T4 antibodies; heterologous anti-lymphocyte globulin; pan-T antibodies; soluble peptide containing a LFA-3 binding domain; streptokinase; TGF-β; streptodornase; FK506; RS-61443; deoxyspergualin; and rapamycin. In certain embodiments, the agent is an antihistamine. An "antihistamine" as used herein is an agent that antagonizes the physiological effect of histamine. Examples of antihistamines are chlorpheniramine, diphenhydramine, promethazine, cromolyn sodium, astemizole, azatadine maleate, bropheniramine maleate, carbinoxamine maleate, cetirizine hydrochloride, clemastine fumarate, cyproheptadine hydrochloride, dexbrompheniramine maleate, dexchlorpheniramine maleate, dimenhydrinate, diphenhydramine hydrochloride, doxylamine succinate, fexofendadine hydrochloride, terphenadine hydrochloride, hydroxyzine hydrochloride, loratidine, meclizine hydrochloride, tripelannamine citrate, tripelennamine hydrochloride, and triprolidine hydrochloride.

Immunosuppressive, anti-allergic, or anti-inflammatory agents may be incorporated into the pharmaceutical composition comprising a viral vector administration regimen. For example, administration of immunosuppressive or anti-inflammatory agents may commence prior to administration of a pharmaceutical composition comprising a viral vector, and may continue with one or more doses thereafter. In certain embodiments, the immunosuppressive or anti-inflammatory agents are administered as premedication to the pharmaceutical composition comprising a viral vector.

It will be appreciated that a pharmaceutical composition comprising a viral vector can be formulated to facilitate administration and promote stability of the active agent. Preferably, pharmaceutical compositions in accordance with the present disclosure comprise a pharmaceutically acceptable, non-toxic, sterile carrier such as physiological saline, non-toxic buffers, preservatives and the like. Of course, the pharmaceutical compositions of the present disclosure can be administered in single or multiple doses to provide for a pharmaceutically effective amount of the polypeptide.

Having now described the present disclosure in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the disclosure. All patents, publications, and articles referred to herein are expressly and specifically incorporated herein by reference.

EXAMPLES

Materials and Methods:

The following are exemplary reagents (Table 2) and general methods provided that are suitable for carrying out methods of the present disclosure, as outlined in Examples 1-6.

Table 2: Exemplary reagents suitable for use in methods described herein.

Description of Material	Vendor	Cat. No.
HEK293 cells	In-house	N/A
Plasmids (Genomic FVIIIXTEN, FVIII, FIX; Gagpol; VSV-G; Rev)	In-house	N/A
Transfection agent: PEIpro Lipofactamine 2000 Lipofactamine 3000	 Polyplus Transfection Thermo-Fisher Scientific Thermo-Fisher Scientific	 115-400 11668019 L300075
VIIISelect resin	Cytiva	Cat No. 17-5450
FreeStyle 293 Expression Production Media	Thermo-Fisher Scientific	12338-002
250 X Cholesterol Lipid Concentrate (CLC)	Thermo Scientific	12351-018
BIOSTAT® STR50 FlexSafe® Bag	Sartorius Stedim	FRS131920 D
Benzonase® endonuclease	Merck Millipore	1.01697.001

Anion Exchange Chromatography media (Sartobind® Q)	Sartorius Stedim	96IEXQ42D1GSS
AKTA Avant 150 Chromatography	GE Healthcare	
HDC® II pre-filter part number	Pall Corporation	NP7J100P1G
Supor® EAV 0.2 µm filter	Pall Corporation	NT7UEAVP1S
Hollow Fiber membrane, 500kDa	Cytiva	ReadyToProcess™ RTPUFP-500-C-6S
Chromogenix Coatest® SP Factor VIII Kit	Diapharma	K824086
CaCl2	Millipore-Sigma	449709-10G
Sodium phosphate	VWR	JT3802-1 and JT3827-1
Tromethane or Tris	Avantor, Millipore	T6066, T5941
Sodium Chloride	JT Baker	BAKR3627
Capto MMC	Cytiva	17531710, 17371601
Capto Adhere	Cytiva	17544410, 17371501
CIMmultus Advanced composite Monolith columns	Bia Separations	411.5114-2, 411.5113-2,

