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(54) **Title:** RECOMBINANT ADENO-ASSOCIATED VIRUS PARTICLE PURIFICATION COMPRISING AN AFFINITY PURIFICATION STEP

(57) **Abstract:** The invention describes a method for obtaining purified recombinant Adeno-Associated Virus particles (rAAV), comprising the steps of: a) performing a depth filtration of a starting material previously obtained from cells producing rAAV particles, the said starting material being selected in a group comprising a cell lysate and a culture supernatant, whereby a rAAV-containing clarified composition is provided; b) submitting the rAAV-containing clarified composition to an affinity purification step, whereby a first rAAV enriched composition is provided; c) submitting the first rAAV enriched composition at least once to: cl) a step of anion-exchange chromatography on a chromatographic support wherein elution is performed by using a salt gradient, preferably a linear salt gradient, and wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided; or c2) a step of density gradient centrifugation, wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided; d) submitting the second rAAV enriched composition to a step of tangential flow filtration, whereby purified recombinant Adeno-Associated Virus particles (rAAV) are provided.



RECOMBINANT ADENO-ASSOCIATED VIRUS PARTICLE PURIFICATION
COMPRISING AN AFFINITY PURIFICATION STEP

FIELD OF THE INVENTION

5 The invention relates to the field of purification of Adeno-Associated Virus particles (rAAVs). In particular, it relates to methods for obtaining purified recombinant Adeno-Associated Virus particles (rAAV). It also relates to purified rAAV particles and compositions thereof which are obtainable using said methods. It further relates to AAV plasmids, and host cells which have been transfected with said plasmids, and also rAAV particles produced by said host cells.

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BACKGROUND OF THE INVENTION

 Gene delivery is a promising method for the treatment of acquired and inherited diseases. A number of viral-based systems for gene transfer purposes have been described, including Adeno-Associated Virus (AAV)-based systems. AAV is a helper-dependent DNA
15 parvovirus that belongs to the genus Dependovirus. AAV requires co-infection with an unrelated helper virus, e.g., adenovirus, herpes virus, or vaccinia, in order for a productive infection to occur. In the absence of a helpervirus, AAV establishes a latent state by inserting its genome into a host cell chromosome. Subsequent infection by a helper virus rescues the integrated viral genome, which can then replicate to produce infectious viral progeny.

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 AAV has a wide host range and is able to replicate in cells from any species in the presence of a suitable helper virus. For example, human AAV will replicate in canine cells co-infected with a canine adenovirus. AAV has not been associated with any human or animal disease and does not appear to alter the biological properties of the host cell upon integration. For a review of AAV, see, e.g., Berns and Bohenzky (1987) *Advances in Virus Research* (Academic
25 Press, Inc.) 32:243-307.

Le meur *et al.* (“*Restoration of vision in RPE65-deficient Briard dogs using an AAV serotype 4 vector that specifically targets the retinal pigmented epithelium*”; *Gene Therapy*; Vol. 14, 292-303; 2007) teaches the use of an rAAV for restoring vision in a RPE65-deficient Dog.

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Petit *et al.* (“*Restoration of vision in the pde6 β -deficient Dog, a Large Animal Model of Rod-cone Dystrophy*”; *Molecular Therapy*”; Vol. 20, no.11, 2019-2030”; 2012) teaches the use of a rAAV for restoring vision in a PDE6 β -deficient Dog.

For all those reasons, the use of Adeno-Associated Virus for gene therapy has been proposed. However, applications are still lacking due to the requirement for preparations having sufficient purity and/or infectivity for clinical use.

Thus, there remains a need for novel methods for purifying Adeno-Associated Virus particles (rAAVs). In particular, there remains a need for scalable methods which are well-defined, reproducible, and within controlled environmental conditions in accordance with Good Manufacturing Practices (GMP).

In particular, there remains a need for methods for purifying Adeno-Associated Virus particles (rAAVs) which require a limited number of steps.

There also remains a need for methods for purifying Adeno-Associated Virus particles (rAAVs) which are suitable for use as a medicament, and/or gene therapy.

Thus, there remains a need for scalable methods for purifying rAAV particles and compositions thereof, with high purity and infectivity.

Methods for purifying Adeno-Associated Virus particles have been reported in the prior Art. However, those methods are not necessarily satisfying for producing rAAV particles which are directly suitable for use as a medicament and/or for gene therapy.

WO2011/094198 teaches a method for purifying AAV particles, including a step of Ion Exchange Column Chromatography and a step of Tangential Flow Filtration.

WO03/097797 teaches a method for purifying virus particles with reduced contaminating DNA levels, which may include steps of cell lysis, depth filtration and/or centrifugation, ultracentrifugation, nuclease treatment, anion exchange chromatography and tangential flow filtration.

On the other hand, many of the available techniques have the disadvantage to require prior extensive treatment of the cell extracts, including density gradient, treatment with nucleases and detergents before the chromatography step. Such additional treatments render the whole process less satisfactory at an industrial scale.

Immunoaffinity chromatography has been reported as a fast, one-step purification method, for purifying adeno-associated viruses (AAV). However, its use is still limited, due to the highly specific nature of antibodies, or fragments thereof, when immobilized onto a chromatography support. What is more, immunoaffinity chromatography supports, when used for one-step purification methods, can be prone to generate a purified rAAV contaminated by a large proportion of empty particles and thus hinder its use for direct administration in gene therapy.

Peptide affinity purification has been reported as an alternative to immunoaffinity, or antibody-based affinity chromatography.

Pulicherla et al. (“Peptide affinity reagents for AAV capsid recognition and purification”; *Gene Ther.*; 18(10):1020-1024; 2011) reports a method for purifying AAV8 capsids which comprises a step of peptide affinity purification using a Pep8-based peptide, followed by an anion-exchange chromatography. However the method has the disadvantage of being restricted to
5 AAV8 capsids, and requires significant optimization for producing clinical grade rAAV particles preparations.

In particular, peptide affinity purification requires to isolate and characterize novel peptide ligands and to generate a GMP chromatography column-compatible with these ligands; a step that may be time-consuming and expansive, and thus a bottleneck for scalability of the whole
10 process.

Thus there remains a need for novel methods that can be scaled up with respect to good manufacturing practice issues, which remain acceptable from an economic point of view, and suitable for use as a medicament or for gene therapy.

There also remains a need for methods for purifying rAAV particles from a starting
15 material containing rAAV particles belonging to a plurality of serotypes.

There also remains a need for methods for purifying rAAV particles with Immunoaffinity chromatography, with reduced empty particles into the purified rAAV particles.

There also remains a need for novel purified rAAV particles and compositions thereof, as such and/or for use as a medicament or for gene therapy, which can be further purified
20 by said processes, and which remain suitable for producing rAAV particles in a given host cell.

SUMMARY OF THE INVENTION

The present invention relates to a method for obtaining purified recombinant Adeno-Associated Virus particles (rAAV), comprising the steps of :

25 a) performing a depth filtration of a starting material previously obtained from cells producing rAAV particles, the said starting material being selected in a group comprising a cell lysate and a culture supernatant, whereby a rAAV-containing clarified composition is provided;

b) submitting the rAAV-containing clarified composition to an affinity purification step, whereby a first rAAV enriched composition is provided;

30 c) submitting the first rAAV enriched composition at least once to:

c1) a step of anion-exchange chromatography on a chromatographic support wherein elution is performed by using a salt gradient, preferably a linear salt gradient, and wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided; or

c2) a step of density gradient centrifugation, wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided;

d) submitting the second rAAV enriched composition to a step of tangential flow filtration, whereby purified recombinant Adeno-Associated Virus particles (rAAV) are provided.

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In particular, the present invention relates to a method for obtaining purified recombinant Adeno-Associated Virus particles (rAAV), comprising the steps of :

a) performing a depth filtration of a starting material previously obtained from cells producing rAAV particles, the said starting material being selected in a group comprising a cell lysate and a culture supernatant, whereby a rAAV-containing clarified composition is provided;

10

b) submitting the rAAV-containing clarified composition to an immunoaffinity purification step, whereby a first rAAV enriched composition is provided;

c) submitting the first rAAV enriched composition at least once to:

15

c1) a step of anion-exchange chromatography on a chromatographic support wherein elution is performed by using a salt gradient, preferably a linear salt gradient, and wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided; or

c2) a step of density gradient centrifugation, wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided;

20

d) submitting the second rAAV enriched composition to a step of tangential flow filtration, whereby purified recombinant Adeno-Associated Virus particles (rAAV) are provided.

The invention also relates to purified rAAV particles that are obtained by performing the method described above. It further relates to a purified rAAV particle composition comprising at least one purified rAAV particle as defined above.

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It further relates to said purified rAAV particles and to compositions thereof, for use for gene therapy and/or for the preparation of a medicament for use for gene therapy.

The invention also relates to an AAV plasmid comprising an expression cassette encoding human PDE6-beta, and to a host cell transfected with said AAV plasmid, and further to rAAV particles which are produced by said host cell.

30

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A: Cartography of the expression cassette of an AAV plasmid encoding human PDE6 β . The nucleic acid sequence coding for the human PDE6 β protein is associated at

its 5' end to a human rhodopsine kinase (RK) promoter and at its 3' end to a bovine growth hormone polyadenylation site (bGHpA), flanked by Inverted Terminal Repeat (ITR) sequences.

Figure 1B: Cartography of the expression cassette of an AAV plasmid encoding

5 **human RPE65.** The nucleic acid sequence coding for the human RPE65 protein is associated at its 5' end to a human RPE65 promoter and at its 3' end to a bovine growth hormone polyadenylation site (bGHpA), flanked by Inverted Terminal Repeat (ITR) sequences

Figure 2: Cartography of the whole AAV plasmid comprising the expression

10 **cassette encoding human PDE6 β .** The expression cassette is delimited by ITR sequences as defined in figure 1. Restriction sites are defined as EcoRV, SpeI and HindIII. The plasmid includes an additional Kanamycine (Kan/neoR) resistance marker and a ColI origin. The directionality of sequences is indicated with arrows according to the standard nomenclature.

15 **Figure 3A: Chromatogram of the complete AVB column step**

Curves represent Absorbance (left axis in mAU) at 280 nm (A line) and 260 nm (B line). Line C represents the conductivity in mS/cm (right axis). Line D represents pH. Empty squares show the sample injection and elution steps.