FVIIIXTEN or FVIII Activity Determination: The chromogenic assay kit Chromogenix Coatest® SP Factor VIII (Cat No. K824086; Diapharma™) can be used for the *in vitro* diagnostic photometric determination of FVIII activity. Briefly, Factor X is activated to Factor Xa by Factor IXa in the presence of calcium and phospholipids. This generation is greatly stimulated by FVIII. Optimal amounts of Ca^{2+} and phospholipids and an excess of Factors IXa and X was used, making the rate of activation of Factor X dependent on the amount of FVIII. Factor Xa hydrolyses the chromogenic substrate (Z-D-Arg-Gly-Arg-pNA) S-2765™, thus liberating the chromophoric group, pNA (p-nitroaniline). The generated Factor Xa and resulting intensity of color is proportional to the FVIII activity in the sample. The color can then be read photometrically at 405nm.

Production of Viral Vector: Enveloped viral vectors LV-FVIIIXTEN or LV-FVIII were produced in human embryonic kidney 293 (HEK293) cells. The cells are adapted to culture media and either: (1) transfected transiently using plasmid DNA and transfection agent such as

PEIpro® (Cat. No. 115-400, Polyplus Transfection®), Lipofactamine® 2000 (Cat. No. 11668019, Thermo Fisher™), Lipofactamine® 3000 (Cat. No. L300075, Thermo Fisher™) or other equivalent reagents, or (2) stable producer cells are induced in shake flasks or bioreactors (disposable systems, conventional stirred tanks with or without adsorption media in the vessel) and run as batch, fed-batch, perfusion or continuous chemostate culture to produce viral particles. The vector producing cells can be cultured in production media such as FreeStyle™ 293 Expression medium, LV-Max™ or equivalent media, generally, serum free and chemically defined. Typically, suspension culture involves perfusion of medium, exchange of media using alternating tangential flow (ATF) or addition of fresh media using sequential addition of nutrients, and is performed to increase cell numbers and product titer beyond the batch culture systems. The product yield, product purity and amount of impurities depends on the ability of the cells to produce vectors, the system of production used, or a combination thereof. The viral vector produced from cells remains in culture supernatant along with other process- and product-related impurities and proteins generated during the process.

Cell Separation and Nucleic Acid Treatment: Vector producing cells, cell debris and other particulate impurities can be separated from supernatant using a filtration step. Suitable filters include those comprising two or more filters in sequence, and may utilize regenerated cellulose fibers, cellulose fibers combined with inorganic filter aids or organic resins, or any combination thereof, and polymeric filters. For example, nylon, polypropylene (PE), polyethersulfone (PES) filters can be used to achieve effective removal of cell debris and other particulate impurities, and result in acceptable recoveries. A multiple stage process can be used to improve vector yield and impurity reduction. An exemplary two or three-stage process would consist of a coarse filter(s) to remove cells, large precipitate and cell debris followed by a polishing second stage filter(s) with nominal pore sizes of 0.65, 0.45, 0.22, or 0.2 micron. The optimal combination may be a function of the depth filter size grading, cell holding capacity and interaction of filter contact surface with viral vector and impurities. Alternatively, a staged operation may be performed for clarification, and may include single stage operations employing a centrifugation followed by a polishing stage filter(s) with nominal pore sizes of 0.65, 0.45, 0.22, or 0.2 micron. Generally, a clarification approach includes a combination of one or more of the following, without limitation, dead-end filtration, microfiltration, centrifugation, and depth filtration, providing a supernatant of suitable clarity or turbidity and absent of particulate matters

that are suitable for subsequent purification steps. For example, two stage depth filtration including 5-10 micron and 1.2-2.0 micron depth filtration followed by a membrane filtration of 0.2-0.65um can be used in combination.

The supernatant contains nucleic acids (DNA/RNA) impurities generated by host cells or a product of the transient transfection process. Such nucleic acids can be degraded by using suitable nucleases, for example Benzonase® Endonuclease (Cat. No. 1.01697.001, Millipore Sigma™) or any other DNase and/or RNase commonly used within the art for the purpose of eliminating unwanted or contaminating DNA and/or RNA. Such nucleases function by attacking and degrading all forms of DNA and RNA (single stranded, double stranded linear or circular) for the preparation of supernatant before or after the cell separation operations are performed. In some embodiments, the concentration of nucleases used is within the range of 1-100 Units/mL, added before or after cell separation unit operation and allowed to incubate with supernatant culture for 5-60 min at 20-37ºC, or potentially over night at 2-8ºC, for digestion of nucleic acids to an acceptable level. Alternatively, in absence of such addition of nucleases, non-vector producing cells responsible for nuclease enzyme secretion can be added, or selective precipitation can be performed using suitable precipitating agents for the removal of DNA/RNA impurities.