20 **Figure 3B: Zoom peak of the eluted AAV from the AVB column step**

Curves represent Absorbance (left axis in mAU) at 280 nm (A line) and 260 nm (B line). Line C represents the conductivity in mS/cm (right axis). Line D represents pH. Empty square shows where the AAVs-containing fractions are eluted.

25 **DETAILED DESCRIPTION OF THE INVENTION**

The invention addresses the aforementioned needs.

The current invention provides a recombinant Adeno-Associated Virus (rAAV) particle purification method that includes two unique features that distinguish it from current "industry-standard" scalable AAV particles purification processes: 1) a modular platform process
30 that can be used for purification of different AAV serotypes / capsid variants with high purity, and which is suitable for producing preparations directly suitable for clinical applications; and 2) a unique sequence of process steps, including an affinity chromatography (Immunoaffinity or peptide affinity chromatography) followed by at least one density gradient centrifugation step, or at least one anion-exchange chromatography preferably performed by using a linear salt gradient.

Surprisingly those characteristics confer unexpected scalability, for purifying a starting material such as a cell lysate or a culture supernatant. In particular, this process has the advantage of being suitable for optimization, in order to prepare purified recombinant Adeno-Associated Virus particles having optimal infectiosity, and which are suitable for gene therapy.

5 The use of one or more additional steps, including anion-chromatography steps and/or density gradient centrifugation, leads to optimal separation of empty and full particles on said support, and is thus suitable for isolating rAAV particles in an improved manner.

Advantageously, this multi-step purification method allows to purify clinical-grade rAAV preparations at an industrial scale.

10 Advantageously, the composition can be passed through the anion-exchange chromatography support using a linear salt gradient. Without wishing to be bound by any particular theory, the inventors are also of the opinion that the linear salt gradient provides more efficient separation of the eluted species, compared to step salt gradients. The difference lies in the lower slope of the gradient, which allows for the formation of a slow increase in salt
15 concentration, and thus optimal separation of rAAV particles within the chromatography support.

To the best of the knowledge of the inventors, it is the first described “*industry-standard*” method for purifying clinical-grade rAAV particles, in particular rAAV particles belonging to the AAV4 serotype, which involves a multi-step purification method comprising an affinity chromatography step, in particular an immunoaffinity chromatography step.

20 Indeed, previously reported methods for purifying rAAV particles involved one-step affinity or immunoaffinity chromatography steps, and/or were not “*industry-standard*” methods.

Also, the methods of the invention are particularly suitable for purifying particular serotypes of rAAVs, due to the higher proportion of full particles in the final product.

25 Also, residual DNA at the end of the process is in accordance with clinical standards, even in the absence of treatment with detergents and nucleases, including DNAses.

Thus, the invention relates to a method for obtaining purified recombinant Adeno-Associated Virus particles (rAAV), and is particularly suitable for purifying rAAV particles belonging to a AAV serotype selected in a group comprising, or consisting of, AAV1, AAV2,
30 AAV3, AAV4, AAV5, AAV8, AAV9, AAV10, and *rhesus macaque*-derived serotypes including AAVrh10, and mixtures thereof; in particular AAV4.

The present invention relates to a method for obtaining purified recombinant Adeno-Associated Virus particles (rAAV), comprising the steps of :

a) performing a depth filtration of a starting material previously obtained from cells producing rAAV particles, the said starting material being selected in a group comprising a cell lysate and a culture supernatant, whereby a rAAV-containing clarified composition is provided;

b) submitting the rAAV-containing clarified composition to an affinity purification
5 step, whereby a first rAAV enriched composition is provided;

c) submitting the first rAAV enriched composition at least once to:

c1) a step of anion-exchange chromatography on a chromatographic support wherein elution is performed by using a salt gradient, preferably a linear salt gradient, and wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is
10 provided; or

c2) a step of density gradient centrifugation, wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided;

d) submitting the second rAAV enriched composition to a step of tangential flow filtration, whereby purified recombinant Adeno-Associated Virus particles (rAAV) are provided.
15

An affinity purification step may consist of a peptide affinity purification step or an immunoaffinity purification step.

According to a first embodiment, the invention relates to a method for obtaining
20 purified recombinant Adeno-Associated Virus particles (rAAV), comprising the steps of :

a) performing a depth filtration of a starting material previously obtained from cells producing rAAV particles, the said starting material being selected in a group comprising a cell lysate and a culture supernatant, whereby a rAAV-containing clarified composition is provided;

b) submitting the rAAV-containing clarified composition to an immunoaffinity
25 purification step, whereby a first rAAV enriched composition is provided;

c) submitting the first rAAV enriched composition at least once to:

c1) a step of anion-exchange chromatography on a chromatographic support wherein elution is performed by using a salt gradient, preferably a linear salt gradient, and wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is
30 provided; or

c2) a step of density gradient centrifugation, wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided;

d) submitting the second rAAV enriched composition to a step of tangential flow filtration, whereby purified recombinant Adeno-Associated Virus particles (rAAV) are provided.
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The starting material may comprise either rAAV particles belonging to one serotype, or belonging to a plurality of serotypes, including rAAV particles belonging to the AAV4, AAV2, AAV10 and/or AAV5 serotype.

In particular, the starting material may comprise either rAAV particles belonging to
5 one serotype, or belonging to a plurality of serotypes, including rAAV particles belonging to the AAV4 and/or AAV5 serotype.

Preferably, the invention relates to a method for obtaining purified recombinant Adeno-Associated Virus particles (rAAV) belonging to the AAV4 serotype, comprising the steps of :

- 10 a) performing a depth filtration of a starting material previously obtained from cells producing said rAAV4 particles belonging to the AAV4 serotype, the starting material being selected in a group comprising a cell lysate and a culture supernatant, whereby a rAAV-containing clarified composition is provided;
- b) submitting the rAAV4-containing clarified composition to an immunoaffinity
15 purification step, whereby a first rAAV4 enriched composition is provided;
- c) submitting the first rAAV4 enriched composition at least once to
- c1) a step of anion-exchange chromatography on a chromatographic support wherein elution is performed by using a salt gradient, preferably a linear salt gradient, and wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is
20 provided; or
- c2) a step of density gradient centrifugation, wherein the rAAV-containing fraction is collected, whereby a second rAAV4 enriched composition is provided;
- d) submitting the second rAAV4 enriched composition to a step of tangential flow filtration, whereby purified recombinant Adeno-Associated Virus particles (rAAV) belonging to
25 the AAV4 serotype are provided.

The purified rAAV particles of the invention comprise a heterologous nucleic acid encoding a desirable gene product. Such products include without limitation, siRNAs, antisense molecules, miRNAs, ribozymes and the like. Other products include nucleic acids encoding
30 hormones, growth receptors, ligands and proteins useful for gene therapy.

According to a second embodiment, the invention relates to a purified rAAV particle obtained by performing the method as described above, and to compositions thereof.

According to a third embodiment, the invention relates to said purified rAAV particles and to compositions thereof, for use for gene therapy.

According to a fourth embodiment, the invention relates to an AAV plasmid comprising an expression cassette encoding human PDE6-beta, to a host cell which has been transfected with said AAV plasmid, and to rAAV particles produced by the host cell, as such and also for use for gene therapy.

5 According to the invention, the expression “*comprising*”, such as in “*comprising the steps of*”, is also understood as “*consisting of*”, such as in “*consisting of the steps of*”.

Methods for obtaining purified recombinant Adeno-Associated Virus Particles

The invention relates to a method for obtaining purified recombinant Adeno-
10 Associated Virus particles (rAAV), comprising the steps of:

a) performing a depth filtration of a starting material previously obtained from cells producing rAAV particles, the said starting material being selected in a group comprising a cell lysate and a culture supernatant, whereby a rAAV-containing clarified composition is provided;

b) submitting the rAAV-containing clarified composition to an affinity purification
15 step, such as an immunoaffinity purification step or a peptide affinity purification step, whereby a first rAAV enriched composition is provided;

c) submitting the first rAAV enriched composition at least once to:

c1) a step of anion-exchange chromatography on a chromatographic support wherein elution is performed by using a salt gradient, preferably a linear salt gradient, and wherein the
20 rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided; or

c2) a step of density gradient centrifugation, wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided;

d) submitting the second rAAV enriched composition to a step of tangential flow
25 filtration, whereby purified recombinant Adeno-Associated Virus particles (rAAV) are provided.

In particular, the affinity purification step is an immunoaffinity purification step.

More particularly, the invention relates to a method for obtaining purified
30 recombinant Adeno-Associated Virus particles (rAAV), comprising the steps of:

a) performing a depth filtration of a starting material previously obtained from cells producing rAAV particles, the said starting material being selected in a group comprising a cell lysate and a culture supernatant, whereby a rAAV-containing clarified composition is provided;

b) submitting the rAAV-containing clarified composition to an immunoaffinity
35 purification step, whereby a first rAAV enriched composition is provided;

c) submitting the first rAAV enriched composition at least once to:

c1) a step of anion-exchange chromatography on a chromatographic support wherein elution is performed by using a salt gradient, preferably a linear salt gradient, and wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is
5 provided; or

c2) a step of density gradient centrifugation, wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided;

d) submitting the second rAAV enriched composition to a step of tangential flow filtration, whereby purified recombinant Adeno-Associated Virus particles (rAAV) are provided.

10 According to some embodiments the method does include a step of treatment with detergents and/or nucleases, including DNAses.

According to some embodiments, the method does not include a step of treatment with detergents and/or nucleases, including DNAses.

15 According to another embodiment, the rAAV particles belong to a AAV serotype selected in a group comprising AAV1, AAV2, AAV3, AAV4, AAV5, AAV8, AAV9, AAV10, and *rhesus macaque*-derived serotypes including AAVrh10 and mixtures thereof; in particular AAV4.

20 According to one alternative embodiment, the rAAV particles consist of rAAV5 particles containing DNA comprising an expression cassette encoding human PDE6-beta.

According to said embodiment, the said expression cassette is of **SEQ ID N° 1**.

According to one embodiment, the rAAV5 particles contain a DNA comprising **SEQ ID N° 2**.

25 According to another alternative embodiment, the rAAV particles consist of rAAV4 particles containing DNA comprising an expression cassette encoding human RPE65.

The terms “*recombinant AAV virion*”, “*rAAV virion*”, “*AAV particle*”, “*full capsids*” and “*full particles*” are defined herein as an infectious, replication-defective virus including an AAV protein shell, encapsidating a heterologous nucleotide sequence of interest which is flanked on both sides by AAV ITRs. A rAAV virion is produced in a suitable host cell which has had
30 sequences specifying an AAV plasmid, AAV helper functions and accessory functions introduced therein. In this manner, the host cell is rendered capable of encoding AAV polypeptides that are required for packaging the AAV plasmid (containing a recombinant nucleotide sequence of interest) into infectious recombinant virion particles for subsequent gene delivery.

35 By “*recombinant virus*” is meant a virus that has been genetically altered, e.g., by the addition or insertion of a heterologous nucleic acid construct into the particle.

By “AAV *virion*” is meant a complete virus particle, such as a wild-type (wt) AAV virus particle (comprising a linear, single-stranded AAV nucleic acid genome associated with an AAV capsid protein coat). In this regard, single-stranded AAV nucleic acid molecules of either complementary sense, e.g., “*sense*” or “*antisense*” strands, can be packaged into any one AAV
5 virion and both strands are equally infectious.'

The term “*host cell*” denote, for example, microorganisms, yeast cells, insect cells, and mammalian cells, that can be, or have been, used as transitory or permanent stable producer cell lines recipients of an AAV helper construct, an AAV plasmid, an accessory function plasmid, or other transfer DNA. The term includes the progeny of the original cell which has been
10 transfected. Thus, a “*host cell*” as used herein generally refers to a cell which has been transfected with an exogenous DNA sequence. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

The term “*rAAV-containing clarified composition*” encompasses any composition
15 including rAAV particles, which is obtained after a step of depth-filtration of a starting material such as a cell lysate or a culture supernatant. A “*rAAV-containing clarified composition*” is distinct from a “*rAAV enriched composition*” and/or purified rAAV particles.

Thus, a “*rAAV-containing clarified composition*” may encompass any composition including rAAV particles which derives directly from a depth-filtered starting material such as a
20 cell lysate or a culture supernatant, and which has not been further submitted to a step of enrichment and/or purification, including which has not been submitted to a step of ion (e.g. cation)-exchange chromatography.

According to said embodiment, the invention relates to a method for obtaining purified recombinant Adeno-Associated Virus particles (rAAV), comprising the steps of :

- 25 a) performing a depth filtration of a cell lysate and/or a culture supernatant, whereby a rAAV-containing clarified composition is provided;
- b) submitting the rAAV-containing clarified composition to an affinity purification step, such as an immunoaffinity purification step or a peptide affinity purification step, whereby a first rAAV enriched composition is provided;
- 30 c) submitting the first rAAV enriched composition at least once to
- c1) a step of anion-exchange chromatography on a chromatographic support wherein elution is performed by using a salt gradient, preferably a linear salt gradient, and wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided; or

c2) a step of density gradient centrifugation, wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided;

d) submitting the second rAAV enriched composition to a step of tangential flow filtration, whereby purified recombinant Adeno-Associated Virus particles (rAAV) are provided.

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According to another embodiment, the invention relates to a method for obtaining purified recombinant Adeno-Associated Virus particles (rAAV) of the invention, wherein the rAAV-containing clarified composition that is submitted to the first step of affinity step (immunoaffinity purification step or peptide affinity purification step), is the composition
10 obtained directly at the end of step a). Thus, according to said embodiment, steps a) and b) are consecutive steps.

By “*consecutive step*” is meant any step that comes after a first step, for which any in-between step of chromatography step, in particular ion (cation)-exchange chromatography, is excluded. On the other hand, and unless stated otherwise, “*consecutive steps*” do not exclude
15 buffer exchange and/or dilution steps, for the purpose of modifying buffer conditions (i.e. concentration of salt; pH) of said compositions.

According to an embodiment, the method of the invention does not comprise a cation-exchange chromatography step.

According to an embodiment, the method of the invention does not comprise an
20 apatite chromatography step.

According to one preferred embodiment, the invention relates to a method for obtaining purified recombinant Adeno-Associated Virus particles (rAAV) of the invention, wherein affinity purification (immunoaffinity or peptide affinity purification) and anion-exchange
25 chromatography steps, or alternatively affinity purification (immunoaffinity or peptide affinity purification) and density gradient centrifugation, are consecutive steps. Thus, according to said embodiment, steps b) and c) are consecutive steps.

According to another embodiment, steps c) and d) are consecutive.

According to another embodiment, steps b) and c) and d) are consecutive.

30 According to another embodiment, steps a) and b) and c) are consecutive.

According to another embodiment, steps a) and b) and c) and d) are consecutive.

Thus, the invention also relates to a method for obtaining purified recombinant Adeno-Associated Virus particles (rAAV), comprising the steps of:

a) performing a depth filtration of a starting material previously obtained from cells producing rAAV particles, the said starting material being selected in a group comprising a cell lysate and a culture supernatant, whereby a rAAV-containing clarified composition is provided;

b) submitting the rAAV-containing clarified composition obtained at step a) to an
5 affinity purification step (immunoaffinity or peptide affinity purification step), whereby a first rAAV enriched composition is provided;

c) submitting the first rAAV enriched composition obtained at step b) at least once to:

c1) a step of anion-exchange chromatography on a chromatographic support wherein elution is performed by using a salt gradient, preferably a linear salt gradient, and wherein the
10 rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided; or

c2) a step of density gradient centrifugation, wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided;

d) submitting the second rAAV enriched composition obtained at step c) to a step of
15 tangential flow filtration, whereby purified recombinant Adeno-Associated Virus particles (rAAV) are provided.

According to another embodiment, steps a) and b) and c) and d) are consecutive and the starting material in step a) is directly obtained from a cell lysate or a culture supernatant.

20

According to said embodiment, the invention relates to a method for obtaining purified recombinant Adeno-Associated Virus particles (rAAV), comprising the steps of :

a) performing a depth filtration of a cell lysate and/or a culture supernatant, whereby a rAAV-containing clarified composition is provided;

b) submitting the rAAV-containing clarified composition obtained at step a) to an
25 affinity purification step (immunoaffinity or peptide affinity purification step), whereby a first rAAV enriched composition is provided;

c) submitting the first rAAV enriched composition obtained at step b) at least once to

c1) a step of anion-exchange chromatography on a chromatographic support wherein
30 elution is performed by using a salt gradient, preferably a linear salt gradient, and wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided; or

c2) a step of density gradient centrifugation, wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided;

d) submitting the second rAAV enriched composition obtained at step c) to a step of tangential flow filtration, whereby purified recombinant Adeno-Associated Virus particles (rAAV) are provided.

5 The above-mentioned methods all include at least: **1)** one step of depth filtration of a starting material; **2)** a first step of affinity (immunoaffinity or peptide affinity) chromatography; **3)** a second step of anion-exchange chromatography or density gradient centrifugation; and **4)** a step of tangential flow filtration. Each individual step will be further defined herebelow.

The inventors are also of the opinion that each additional step contributes to the
10 scalability and high purity of the purified recombinant Adeno-Associated virus particles.

Depth filtration

Depth filtration step allows for discarding a major part of contaminant DNA and proteins. This step renders possible the purification of rAAV particles through immunoaffinity
15 chromatography directly from a rAAV-containing clarified composition.

According to one embodiment, the starting material used at step a) is a cell lysate obtained by contacting a culture of cells producing rAAV particles with a composition comprising at least a detergent or a surfactant, and preferably a detergent.

According to one embodiment, the starting material used at step a) is a cell lysate
20 obtained by contacting a culture of cells producing rAAV particles with a composition which may or may not comprise a detergent or a surfactant, but which is not treated with a nuclease such as a DNase.

Examples of suitable detergents for cell lysis include Triton X-100, Triton X-114, NP-40, Brij-35, Brij-58, Tween 20, Tween 80, Octyl glucoside, Octyl thioglucoside, SDS,
25 CHAPS and CHAPSO.

According to one preferred embodiment, step a) is performed by using a depth filter membrane comprising a layer of borosilicate glass microfibers and a layer of mixed esters of cellulose.

According to one exemplary embodiment, step a) is performed using a Polysep™ II
30 (Millipore®) filter.

Affinity chromatography

The term “*affinity chromatography*” or “*affinity purification*” as used herein designates any method that uses specific binding interactions between molecules. A particular
35 ligand is chemically immobilized or “coupled” to a solid support so that when a complex mixture

is passed over the column, those molecules having specific binding affinity to the ligand become bound. After other sample components are washed away, the bound molecule is stripped from the support, resulting in its purification from the original sample.

The term “*immunoaffinity chromatography*” as used herein designates any method
5 that uses immobilized antibodies, or fragments thereof, in affinity chromatography.

The term “*antibodies or fragments thereof*” may include monoclonal and polyclonal antibodies, naturally and non-naturally occurring antibodies, whole antibodies and fragments thereof, including fragment antigen-binding such as Fv, Fab and F(ab')₂ regions, complementarity determining regions (CDRs), single-domain antibodies, nanobodies, and mixtures thereof.

10 The term “*fragment thereof*” may encompass any fragment of an antibody that can be obtained by deleting part of the original antibody, including in a non-limitative manner any antibody of which the Fc region or parts of the variable region (including CDRs) have been deleted.

The term “*peptide affinity chromatography*” as used herein designates any method
15 that uses immobilized peptide in affinity chromatography. Peptide affinity chromatography is well known in the art (see for example Pulicherla et al. 2011. Gen Ther 18(10): 1020-1024).

When immobilized onto the chromatography support, the term encompasses any of the aforementioned variants as long as it retains its ability to bind to at least one epitope at the surface of the rAAV particles which are purified.

20 In particular, such antibodies or fragments thereof may include isotypes of the IgA, IgD, IgE, IgG and IgM subclasses.

According to one particular embodiment, the antibodies or fragments thereof are monoclonal.

25 Antibodies which are considered by the invention may be naturally-occurring or non-naturally occurring. They may be of human or non-human origin.