Purification of Viral Vector: The purification of viral vector involves separating functional vector from supernatant comprising viral vector product as well as other protein- and non-protein-related impurities, including the transgene protein contaminants mediated by the promoter upstream of the transgene expression cassette (e.g., CMV promoter). The supernatant undergoes stages of purifications, for example, potentially multiple stages of chromatography, TFF and microfiltration processes, as outlined in FIGs. 1A and 1B. Subsequent purification is performed to achieve the drug substance.

In some embodiments, two or more sequential chromatography steps may be useful to achieve higher viral vector particle purity and assure removal of contaminating proteins, nucleic acids, endotoxins, and other product-related variants. High viral vector particle purity is a requirement for the manufacture of enveloped viral vector for use in human subjects to treat diseases. Sequential chromatography steps can use different resins performed sequentially in varying orders, optimized for higher recovery of functional viral vector and minimizing impurities in the final drug product. For example, vector particle purification achieved by two

sequential chromatography steps, as shown in FIG. 1B, may employ Viral Vector Capture Chromatography (First Chromatography Purification) followed by a transgene protein encoded by the Transgene Impurity Capture Chromatography (Second Chromatography Purification). The viral vector capture chromatography step and the transgene impurity capture chromatography step can be performed in any order, e.g., in reverse order to what is shown in FIG. 1B where the transgene impurity capture chromatography step is performed first, followed by the viral vector capture chromatography step.

In certain embodiments, the supernatant is applied to a first anion exchange column. In certain embodiments, the anion exchange column comprises a combination of membrane and DEAE or Q chemistry. A number of suitable anion exchangers for use with the present invention includes, without limitation, CIM Monolith (Q or DEAE, strong or weak anion-exchanger, BIA Separations), Sartobind Q (Q or DEAE, strong or weak anion exchanger, Sartorius Stedim), TOYOPEARLE 650C (DEAE, UNOSPHERE Q or DEAE, strong or weak anion-exchanger, BioRad, Hercules); POROS 50 (HQ, strong-anion-exchanger, Life Technologies), POROS 50 (XS, strong-anion-exchanger, Life Technologies), POROS 50 (D, weak anion-exchanger, Life Technologies), POROS 50 (PI, weak anion-exchanger, Life Technologies), SEPHAROSE (DEAE, weak anion-exchanger, Cytiva); SEPHAROSE (Q, Strong anion-exchanger, Cytiva), and Capto Q (Srong anion-exchanger, Cytiva).

The anion exchange column is first equilibrated using standard buffers and according to the manufacturer's specifications or buffers well known to those of skill in the art. Such buffers may comprise, without limitation, Tris, phosphate, histidine, salt and other excipients at a pH value close to physiological condition. For example, the column can be equilibrated with a buffer comprising 10-50 mM Tris-HCl, 2 mM $MgCl_2$, 150 mM NaCl, pH 7.2, or a buffer comprising 10-50 mM Phosphate, 2 mM $MgCl_2$, 150 mM NaCl, pH 7.2. Sample is then loaded and two elution buffers are applied, one low salt buffer and one high salt buffer. Fractions are collected following each of the low salt and high salt washes and protein is detected in the fractions using standard techniques, such as monitoring UV absorption at 260 and/or 280 nm. Using an anion exchanger, the protein peaks from the higher salt eluate contain viral vector particles, which are diluted using a low salt or no salt containing elution buffer and processed for the next step. Alternatively, the vector containing product fraction is diluted and stored at 2-15^{0}C prior to the next operation step. Appropriate buffers for use in the viral vector capture chromatography step

comprising use of anion-exchange columns are well known in the art and are generally cationic or zwitterionic in nature. Such buffers include, without limitation, buffers with the following buffer ions: Triethanolamine; Tris; Sodium or Potassium Phosphate; Bis-Tris; Bis-Tris propane; N-methyldiethanolamine, and the like.

In certain embodiments, the eluate of the viral vector chromatography step can be further processed by a transgene impurity capture chromatography step, wherein undesired impurities including transgene protein contaminants encoded by the viral vector are captured. The purified composition comprising the viral vector is received in the flow-through product, wherein the viral vector runs through the chromatography device without specifically binding to a transgene impurity capture chromatography matrix.

The transgene impurity capture chromatography step comprises use of polymers having sufficient matrix crosslinking such that interaction of a transgene protein contaminant takes place, wherein also present are any of a number of binding groups including, for example, those that are affinity, ion-exchange, multimodal, mixed-mode or hydrophobic in nature. The second transgene impurity capture chromatography step involves capture of impurities including transgene protein contaminants mediated by viral vector, and uses one or a combination of affinity (e.g. VIIISelect available from Cytiva), ion-exchange (e.g. Cation-exchange available from multiple vendors), multimodal/mixed-mode (e.g. Capto MMC available from Cytiva) and hydrophobic interaction (e.g. Capto Adhere available from Cytiva) chromatography resins.

In certain embodiments, the resulting sample obtained after a viral vector capture chromatography step is loaded onto a transgene impurity capture chromatography matrix after dilution of the sample to enable appropriate impurity binding and viral vector unbinding condition into the column. For example, the loading sample may comprise, without limitation, 10-50 mM Tris-HCl or 10-50 mM Phosphate, with 2 mM $MgCl_2$, 10 mM to 120 mM $CaCl_2$, pH 6.5 to 7.5 with diluted sample conductivity in the range of 20 to 40 mS/cm. The transgene impurity capture chromatography resin is equilibrated with an equilibration buffer known to those of skill in the art, for example, a buffer comprising, without limitation, 10-50 mM Tris or 10-50 mM Phosphate containing 0 to 2 mM $MgCl_2$, 10-30 mM $CaCl_2$, 10 to 70 mM NaCl, pH 7.2.

Example 1: Transgene Protein Encoded by the Viral Vector

LV-FVIIIXTEN / LV-FVIII are lentiviral vectors developed to express FVIIIXTEN / FVIII proteins respectively, in blood after post-administration of viral vector for use in the treatment of Hemophilia A. FVIIIXTEN / FVIII activity was observed during LV-FVIIIXTEN / LV-FVIII viral vector production process, respectively in the bioreactor cell culture, clarified supernatant, after purification chromatography, drug substance and in the purified drug product. Purified enveloped viral vector produced in a bioreactor and purified according to a process as shown in FIG. 1A, was found to contain a significant level of protein impurity in the drug product. Without being bound to any theory, the protein impurity may be present due to the expression of transgene proteins mediated by the CMV promoter upstream of the FVIIIXTEN / FVIII expression cassette. The protein impurity (e.g., transgene protein contaminant) could not be purified efficiently using standard (i.e. conventional) downstream process workflows such as the one shown in FIG. 1A. The activity of transgene protein per 1E9 TU of functional viral vector was found to be consistent across the stages of the purification process, and the final product sample, as shown in FIG. 2. Without being bound to any theory, the purification chromatography column utilized to capture viral vector particles was found to be less effective in separating enveloped viral vector from transgene protein contaminants, likely due to the transgene protein contaminant co-purifying with viral vector product.

Example 2: Reduction of Contaminating Transgene Protein Product During Purification

To reduce contaminating transgene protein product, a second purification chromatography resin or media was investigated for capturing transgene protein encoded by viral vector to improve overall viral vector purification in the downstream process workflow, as shown in FIG. 1B. In FIGs. 3A and 3B, Exp 1 and 2 were performed with the addition of a second purification chromatography column. The column XK16/20 (GE Healthcare, Cat No. 17-5450) was packed with VIIISelect resin (Cat No. 17-5450-01) to a bed height of 11.5cm with a Column Volume (CV) of 23 mL. The column and VIIISelect resin were purchased from Cytiva, USA. The starting material was produced in suspension culture using a single use stirred tank bioreactor utilizing HEK293 cells (in-house), FreeStyle 293 Expression (Cat. No. 12338-002, Thermo Fisher Scientific) production media, plasmids (in-house) and PEIpro transfection agent (Cat. No. 115-400, Polyplus Transfection) using a quad transfection process.