According to one exemplary embodiment, the antibodies are single-chain antibodies, such as the ones obtained by immunization of camelids including dromedaries, camels, llamas, and alpacas; or sharks.

30 Antibodies and fragments thereof may be obtained and immobilized onto supports by using a variety of techniques that range from covalent attachment to adsorption-based methods, as described for instance in Moser & Hage (« Immunoaffinity chromatography : an introduction to applications and recent developments » ; Bioanalysis ; 2(4) : 769-790 ; 2010).

Monoclonal antibodies may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not
35 limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-

hybridoma technique (Kohler, et al.,1975, Nature 256:495-497; Kozbor, et al., 1985, J. Immunol. Methods 81 :31-42; Cote, et al., 1983, Proc.Natl.Acad.Sci. 80:2026-2030; Cole, et al, 1984, Mol Cell Biol. 62:109-120).

A number of suitable immunoaffinity chromatography supports for use with the present invention are known and include without limitation, Affi-Gel (Biorad); Affinica Agarose/Polymeric Supports (Schleicher and Schuell); AvidGel (BioProbe); Bio-Gel (BioRad); Fractogel (EM Separations); HEMA-AFC (Alltech); Reacti-Gel (Pierce); Sephacryl (Pharmacia); Sepharose (Pharmacia); Superose (Pharmacia); Trisacryl (IBF); TSK Gel Toyopearl (TosoHaas); Ultragel (IBF); AvidGel CPG (BioProbe); HiPAC (ChromatoChem); Protein-Pak Affinity Packing (Waters); Ultrafinity-EP (Bodman) and Emphaze (3M Corp./Pierce).

Other chromatography supports include affinity monolith chromatography supports, and POROS® affinity chromatography supports.

Preferably, antibodies and fragments thereof may be immobilized on an agarose matrix.

Thus, according to one embodiment, the method comprises a step b) that is performed by using a chromatography support onto which antibodies or fragments thereof directed to the said rAAV particles, are immobilized.

In particular the antibodies or fragments thereof are immobilized to an agarose matrix by a hydrophilic spacer arm.

According to one embodiment, step b) is performed using an antibody that binds specifically to at least one epitope that is present on the AAV particles. In one embodiment, said epitope belongs at least to serotypes selected in a group comprising AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9 and AAVrh10.

In one embodiment, said epitope belongs at least to serotypes selected in a group comprising or consisting of AAV1, AAV2, AAV3 and AAV5.

According to one particular embodiment, step b) is performed using a single-chain camelid antibody, or VHH antibody, that binds specifically to at least one epitope that is present on the AAV particles. In one embodiment, said epitope belongs at least to serotypes selected in a group comprising AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9 and AAVrh10, which includes an epitope selected from the group comprising or consisting of: AAV1, AAV2, AAV3 and AAV5.

Still, according to one exemplary embodiment, step b) is performed using an AVB Sepharose™ chromatography (GE Healthcare) support.

According to one preferred embodiment, the pH of the rAAV-containing clarified composition, in particular the rAAV4, rAAVrh10 or rAAV2-containing clarified composition, is

at a neutral to basic pH, prior to loading on the Immunoaffinity column, which includes a pH ranging from 6.0 to 8.0.

According to one most preferred, the rAAV-containing clarified composition is a rAAV4-containing clarified composition.

5 Still, according to one exemplary embodiment, a rAAV-containing clarified composition is loaded on an AVB column equilibrated with Dulbecco's Phosphate Buffered Saline (DPBS) supplemented with Calcium and Magnesium, or Phosphate Buffered Saline (PBS).

Still, according to one exemplary embodiment, the first rAAV enriched composition is eluted from the Immunoaffinity column with an acid pH. An acid pH may refer to any pH
10 below 7.0, including below 5.0, or even below 3.0.

According to said exemplary embodiment, the first rAAV enriched composition is eluted from the Immunoaffinity column with an acid pH, wherein said pH is below 3.0, which includes a pH of about 2.0.

The above-mentioned immunoaffinity supports and methods are particularly
15 convenient in methods of the invention, for purifying rAAV particles belonging to the AAV4, AAV10 (i.e. AAVrh10) or AAV2 serotype; in particular rAAV particles belonging to the AAV4 serotype.

Once eluted, the pH of the first rAAV enriched composition is preferably neutralized in a manner suitable for obtaining a first rAAV enriched composition with a neutral or basic pH,
20 which includes a pH of 8.0 or above, which includes 8.5. The reason is that rAAV particles, including rAAV4 particles, tend to lose their integrity and/or infectivity if maintained in a composition having an acid pH.

According to one exemplary embodiment, the first rAAV enriched composition is neutralized in a Tris buffer having a pH of about 8.5.

25 According to another exemplary embodiment, the first rAAV enriched composition is neutralized in a Tris buffer having a pH of about 8.0.

Advantageously, before, during or after neutralization, the first rAAV enriched composition can be supplemented with a non-ionic surfactant, for example Pluronic® F-68(Gibco).

30 The non-ionic surfactant can be present in an amount ranging from 0.0001% to 0.1% (v/v) of the total volume of the composition; which includes in an amount ranging from 0.0005% to 0.005% (v/v) of the total volume of the composition; which includes about 0.001% (v/v) of the total volume of the composition.

Advantageously the Adeno-Associated Virus particles (rAAV) enriched composition is concentrated in the presence of a non-ionic surfactant, for example Pluronic® F-68 (Gibco), and in amount as defined above.

Also advantageously the Adeno-Associated Virus particles (rAAV) containing
5 composition is diafiltered and concentrated against a Saline Ocular Solution, which may further comprise a non-ionic surfactant, as defined above, and in an amount as also defined above.

The use of a non-ionic surfactant, as defined above, and in the other steps, further contributes to the efficiency and scalability of the method. In particular, the use of a non-ionic surfactant, as defined above, prevents the aggregation or adherence of rAAV particles, before,
10 during and after purification.

Anion-Exchange chromatography

A number of suitable anion exchangers for use with the present invention are known and include without limitation, MACRO PREP Q (strong anion- exchanger available from
15 BioRad, Hercules, Calif.); UNOSPHERE Q (strong anion- exchanger available from BioRad, Hercules, Calif.); POROS 50HQ (strong anion-exchanger available from Applied Biosystems, Foster City, Calif.); POROS 50D (weak anion-exchanger available from Applied Biosystems, Foster City, Calif.); POROS 50PI (weak anion- exchanger available from Applied Biosystems, Foster City, Calif.); SOURCE 30Q (strong anion-exchanger available from GE Healthcare, N.J.);
20 DEAE SEPHAROSE (weak anion-exchanger available from GE Healthcare, Piscataway, N.J.); Q SEPHAROSE (strong anion-exchanger available from GE Healthcare Biosciences, Piscataway, N.J.), Capto Q and Capto Adhere (GE Healthcare, N.J.).

Examples of suitable monolithic chromatographic supports are known in the Art, and include in a non-limitative manner the monolithic column CIMmultus® QA, CIM® QA, CIM
25 DEAE and CIMmultus® DEAE (Bia Separations).

Chromatography monoliths are one-piece porous solids made of fused micrometer-sized globules of silica or an organic polymer that can be synthesized directly inside a chromatography tube. This makes this support very resistant and ready-to-use, with no package parameters variability.

30 Monoliths are homogenous columns with a continuous porous bed matrix, consisting of interconnected perfusion channels. These channels are relatively large (1–5 µm) by comparison to the typical pore sizes of packed-bed particle-based chromatography (5–100 nm). One benefit of this is that it increases the potential binding capacity of macromolecules such as virus because of high surface of accessibility. These supports generate low counter pressure even at high flow
35 rates, and low shear rate.

By comparison to packed-bed columns, the improved mass transport of monolithic supports results in efficient separation of the macromolecules.

From a practical point of view, the use of these columns allows to work with a viral product (even when it is a cellular lysate) at high flow rate compared to packed-bed columns, with a high binding capacity and a good resolution, independent of the flow rate. The high resolution of these supports contributes to the reduction of the purification steps and the scalability of the whole process.

According to some embodiments, the method for obtaining purified recombinant Adeno-Associated Virus particles (rAAV) comprises only one anion-exchange chromatography step.

According to some embodiments, the method for obtaining purified recombinant Adeno-Associated Virus particles (rAAV) comprises more than one anion-exchange chromatography step, which includes in particular two, three or even four anion-chromatography steps. When the method comprises more than one anion-exchange chromatography step, the aforementioned steps may be achieved on the same type of support or on different supports.

According to an exemplary embodiment, the at least one chromatographic support at step c1) is a monolithic chromatographic support.

The anion exchange column is first equilibrated using standard buffers and according to the manufacturer's specifications. For example, the column can be equilibrated with, e.g., a 5 to 50 mM, preferably 7-20 mM, such as 20 mM, Tris buffer. Sample is then loaded and two elution buffers are used, one low salt buffer and one high salt buffer.

Fractions are collected following a progressive mix of the low salt and high salt buffers, generating a salt gradient, and the eluted material is detected in the fractions using standard techniques, such as monitoring UV absorption at 260 and 280 nm. Using an anion exchanger, the protein peaks from the lower salt eluate contain AAV empty capsids and the higher salt fractions contain AAV particles.

In particular, on the anion exchange column, AAV particles can be further purified using an appropriate buffer at a pH of from about pH 5 to pH 12, preferably pH 6 to pH 10, and even more preferably pH 7 to pH 9.5, such as pH 7.1, 7.2, 7.3, 7.4 - 8.0, 8.1, 8.2, 8.3, 8.4, 8.5 - 9.0, 9.1, 9.2, 9.3, 9.4, 9.5, or any pH between the stated ranges.

Appropriate buffers for use with the 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: N-methylpiperazine; piperazine; Bis-Tris; Bis-Tris

propane; Triethanolamine; Tris; N-methyldiethanolamine; 1,3-diaminopropane; ethanolamine; acetic acid, and the like. To elute the sample, the ionic strength of the starting buffer is increased using a salt, such as NaCl, KCl, sulfate, formate or acetate, at an appropriate pH.