The second purification chromatography resin captured specific transgene protein contaminants and allowed viral vector to flow through the chromatography column without viral particles being specifically bound to the column. The one-column process was compared to the two-column process involving VIIISelect affinity resin as second chromatography resin to capture transgene protein contaminant, and it was found that the two-column process enabled lower level of FVIIIXTEN or FVIII protein contaminant in the drug product. The level of transgene protein contaminant was reduced from 20 IU/mL to 3 IU/mL in the LV-FVIII sample (Exp 1) and from 129 IU/mL to 63 IU/mL in the LV-FVIIIXTEN sample (Exp 2) (FIG. 3A). This equates to a total reduction of about 51% and 48% in transgene protein contaminant level in the LV-FVIII and LV-FVIIIXTEN viral vector samples, respectively (FIG. 3B). In FIG. 3A and 3B, the LV-FVIII and LV-FVIIIXTEN samples for loading into VIIISelect affinity resin were formulated in 10 mM sodium phosphate, 100 mM NaCl, 3% sucrose (w/v), and 0.05% Poloxamer (w/v), pH 7.2.

Example 3: Enhanced Reduction of Contaminating Transgene Protein During Purification

To improve transgene impurity capture in the two-column purification work flow, addition of $CaCl_2$ was investigated for potential improvement in viral vector purification. 10mM of $CaCl_2$ was either added in the viral vector sample before capture chromatography (+CaCl2) or not added (-CaCl2), and the load sample was incubated for 10 minutes prior to subjecting the sample to the purification column using VIIISelect affinity chromatography media. In the second chromatography purification step, affinity chromatography was performed using 450 uL of VIIISelect resin in a column. The column was equilibrated with 10 volumes of resin with formulation buffer containing histidine for FVIII (Exp 3) or phosphate for FVIIIXTEN (Exp 4) for separation. In an experiment, the viral vector sample was mixed with 500 ul of resulting sample and 10mM $CaCl_2$ (+CaCl2) or without $CaCl_2$ (-CaCl2) and loaded into the column. The formulation buffer was loaded to recover vector product in flow-through after a 10 minute contact time.

Remaining FVIIIXTEN or FVIII activity in the flow-through sample was measured by chromogenic assay (See above materials and methods). FIG. 4 shows the remaining FVIIIXTEN or FVIII activity in the flow-through in formulation buffer containing histidine, where the viral vector sample was mixed with LV-FVIII or flow-through in formulation buffer containing phosphate, where the viral vector was LV-FVIIIXTEN.

As shown in FIG. 4, bars labeled "One Column Process" indicate downstream workflow involving one dedicated vector purification chromatography step, whereas bars labelled "Two Column Process" indicates downstream workflow involving two dedicated vector purification chromatography steps. In Exp 3, FVIII protein activity was reduced from 15.1 IU/mL to 2.4 IU/mL in the +CaCL2 condition, and was reduced to 4.8 IU/mL in the -CaCl2 condition. In Exp 4, FVIIIXTEN protein activity was reduced from 168.0 IU/mL to 13.4 IU/mL in the +CaCl2 condition, and reduced to 28.0 IU/mL in the -CaCl2 condition. As shown in FIG. 4, the presence of $CaCl_2$ in the pre-column samples provides improvement in viral vector purification.

Example 4: Enhanced Reduction of Contaminating Transgene Protein Using Recycling Mode

Transgene protein contaminant reduction was investigated for further reduction using recycle mode technique, where the sample containing the transgene protein contaminant was passed through transgene impurity capture purification chromatography column multiple times. The load sample comprising LV-FVIII viral vector and FVIII protein impurity present in histidine buffer (i.e. Load) was spiked with 10mM $CaCl_2$ (+CaCl2), incubated for 10 min, and passed through an VIIISelect affinity chromatography column. The prepared load sample was run through the column at 6 min Residence Time (RT) and the flow-through of the column called "FT_Pass 1" was collected and analyzed for impurity activity or concentration. Then, the "FT_Pass 1" was adjusted to 10 mM $CaCl_2$ concentration, incubated for 10min and loaded again into the same column without the column being cleaned or regenerated. The flow through of the second pass is called "FT_Pass 2." Similarly, the FT_Pass 2 load sample was passed a third time through the column after adjustment of $CaCl_2$ concentration and incubation time in a manner similar to previous passes. The third pass flow through was collected as "FT_Pass 3." The load, FT_Pass 1, FT_Pass 2 and FT_Pass 3 samples were used to determine the content of impurity activity. As shown in FIG. 5, the total activity amount present in the sample to be purified by the transgene impurity capture chromatography was found to be 98 IU. This activity was reduced to 52 IU (FT_Pass 1), 42 IU (FT_Pass 2) and 10 IU (FT_Pass 3), after the multiple cycles. The overall reduction in amount of contaminating FVIII protein activity was found to be 90% after three cycles.