Advantageously, all the buffers used during, before or after the anion-exchange
5 chromatography comprise a non-ionic surfactant, for example Pluronic® F-68 (ThermoFisher Scientific), in an amount ranging from 0.0001% to 0.1% (v/v) of the total volume of the buffer composition; which includes in an amount ranging from 0.0005% to 0.005% (v/v) of the total volume of the buffer composition; which includes about 0.001% (v/v) of the total volume of the buffer composition.

10 The nature of the resins used (i.e. strong or weak ion exchangers) and the conditions of salt concentration, buffer used, and pH, will vary on the AAV capsid variant (i.e. AAV capsid serotype or pseudotype). While the known AAV capsid variants all share features such as size and shape, they differ in fine details of molecular topology and surface charge distribution. Hence, while all capsid variants are expected to be amenable to purification by anion exchange
15 chromatography, and relevant methods can be determined in a systematic manner using chromatography resin and buffer screening experiments, different conditions will be required for each AAV capsid variant to achieve efficient AAV particle purification. The determination for such conditions is readily apparent to the skilled artisan.

Advantageously, at the end of step b), the pH of the rAAV-containing clarified
20 composition is adjusted at a basic pH so as to ensure optimal retention of the rAAV particles on the at least one anion-chromatography support used at step c).

A pH of 8.5, or above 8.5, which includes 9, is particularly convenient for the binding of rAAV4 particles, or rAAV4-containing clarified compositions, to the anion-exchange column.

25 Thus, according to one exemplary embodiment, at the end of step b), the optimal pH of a rAAV4-containing clarified composition for binding to the anion-exchange column is adjusted to pH 8.5, or above 8.5, which includes 9.

A pH of 8.5, or above 8.5, which includes 9, is particularly convenient for the binding of rAAVrh10 particles, or rAAVrh10-containing clarified compositions, to the anion-exchange
30 column.

Thus, according to one exemplary embodiment, at the end of step b), the optimal pH of a rAAVrh10-containing clarified composition for binding to the anion-exchange column is adjusted to pH 8.5, or above 8.5, which includes 9.

A pH of 8.5, or above 8.5, which includes 9, is particularly convenient for the binding
35 of rAAV2 particles, or rAAV2-containing clarified compositions, to the anion-exchange column.

Thus, according to one exemplary embodiment, at the end of step b), the optimal pH of a rAAV2-containing clarified composition for binding to the anion-exchange column is adjusted to pH 8.5, or above 8.5, which includes 9.

5

Appropriate buffers for use with the 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: N-methylpiperazine; piperazine; Bis-Tris; Bis-Tris propane; Triethanolamine; Tris; N-methyldiethanolamine; 1,3-diaminopropane; ethanolamine; 10 acetic acid, and the like. To elute the sample, the ionic strength of the starting buffer is increased using a salt, such as NaCl, KCl, sulfate, formate or acetate, at an appropriate pH.

Prior to elution, the anion-exchange chromatography may also include an optional step of treating the support, or anion-exchange column, with a lower salt concentration than what is generally used for the elution step. Such treatment may also be referred herein as a “pre- 15 elution” step.

Thus, in one embodiment of the invention, the anion exchange column is first treated with a low salt concentration, e.g., 5-100 mM of salt, in particular 5-100 mM of NaCl, such as 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 - 100 mM, or any concentration within these ranges. For example the salt concentration in a “first pre-elution step” may initially correspond to the salt 20 concentration of the buffer used for binding the rAAV particles to the column after loading.

In some embodiments, and following initial treatment, the column is then treated with a higher salt concentration in order to elute impurities, such as a higher NaCl concentration, or with another buffer with a greater ionic strength. One example for use as the second buffer is a sodium acetate buffer or a Tris-based buffer. This additional step may also be referred herein as a 25 “second pre-elution” step. In that case, one may take care that the salt concentration remains low enough to not elute the AAV particles from the column.

After additional impurities are eluted from the column, the AAV particles can then be recovered using a higher concentration of salt in the elution step.

In some embodiments, the elution pH on the chromatographic support is adjusted 30 depending on the rAAV particle that is purified. Elution at an optimal pH has the advantage of maintaining optimal infectivity of a given particle.

Elution of rAAV particles from the chromatographic support at step c), in particular elution of rAAV4 particles, can also be adjusted at pH 8.

Preferably, elution of rAAV particles from the chromatographic support is performed by using a linear salt gradient. The salt may be selected from the group consisting of : NaCl, KCl, sulfate, formate and acetate ; and preferably NaCl.

5 The term “*slope of the linear salt gradient*” refers to the slope between two points (A,B) of the linear gradient, and depends both on the concentration of salt in the chromatography support and the column volume (CV) that is eluted over time, according to the following formula:

$$\text{slope (A, B) = (concentration of salt in B – concentration of salt in A) /} \\ \text{(column volume in B – column volume in A).}$$

10 Thus, the slope of the linear salt gradient can be expressed in M / CV or in mM / CV, and is preferably a shallow salt gradient according to standard protocols.

According to one embodiment, the slope of the linear salt gradient in step c) is equal or less than 0,1 M / CV, in particular ranging from 0,015 to 0,09 M /CV.

15 According to one embodiment, the slope of the linear salt gradient in step c) is equal or less than 0,07 M / CV, in particular ranging from 0,01 to 0,06 M /CV.

Advantageously, the method may include an additional step of dilution of the rAAV enriched composition before or after the anion-exchange chromatography step. A step of dilution may be, for instance, in the form of a buffer exchange step.

20 According to one preferred embodiment, at the end of step c), the second rAAV enriched composition is stored under a frozen form until its use for performing step d).

Density gradient centrifugation.

25 In density gradient centrifugation, the density of the suspension medium varies in a known manner from one end of the density gradient container, e.g of the centrifuge tube, to the other end. When the particle under the influence of centrifugal force reaches the point of its isopycnic density, i.e., when the density of the surrounding liquid is equal to the density of the rAAV particle, the rAAV particle will cease to migrate along the force vector.

30 Solute systems used to establish density gradients for centrifugation which are considered by the invention include, in a non-limitative manner, inorganic salts (cesium chloride, potassium bromide, sodium chloride), sucrose and several commercially available solutes such as Ficoll®, a synthetic polysaccharide made by crosslinking sucrose; Percoll™, a suspension of silica particles coated with polyvinylpyrrolidone; and Nycodenz®, a derivative of the synthetic molecule metrizoic acid (metrizamide). Iodixinol, or Iodixanol, a dimer of Nycodenz®, is also
35 used widely.

Thus, step c) may include a step of submitting the first rAAV enriched composition obtained at step b) at least once to a step of density gradient centrifugation, wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided;

5 According to one embodiment, a step of density gradient centrifugation is selected from: cesium chloride (CsCl) gradient centrifugation; iodixanol gradient centrifugation; sucrose gradient centrifugation; Percoll™ gradient centrifugation; Ficoll® gradient centrifugation; and most preferably cesium chloride (CsCl) gradient centrifugation.

10 Advantageously, prior to density-gradient based centrifugation, the first rAAV enriched composition may be precipitated in order to obtain a suitable volume of material for performing the density gradient centrifugation step, in particular in the presence of Polyethylene Glycol, such as PEG 8000.

It may also be diafiltered and concentrated by Tangential Flow Filtration (TFF).

15 Thus, according to one embodiment, the first rAAV enriched composition is precipitated, in the presence of PEG 8000, for instance PEG 8000 at a final concentration of about 5 to 10% (w/v). Additionally, the sample can then be centrifugated prior to density-gradient based centrifugation, so as to facilitate the collection of the precipitated rAAV particles for their further processing.

20

According to a most general embodiment, density gradient-based centrifugation methods comprise at least the steps of:

i) providing a density-gradient forming solution which includes the sample of interest;

25 ii) centrifuging the solution provided at step (i) in a manner suitable for producing a density gradient having at least one layer comprising a second rAAV enriched composition;

iii) optionally recovering the layer comprising the second rAAV enriched composition.

30 Accordingly, the sample of interest may comprise, or may consist of, the first rAAV enriched composition.

According to the invention, a “*density-gradient forming solution*” is a solution which is either in the form of a continuous or discontinuous gradient prior to centrifugation; or
35 alternatively for which the gradient is self-formed during centrifugation.

A “*density-gradient forming solution*” may be selected from the group consisting of a cesium chloride (CsCl), Iodixanol, sucrose, Percoll™, and Ficoll® solution; and most preferably cesium chloride (CsCl) solution or Iodixanol.

Iodixanol is preferred, including for reglementary reasons.

5

In some embodiments of Step i), the sample of interest may be simply in admixture with the density gradient forming solution.

In some other embodiments of Step i), the sample of interest may be layered on the top of the density gradient forming solution, i.e. at the interface between the said gradient forming
10 solution and the atmosphere.

Step ii) of centrifuging the solution in a manner suitable for producing a density gradient having at least one layer comprising a second rAAV enriched composition can thus be achieved by centrifuging a density-gradient forming solution which is either in the form of a continuous or discontinuous gradient prior to centrifugation; or alternatively for which the
15 gradient is self-formed during centrifugation.

According to said embodiment, the solution provided at step (i) can already be in the form of a continuous or discontinuous gradient of density. For instance, the solution provided at step (i) can be in the form of a set of layers having, from the top to the bottom, a plurality of
20 layers having increasing densities.

The preparation of continuous and discontinuous density gradients is well known in the Art.

According to another embodiment, the density-gradient forming solution provided at
25 step (i) can be a solution, for instance an isopycnic solution, of which the density gradient is self-formed during centrifugation.

According to said embodiment, the density gradient can be formed during the step (ii) of centrifuging. For instance the formation of a density gradient during centrifugation may occur by starting from an isopycnic Cesium Chloride (CsCl) solution.

30

According to one embodiment, the AAV-containing solution provided at step (i) is a CsCl solution having a density ranging from about 1 to 1.6 g/cm³, which includes 1, 1.1, 1.2, 1.3, 1.4, and 1.5 to 1.6 g/cm³, which includes 1.3 to 1.5 g/cm³, which includes 1.376 g/cm³.

According to one embodiment, the sample of interest at step (i) is present at the top layer of the said solution before centrifugation.

Step ii) of centrifuging the solution is known in the Art and consists of applying a centrifugal field to said solution, at least until a gradient has formed from top to bottom.

The centrifugal force is generally applied by spinning a centrifuge tube containing the solution of step (i) for a given time (i.e. a few hours), for a given intensity (estimated in round per minutes or rpm ; or alternatively in g) and at a given temperature in a centrifuge rotor.

One example of a preferred protocol is described in Example 1.

Another example of a preferred protocol is described in Example 2.

According to one embodiment, the rotor used at step (ii) is a SW41 rotor.

According to one embodiment, step ii) consists of centrifuging the solution provided at step (i) in a SW41 rotor at about 30,000 to 50,000 rpm, which includes 38,000 rpm, for about 24 to 72 hours, which includes 48 hours, and at a temperature ranging from about 4°C to 20°C in temperature, which includes 15°C, in order to separate full particles from “empty” ones. The layer comprising the second rAAV enriched composition can be recovered at step (iii) based on the banding density of purified rAAVs.

The determination of banding densities of purified rAAVs is also known in the Art and can be determined either by comparison to a reference value or empirically.

The density of rAAV particles can be expressed in g/cm³.

For reference, densities of known rAAV particles, either full or empty, are described in Qu *et al* (“Separation of adeno-associated virus type 2 empty particles from genome containing vectors by anion-exchange column chromatography; Journal of Virological Methods; 2006) : ≈ 1.32 g/mL for empty capsids, 1.40 g/mL for genome containing capsids.

Overall densities for one given preparation may depend both on the serotype of the rAAV, and the presence of full or empty particles in the preparation.

In particular, the resolution of the resulting gradient will depend upon various parameters, including in a non-limitative manner the nature of the solution used at step (i), the parameters used at step (ii) for centrifuging the solution and the banding densities of rAAVs which have to be recovered.

For instance, Iodixanol is an iodinated density gradient medium which is known in the Art and used for clinical use. Because the apparent density of macromolecules in iodixanol is different from that in CsCl, the banding density of purified rAAVs must be determined

empirically. For rAAV capsids, density is of about 1.40 g/mL in CsCl and 1.266 g/mL in Iodixanol.

At step (iii), and after fractionation of the gradient, the positive fractions can also be
5 identified by quantitative PCR, and optionally pooled.

Optionally, the rAAV particles-containing fractions can also be concentrated and/or dialyzed further, for instance using the Tangential Flow Filtration.

Optionally, the rAAV particles-containing fractions can also be submitted to an additional density gradient centrifugation.

10

Protocols for purifying rAAV particles preparations using density gradient centrifugation are known in the Art, and are further described, for instance, in Zolotukhin *et al.* (« Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield » ; Gene Therapy (1999).

15

Tangential Flow Filtration and subsequent steps

Tangential Flow Filtration is a polishing step which allows to discard small-sized particle-related impurities through cycles of concentration and diafiltration through the pores of the filter. This polishing step has the other advantage of being suitable for changing the buffer of
20 the eluted fractions and for concentrating the rAAVs.

Tangential Flow Filtration is achieved preferably using, as a filter, a hollow fiber filter.

According to one embodiment, step d) is performed by using a filter membrane
25 having a molecular weight cut-off value equal or inferior to 150 kDa, in particular ranging from 20 kDa to 150 kDa, preferably 25 kDa to 150 kDa, which includes about 30 kDa.

The man skilled in the Art will recognize that the molecular weight cut-off must be adjusted depending on the type of rAAV particle that is purified and on the of elution parameters (pH, salt).

30

According to some embodiment, salts and/or detergents and/or surfactants and/or nucleases can added during, before or after the Tangential Flow Filtration, in particular during or before the Tangential Flow Filtration.

According to one embodiment, the method may further include a step of treatment with detergents, surfactants, and/or nucleases, including DNAses, during, before or after the
35 Tangential Flow Filtration.

Advantageously the Adeno-Associated Virus particles (rAAV) containing composition is diafiltered and concentrated in the presence of a non-ionic surfactant, for example Pluronic® F-68 (Gibco).

The non-ionic surfactant can be present in an amount ranging from 0.0001% to 0.1% (v/v) of the total volume of the composition; which includes in an amount ranging from 0.0005% to 0.005% (v/v) of the total volume of the composition; which includes about 0.001% (v/v) of the total volume of the composition.

Also advantageously the Adeno-Associated Virus particles (rAAV) containing composition is diafiltered and concentrated against a Saline Ocular Solution, which may further comprise a non-ionic surfactant as defined above, and in an amount as also described above.

According to one embodiment, the purified recombinant Adeno-Associated Virus particles (rAAV) obtained at step c) or d) are submitted to an additional gradient, including a step of differential centrifugation, including density gradients, in particular selected from: cesium chloride (CsCl) gradient centrifugation; iodixanol gradient centrifugation; sucrose gradient centrifugation.

According to one preferred embodiment, the method may further include an additional step of Tangential Flow Filtration after said additional gradient.

According to one embodiment, the purified recombinant Adeno-Associated Virus particles (rAAV) obtained at step d) are sterilized.

According to one embodiment, the purified recombinant Adeno-Associated Virus particles (rAAV) are submitted to a step of sterile filtration over a filter membrane having a pore size of 0.2 µm or less.

The invention also relates to purified rAAV particles obtained by performing a method as defined above.

Characterization of the purified rAAV particles

Advantageously, the above-mentioned method can be used for obtaining purified recombinant Adeno-Associated Virus particles (rAAV) which are suitable for gene therapy, and/or for preparing a medicament for gene therapy.

According to one preferred embodiment, said purified rAAV particles are suitable for use for gene therapy.

The purity of adeno-associated virus (AAV) particles preparations also has important implications for both safety and efficacy of clinical gene transfer.

The method used to purify a rAAV particle can dramatically influence the purity of the preparation in terms of the amount of host cell protein contamination and of ratio of full/total particles.

Vector particle concentration can be assessed by quantitative PCR (genome
5 containing particles) or by ELISA (total vector particles) as shown in **Example 1**

The purity of the preparation, is most commonly assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), as shown in **Example 1**.

Bands corresponding to the viral structural (capsid) proteins, VP1, VP2 and VP3 may be visualized after staining and their size and relative intensity assessed with respect to
10 contaminating proteins. For reference, VP1, VP2 and VP3 belonging to the AAV4 and AAV5 serotypes are encoded by nucleic acids of sequences **SEQ ID N° 10 to 15**.

VP1, VP2, and VP3 belonging to the AAV4 serotype can be encoded respectively by nucleic acids of sequences **SEQ ID N°10 to 12**.

VP1, VP2, and VP3 belonging to the AAV5 serotype can be encoded respectively by
15 nucleic acids of sequences **SEQ ID N°13 to 15**.

The above-mentioned sequences are given as references.

Thus, the term “*purity*” refers to the absence of general impurities. Purity is expressed as a percentage, and relates to the total amount of VP1, VP2 and VP3 proteins, in comparison to the total amount of detected proteins in a Coomassie Blue stained polyacrylamide gel.

20 The term “*general impurities*” refers to impurities which were present in the starting material but which are not considered as particle-related impurities. Thus, general impurities encompass impurities which are derived from the host cells but which are not AAV particles.

The term “*particle-related impurities*” refers to all types of AAV particles other than bona fide recombinant AAV particles. Particle-related impurities include empty AAV capsids
25 (also referred to as “*empties*”, or “*empty particles*”, and AAV particles containing polynucleotide sequences other than the intended particle genome (also referred to “*AAV- encapsidated nucleic acid impurities*” or “*AAV-encapsidated DNA impurities*”).

Residual DNA or Host Cell DNA can be present as contaminants in rAAV preparations. Because residual DNA can have negative effects, manufacturers must ensure that
30 final products derived from host cells contain acceptable levels of residual DNA.

Residual DNA testing can be assessed by quantitative PCR, including real-time PCR, using the protocol described in **Example 1**. Residual DNA is expressed in ng per mL, or alternatively in ng per dose.

Preferably, residual DNA is determined by qPCR by determining the relative quantity of an E1A amplicon DNA in the composition, in comparison to a standard curve established with a known amount of E1A amplicon DNA.

A « dose » is defined as the volume of preparation which corresponds to $1 \cdot 10^{12}$ vector genome (vg).

Advantageously, compositions of the invention can be further characterized by their ratio of empty rAAV particles/full rAAV particles.

The terms “*empty capsid*” and “*empty particle*” refer to an AAV virion that includes an AAV protein shell but that lacks in whole or part the polynucleotide construct comprising the heterologous nucleotide sequence of interest flanked on both sides by AAV ITRs. Accordingly, the empty capsid does not function to transfer the gene of interest into the host cell.

The ratio of empty rAAV particles/full rAAV particles can be assessed by SDS-PAGE Gel, using the protocol described in **Example 1**.

Infectious particle concentration can also be assessed using the protocol of ICA described in **Example 1**.

Thus, a composition of the invention comprises at least one purified rAAV particle using either one of the above mentioned methods, and preferably has at least one of the following characteristics:

- a purity equal or superior to 90%, and preferably superior to 99%; or even 100%;
- an amount of residual cellular DNA equal or inferior to 50 ng per dose.

An amount of residual cellular DNA equal or inferior to 50 ng per dose includes equal or inferior to 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, or 5 ng per dose.

In particular, a composition of the invention comprises at least one purified rAAV particle using either one of the above mentioned methods, and has the following characteristics:

- a purity equal or superior to 99%; or even 100% ; and
- an amount of residual cellular DNA equal or inferior to 25 ng per dose, which includes 15 ng per dose.

Most preferably, the purity of the composition is superior to 99%, which includes a purity of 100%.

According to a preferred embodiment, a purified rAAV particle composition of the invention is a composition comprising rAAV particles purified according to the invention, optionally supplemented with a non-ionic surfactant.

According to a preferred embodiment, a purified rAAV particle composition of the invention is a Saline Ocular Solution comprising rAAV particles purified according to the invention, optionally supplemented with a non-ionic surfactant.

According to an exemplary embodiment, the composition is obtained directly from one of the above-mentioned methods.

Saline Ocular Solutions which are suitable for the invention may be obtained from BD Medical, such as for example BD Standard Solution or BD Aqueo Premium™).

According to said exemplary embodiment, the non-ionic surfactant is preferably Pluronic® F-68 (Gibco®).