Example 5: Reduction of Contaminating Transgene Protein During Purification Using Tris Buffer System Without Excipients

The purification of vector was investigated for further removal of contaminating transgene protein using a second chromatography purification step in an improved downstream workflow scheme (e.g., as shown in FIG. 1B, FIG. 6A and FIG. 6B). In the improved scheme, the vector product is subjected to a chromatography media specifically designed for removal of contaminating transgene protein impurity. The step of removal of contaminating transgene protein impurity may be the second chromatography purification step (as shown in FIG. 6A), or it may be the first chromatography step (as shown in FIG. 6B), in an improved downstream workflow scheme of the invention.

Figure 7 shows the impact of the concentration of salt in the loading sample on the effectiveness of purification of a second chromatography purification, wherein the second chromatography purification comprises the use of VIIISelect resin. In this experiment, a first chromatography purification using an anion exchange (AEX) column was performed, and the vector product was eluted from the AEX column with a buffer containing 400mM NaCl. The AEX eluate (i.e., load sample for the second chromatography purification comprising VIIISelect resin) was then adjusted to have a salt concentration of either 400 mM NaCl (Exp5), or 100 mM NaCl (Exp6). In Exp5, the adjusted load sample contained 400 mM NaCl, 2.5 mM $CaCl_2$, and 2.0 mM $MgCl_2$ in Tris-HCl buffer, pH 7.2. In Exp6, the load sample contained 100 mM NaCl, 2.5 mM $CaCl_2$, and 2.0 mM $MgCl_2$ in Tris-HCl buffer, pH 7.2.

FIG. 7A shows the level of FVIIIXTEN as a function of total functional lentiviral vector recovered, detected before (black bars; "VIIISelect Load"), and after (grey bars; "VIIISelect Product") the VIIISelect purification step. Adjustment of the VIIISelect load sample was performed according to Exp5 and Exp6 described above. As shown in FIG. 7A, when the VIIISelect purification step was performed using a load sample containing 400 mM NaCl (Exp5), the VIIISelect purification step resulted in a decrease of FVIIIXTEN activity from 194.6 IU/1E9 transducing units (TU), to 15.3 IU/1E9 TU, representing a 92% reduction of FVIIIXTEN contaminating transgene protein. In comparison, when the VIIISelect purification step was performed using a load sample containing 100 mM NaCl (Exp5), the VIIISelect purification step resulted in a decrease of FVIIIXTEN activity from 127.6 IU/1E9 TU, to 64 IU/1E9 TU, representing a 50% reduction of FVIIIXTEN contaminating transgene protein. Using Tris

buffered load samples, close to 2-fold improvement in the removal of contaminating FVIIIXTEN was achieved when load samples were adjusted to 400 mM NaCl (Exp5) as compared to 100 mM NaCl (Exp6).

FIG. 7B shows the results of adjusting load samples to 400 mM NaCl (Exp5) or 100 mM NaCl (Exp6) on FVIIIXTEN level measured as a function of total lentiviral particle number (IU/1E6 ng P24). As shown in FIG. 7B, when the VIIISelect purification step was performed using a load sample containing 400 mM NaCl (Exp5), the VIIISelect purification step resulted in a decrease of FVIIIXTEN activity from 167.9 IU/1E6 ng P24 to 16.3 IU/1E6 ng P24, representing a 90% reduction of FVIIIXTEN contaminating transgene protein. In comparison, when the VIIISelect purification step was performed using a load sample containing 100 mM NaCl (Exp5), the VIIISelect purification step resulted in a decrease of FVIIIXTEN activity from 125.6 IU/1E6 ng P24 to 75.8 IU/1E6 ng P24, representing a 40% reduction of FVIIIXTEN contaminating transgene protein.