The non-ionic surfactant (i.e. Pluronic® F-68) can be present in an amount ranging from 0.0001% to 0.1% (v/v) of the total volume of the composition; which includes in an amount ranging from 0.0005% to 0.005% (v/v) of the total volume of the composition; which includes about 0.001% (v/v) of the total volume of the composition.

Compositions of the invention, including compositions for administration and/or of use for gene therapy, as further described herebelow are preferably sterile.

rAAV particles for use for gene therapy

The invention further relates to a method for obtaining purified recombinant Adeno-Associated Virus particles (rAAV), for use for gene therapy and/or for use for the preparation of a medicament suitable for gene therapy.

Advantageously, such rAAV particles and compositions thereof are also suitable, as such, for use for gene therapy, and/or for use for the preparation of a medicament suitable for gene therapy.

By “*gene therapy*” is meant the administration of a nucleic acid sequence to an individual, for treating and/or preventing and/or reducing the likelihood of the occurrence of a disease. Several approaches have been proposed in the Art. In view of the above, rAAV particles are used as vehicles for delivering said nucleic acid sequence to the individual to be treated.

One may replace a mutated gene that causes disease with a healthy copy of the gene.

One may inactivate (“*knocking-out*”) a mutated gene that is functioning improperly.

One may introduce a new gene into the body for treating and/or preventing and/or reducing the likelihood of the occurrence of a disease.

The reported methods and plasmids are particularly efficient for use for preparing a medicament for eye-diseases, including sight loss, and more particularly rod-cone dystrophies due to rod-specific defects.

In particular, said disease may be selected from phosphodiesterase 6 (PDE6)-related diseases, including PDE6 β -related diseases, and Retinal Pigment Epithelium-specific 65 kDa protein (RPE65)-related diseases.

RPE65-related diseases include Leber's congenital amaurosis and retinitis pigmentosa. PDE6-related diseases include Rod-cone Dystrophy, and retinitis pigmentosa

The individual to be treated includes humans and non-human mammals.

AAV particles and compositions thereof may be administered topically or parenterally, which includes intraorbital and intraocular administration. Intraocular administration further includes intravitreal, subretinal and intracorneal administration.

Thus, compositions comprising said AAV particles are preferably suitable for topical or parenteral administration, which includes intraocular, (preferably intravitreal and subretinal), and intracorneal administration.

rAAV particles of the invention preferably include an expression cassette encoding human PDE6 β , or human RPE65 gene, respectively of sequences **SEQ ID N°1 and 7**, and as further described herebelow.

According to one preferred embodiment, a purified rAAV particle can be obtained by performing any one of the methods described herein. A purified rAAV particle composition comprises at least one of said purified rAAV particles. Purified rAAV particles and compositions thereof are suitable for use for gene therapy.

AAV Plasmid

AAV plasmids for producing said rAAV particles are further disclosed.

The construction of infectious recombinant AAV (rAAV) virions has been described. See, e.g., U.S. Pat. Nos. 5,173,414 and 5,139,941; International Publication Numbers WO 92/01070 (published Jan. 23, 1992) and WO 93/03769 (published Mar. 4, 1993); Lebkowski et al. (1988) Molec. Cell. Biol. 8:3988-3996; Vincent et al. (1990) Vaccines 90 (Cold Spring Harbor Laboratory Press); Carter, B. J. (1992) Current Opinion in Biotechnology 3:533-539; Muzyczka, N. (1992) Current Topics in Microbiol, and Immunol. 158:97-129; and Kotin, R. M. (1994) Human Gene Therapy 5:793-801.

By an “AAV *plasmid*” is meant a plasmid derived from an adeno-associated virus serotype, including without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, and AAV10, and *rhesus macaque*-derived serotypes including AAVrh10. AAV plasmids can have one or more of the AAV wild-type genes deleted in whole or part, preferably
5 the rep and/or cap genes, but retain functional flanking ITR sequences. Functional ITR sequences are necessary for the rescue, replication and packaging of the AAV virion. Thus, an AAV plasmid is defined herein to include at least those sequences required in cis for replication and packaging (e.g., functional ITRs) of the virus. The ITRs need not be the wild-type nucleotide sequences, and may be altered, e.g., by the insertion, deletion or substitution of nucleotides, so long as the
10 sequences provide for functional rescue, replication and packaging. Also by an “AAV *particle*” is meant the protein shell or capsid, which provides an efficient vehicle for delivery of a nucleic acid to the nucleus of target cells.

In particular, the AAV particle is derived from an adeno-associated virus selected from AAVs belonging to the AAV4, AAV5, AAVrh10 and/or AAV2 serotypes.

15 Preferably, the AAV particle is derived from an adeno-associated virus selected from AAVs belonging to the AAV4 or AAV5 serotypes

“AAV *helper functions*” refer to AAV-derived coding sequences which can be expressed to provide AAV gene products that, in turn, function in trans for productive AAV replication. Thus, AAV helper functions include both of the major AAV open reading frames (ORFs),
20 rep and cap. The Rep expression products have been shown to possess many functions, including, among others: recognition, binding and nicking of the AAV origin of DNA replication; DNA helicase activity; and modulation of transcription from AAV (or other heterologous) promoters. The Cap expression products supply necessary packaging functions. AAV helper functions are used herein to complement AAV functions in trans that are missing from AAV plasmids.

25 The term “AAV *helper construct*” refers generally to a nucleic acid molecule that includes nucleotide sequences providing AAV functions deleted from an AAV plasmid which is to be used to produce a transducing particle for delivery of a nucleotide sequence of interest. AAV helper constructs are commonly used to provide transient or stable expression of AAV rep and/or cap genes to complement missing AAV functions that are necessary for AAV replication; however,
30 helper constructs lack AAV ITRs and can neither replicate nor package themselves. AAV helper constructs can be in the form of a plasmid, phage, transposon, cosmid, virus, or virion. A number of AAV helper constructs have been described, such as the commonly used plasmids pAAV/Ad and pIM29+45 which encode both Rep and Cap expression products. See, e.g., Samulski et al. (1989) J. Virol. 63:3822-3828; and McCarty et al. (1991) J. Virol. 65:2936-2945. A

number of other plasmids have been described which encode Rep and/or Cap expression products. See, e.g., U.S. Pat. Nos. 5,139,941 and 6,376,237.

The term “*transfection*” is used to refer to the uptake of foreign DNA by a cell, and a cell has been “*transfected*” when exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are generally known in the art. See, e.g., Graham *et al.* (1973) *Virology*, 52 :456, Sambrook *et al.* (1989) *Molecular Cloning*, a laboratory manual, Cold Spring Harbor Laboratories, New York, Davis *et al.* (1986) *Basic Methods in Molecular Biology*, Elsevier, and Chu *et al.* (1981) *Gene* 13:197. Such techniques can be used to introduce one or more exogenous DNA moieties into suitable host cells.

A “*nucleic acid*”, “*nucleotide sequence*” or “*polynucleotide sequence*” refers to a DNA or RNA sequence. The term comprises sequences that include any of the known base analogues of DNA and RNA such as, but not limited to, 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxymethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, Buracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine.

A “*coding sequence*” or a sequence which “*encodes*” a selected polypeptide is a nucleic acid molecule which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A transcription termination sequence may be located 3' to the coding sequence.

The term DNA “*control sequences*” refers collectively to promoter sequences, polyadenylation signals, transcription termination sequences, upstream regulatory domains, origins of replication, internal ribosome entry sites (“IRES”), enhancers, and the like, which collectively provide for the replication, transcription and translation of a coding sequence in a recipient cell. Not all of these control sequences need always be present so long as the selected coding sequence

is capable of being replicated, transcribed and translated in an appropriate host cell. The term "promoter" is used herein in its ordinary sense to refer to a nucleotide region comprising a DNA regulatory sequence, wherein the regulatory sequence is derived from a gene which is capable of binding RNA polymerase and initiating transcription of a downstream (3' -direction) coding sequence. Transcription promoters can include "*inducible promoters*" (where expression of a polynucleotide sequence operably linked to the promoter is induced by an analyte, cofactor, regulatory protein, etc.), "*repressible promoters*" (where expression of a polynucleotide sequence operably linked to the promoter is induced by an analyte, cofactor, regulatory protein, etc.), and "*constitutive promoters*".

10 The term "*heterologous*" denotes sequences that are not normally joined together, and/or are not normally associated with a particular cell. Thus, a "*heterologous*" region of a nucleic acid construct or a plasmid is a segment of nucleic acid within or attached to another nucleic acid molecule that is not found in association with the other molecule in nature. The transgene comprising the heterologous nucleic acid can encode a number of useful products. 15 These can include siRNA, antisense molecules, and miRNAs for example. Alternatively, transgenes can encode hormones and growth and differentiation factors.

AAV plasmids can be engineered to carry a heterologous nucleotide sequence of interest (e.g., a selected gene encoding a therapeutic protein, an antisense nucleic acid molecule, a ribozyme, a miRNA or the like) by deleting, in whole or in part, the internal portion of the AAV 20 genome and inserting the DNA sequence of interest between the ITRs. The ITRs remain functional in such plasmids allowing replication and packaging of the rAAV containing the heterologous nucleotide sequence of interest. The heterologous nucleotide sequence is also typically linked to a promoter sequence capable of driving gene expression in the patient's target cells under the certain conditions. Termination signals, such as polyadenylation sites, can also be 25 included in the plasmid.

The term "*expression cassette*" refers to a DNA nucleic acid sequence including at least one gene of interest, one open reading frame and a 3' untranslated region that, in eukaryotes, usually contains a polyadenylation site.

According to a general embodiment, an expression cassette according to the invention 30 may thus comprise, or consist of, a DNA nucleic acid sequence having the general formula:

$$[5'ITR] - [Gene\ of\ Interest] - [PolyA]_z - [3'ITR] -$$

wherein [5'ITR] and [3'ITR] are inverted terminal repeats (ITR),

wherein [Gene of Interest] is any gene coding for a protein of interest,

wherein [PolyA] is a poly-adenylation signal with z being 0 or 1, and

wherein each of [5'ITR], [Gene of Interest], [PolyA], and [3'ITR], can be optionally separated by one additional linker sequence.