Example 6: Reduction of Contaminating Transgene Protein During Purification Using Phosphate Buffer System With Excipients

The purification efficiency of a second chromatography purification step using VIIISelect resin for reduction of FVIIIXTEN contaminating transgene protein was investigated using a phosphate buffer system containing excipients. In this experiment, the load sample was adjusted to contain 400 mM NaCl and 1mM $CaCl_2$, as well as 10 mM sodium phosphate, sucrose (3% w/v) and poloxamer (0.05% w/v). As shown in FIG. 8, the total activity of FVIIIXTEN detected decreased from 1768 IU pre-purification, to 423 IU post-purification, representing a 76% reduction of FVIIIXTEN contaminating transgene protein in a single pass, when the sample was allowed to incubate for 10 min residence time in the VIIISelect column. With regards to functional vector recovery of a 400mM NaCl phosphate buffered load, the FVIIIXTEN activity detected was reduced from 324 IU/1E9 TU pre-purification, to 86 IU/1E9 TU post-purification, representing a 73% reduction in impurity level; FVIIIXTEN activity detected as a function of total lentiviral particle number was reduced from 766 IU/1E6 ng P24 pre-purification, to 199 IU/1E6 ng P24 post-purification, representing a 74% reduction in impurity level.

Example 2, exp2 (see, FIG. 3A and FIG. 3B), using the same phosphate buffered load sample containing 100mM NaCl (no adjustment of NaCl concentration was performed), the two-

column process only resulted in 51% reduction of FVIIIXTEN activity. In comparison, in this experiment, where the phosphate buffered load sample was adjusted to 400 mM NaCl, over 70% reduction in FVIIIXTEN activity was observed, representing a significant enhancement of the reduction in the level of FVIIIXTEN contaminating transgene protein.

Example 7: Reduction of Contaminating Transgene Protein During Purification Using Tris and Phosphate Buffers at Salt Concentration from 200 mM to 600 mM

The purification of vector was investigated for removal of contaminating transgene protein using a chromatography purification step in an improved downstream workflow scheme (e.g., as shown in FIG. 1B, FIG. 6A and FIG. 6B). In the improved scheme, the vector product is subjected to a chromatography media specifically designed for removal of contaminating transgene protein impurity present in either Tris or Phosphate buffer containing a range of salt concentration. The removal step of the contaminating transgene protein impurity may be the second chromatography purification step (as shown in FIG. 6A), or it may be the first chromatography step (as shown in FIG. 6B), in an improved downstream workflow scheme of the invention.

FIG. 9 shows the impact of the concentration of salt in the loading sample on the effectiveness of purification of a second chromatography purification step, wherein the second chromatography purification step comprises the use of VIIISelect resin. In this experiment, a first chromatography purification step using an anion exchange (AEX) column was performed, and the vector product was eluted from the AEX column with an elution buffer comprising either Tris or Phosphate and a total salt concentration of from 200 mM to 600 mM. The AEX eluate (i.e., load sample for the second chromatography purification comprising VIIISelect resin) did not require adjustment if the AEX pool salt concentration was < 600 mM but did require adjustment if the AEX pool salt concentration was > 600 mM NaCl. The adjusted load sample contained either a range of 200 to 600mM NaCl, 2.0 mM MgCl2 in Tris-HCl buffer, pH 7.2 + 0.3; or a range of 200 to 600 mM NaCl, 2.0 mM MgCl2 in Phosphate buffer, pH 7.2 + 0.3. The

adjusted load sample was then applied to second chromatography resin to bind protein impurity and allow the enveloped vector to pass through it as purified vector product.

As shown in FIG. 9, the amount of FVIII protein activity dropped from the activity values above 10 IU to below 1 IU demonstrating at least a 10-fold reduction in contaminating transgene activity, while reducing the protein impurity activity values in vector product sample below the limit of quantitation (<LoQ) of an activity assay. Thus, removal of transgene protein impurities from the sample was achieved by >90% irrespective of whether Tris buffer (compare solid black to lined black bars) or Phosphate buffer (compare solid grey to lined grey bars) was used as a loading buffer in the VIIISelect chromatography step. Furthermore, removal of contaminating transgene product was consistently achieved irrespective of buffer species and salt concentrations. Accordingly, these experiments demonstrate the effectiveness of the improved purification process for removing transgene contaminant without significant loss of functional enveloped viral vector, and demonstrate the general suitability of a variety of buffer species and salt concentrations in the improved purification process.