In the sense of the invention, a “gene coding for a protein of interest” comprises at least one nucleic acid sequence coding for the protein of interest, and preferably at least one promoter.

Preferably the gene of interest is a nucleic acid coding for human PDE-6 β or human RPE65.

According to one embodiment, the AAV plasmid comprises an expression cassette comprising a nucleic acid coding for human PDE-6 β , of **SEQ ID N° 1**.

10 According to one alternative embodiment, the AAV plasmid comprises an expression cassette comprising a nucleic acid coding for human RPE65, of **SEQ ID N° 7**.

According to one particular embodiment, the AAV plasmid comprises a nucleic acid coding for human PDE6- β , of **SEQ ID N° 2**.

15 According to one embodiment, the [5'ITR] and [3'ITR] are inverted terminal repeats derived from AAV-2, which are preferably and respectively of sequences **SEQ ID N°3 and 4**.

Advantageously, the AAV plasmid includes a polyadenylation site, an ITR derived from AAV2, and an expression cassette encoding human PDE6-beta.

20 Preferably, the expression of the human PDE6-beta gene is under the control of the promoter for Rhodopsine Kinase, preferably of sequence **SEQ ID N°5**.

According to an alternative embodiment, the AAV plasmid includes a polyadenylation site, an ITR derived from AAV2, and an expression cassette encoding human RPE65.

25 Preferably, the expression of the human RPE65 gene is under the control of the RPE65 promoter, preferably of sequence **SEQ ID N°8**.

According to one embodiment, the polyadenylation signal is derived from the Bovine Growth Hormone (bGH) and is preferably of sequence **SEQ ID N°6**.

The above-mentioned embodiments may be considered individually or in combination.

30 According to one most preferred embodiment, the AAV plasmid comprises an expression cassette comprising a nucleic coding for PDE6 β as disclosed above and as illustrated in figure 1A.

According to one alternative embodiment, the AAV plasmid comprises an expression cassette comprising a nucleic coding for RPE65 as disclosed above and as illustrated in figure 1B.

The invention further relates to a host cell which has been transfected with a AAV plasmid as defined above, and to a rAAV particle produced by said host cell.

The invention further relates to rAAV particles produced by the host cell, for use for gene therapy.

5 The invention further relates to rAAVs particles including an AAV plasmid as described above. In particular, the invention further relates to rAAV particles selected in a group comprising AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV 9, AAV10, and *rhesus macaque*-derived serotypes including AAVrh10.

In particular, rAAV particles are selected from rAAV particles belonging to the
10 AAV4, AAV5, AAV10 (i.e. AAVrh10) and/or AAV2 serotype.

Preferably, rAAV particles are selected from rAAV particles belonging to the AAV4 and/or AAV5 serotype.

In particular, the invention relates to rAAV5 particles containing DNA comprising an expression cassette encoding human PDE6-beta as defined above.

15

EXAMPLES

EXAMPLE 1: Two-step chromatography for purifying AAV4 particles using a first step of Immunoaffinity chromatography.

20

A. MATERIAL & METHODS

A1. Harvest of the cell lysate.

Transfected cells are harvested at 96 +/- 5 hours post transfection by rapping the CellStacks 10 (CS10) on all sides. The entire suspension is transferred in a BioProcess Container (BPC). A major part of the viral particle is released in the supernatant.

25

At this step the major part of the viral vector is already released in the supernatant but a 0.5% (v/v) Triton X-100 treatment (1 hour, stirring, at room temperature) is necessary to optimize the extraction of AAV4 from the cells, to contribute to detach the particles from impurities, and finally to optimize the recovery of AAV4 in the supernatant and to optimize the next step of depth filtration.

30

A2. Clarification of the cell lysate.

The suspension obtained after the action of Triton X-100 is filtered through a Millipore depth filter Polysep II 1/0.2 µm to discard the cell debris. At the end of the filtration step, the filtration unit is emptied by pushing with air. This is the clarified lysate.

35

During this step a great part of DNA and soluble proteins are discarded as well.

A3. Chromatographic immuno-affinity purification step

This step is performed with an Akta Pilot (GE Healthcare) controlled by a Unicorn software. The clarified lysate is injected at 150 cm/h linear flow rate through an AVB column 185
5 mL of packed matrice equilibrated with DPBS supplemented with Calcium and Magnesium. During this step the AAV binds specifically to the column. Impurities do not bind to the column and are discarded in the flowthrough. To wash the unspecific bound material, the column is then rinsed with DPBS.

AAV is eluted with a PBS pH 2.0 buffer. Eluted fractions are simultaneously
10 neutralized with 10% of Buffer Tris 1M, MgCl₂ 20 mM, CaCl 10 mM, pH 8.5.

Eluted fractions neutralized with Tris buffer are frozen at <-70°C, or even <-80°C until titration of AAV by QPCR to detect the amount of AAV in each fraction.

A4. 1st Alternative: CsCl gradient after immuno-affinity purification step.

15 Fractions eluted from the AVB column and containing the AAV are pooled. To precipitate AAV and to obtain a suitable volume for gradient, PEG 8 000 is added to obtain 8% final. After an overnight precipitation with PEG 8% (w/v), the AAV containing suspension is centrifuged at 4 000 rpm for 1 hour at 4°C.

The AAV-containing pellet is resuspended in 12 mL of 1,376 g/cm³ density CsCl,
20 transferred to an UltraClear tube for SW41 rotor. The gradient is centrifuged at 38,000 rpm for 48 hours at 15°C to separate full particles from “empty” one. The enriched-full particles band is collected with a 5 or 10 mL syringe. The viral suspension is then subjected to Tangential Flow Filtration (TFF).

A5. 2nd Alternative: CIMmultus anion-exchange chromatography after after immuno-affinity purification step.

This step is realised with an Akta Pilot (GE Healthcare) controlled by a Unicorn software.

Fractions eluted from the AVB column and containing the AAV are pooled and
30 submitted to a TFF step for diafiltration. The goal of this TFF step is to change the buffer of neutralized eluted fractions from AVB to be able to bind AAV to the CIMmultus QA column.

The diafiltered AAV is then injected through a Monolith CIMmultus QA column equilibrated with buffer 1 : Tris 20 mM, NaCl 10 mM, MgCl₂ 2 mM, CaCl₂ 1 mM, pH 8.5 to promote the binding of AAV to the column.

At the end of the injection of the AAV containing suspension, the column is washed with buffer 1 to discard everything that could be bound unspecifically.

The elution step is a NaCl salt gradient with a very shallow slope which is the result of mixing buffer 1 (Tris 20 mM, NaCl 10 mM, MgCl₂ 2 mM, CaCl₂ 1 mM, pH 8,5) with buffer 2
5 (Tris 20 mM, NaCl 1 M, MgCl₂ 2 mM, CaCl₂ 1 mM, pH 8,5). The slow increase of salt allows to elute gradually AAV and to separate sub populations as full and empty particles if sub populations are present.

Eluted fractions are collected in polypropylene bottles and stored at <-70°C, or even <-80°C, until the TFF.

10

A6. Tangential Flow Filtration (TFF).

Selected fractions are pooled (around 1L in volume). TFF is done using a mPES hollow fiber with a cut off of 30 KDa. AAV is diafiltered and concentrated against Saline Ocular Solution (SS0).

15

The pooled fractions are first slightly concentrated, diafiltered against 10 volumes of buffer and finally concentrated to reach the expected concentration. TFF allows to discard the remaining small size impurities as well as to formulate the viral vector in the right buffer for injection and to concentrate to the right level. The filtered purified and concentrated viral vector is stored at <-70°C, or even <-80°C, until titration.

20

A7. Sterile Filtration.

AAV is diluted with SSO (Saline Ocular Solution) to obtain the precise concentration necessary for the injection to patients and sterile filtered with a 0.2 µm PES filter before to be aliquoted in final container according to the expected volume. Doses are stored at < -80 °C until
25 injection.

A8. Vector genome concentration by qPCR (vg/mL)

This method consists in the determination of genome-containing particles by quantitative PCR (qPCR) targeting the expression cassette of the transgene (the transgene, the
30 poly(A), the ITR or the promoter). The rAAV samples are first digested with DNase in order to eliminate non-encapsidated DNA. Afterwards, a Proteinase K treatment degrades AAV capsids and releases the viral DNA for the subsequent qPCR quantification.

The vector plasmid containing the targeted amplicon is used to generate the standard curve for the qPCR. This plasmid is linearized by restriction endonuclease digestion, aliquoted
35 and stored at ≤ -18°C to ensure stability and reproducibility of the standard curve in each assay.

The calculation of 1×10^{10} copies of amplicon is based on the following formula:

$$\text{Quantity of DNA (g)} = (660 \times \text{plasmid size (bp)} \times 1 \times 10^{10} \text{ copies}) / (6.02 \times 10^{23})$$

The relative quantity of vector genome DNA is assigned by extrapolation of copy
5 number per reaction from the standard curve containing a known amount of amplicon DNA.

The results are reported in vector genome per mL.

A9. Infectious particles concentration by ICA (ip/mL)

The Infectious Center Assay (ICA) allows the quantification of infectious particles in
10 a rAAV lot. This assay involves the infection of a permissive cell line stably carrying the AAV2
rep and *cap* sequences (HeLaRC32, ATCC CRL-2972) with increasing serial dilutions of the
rAAV vector and with wild-type Adenovirus (type 5). Thus, infectious rAAV particles entering
into the cells will be able to replicate. The replication events are then detected by
chemiluminescence and quantified following hybridization with a transgene specific probe
15 (Salvetti et al., Hum Gene Ther, 1998).

Twenty-six hours post-infection, the cells are harvested, lysed and blotted on a nylon
membrane. An hybridization is performed with a specific transgene probe labeled with
fluorescein. The signal is then amplified with an anti-fluorescein antibody coupled with Alkaline
Phosphatase and detected by chemiluminescence.

20 Finally, the replication events are quantified by dot counting after revelation on a ra-
diographic film.

A10. Vector capsid purity and identity by SDS-PAGE and Coomassie blue staining.

25 The SDS-PAGE method is an electrophoresis in a polyacrylamide gel in denaturing
conditions that separates the proteins and the other components of the sample depending on their
molecular weight and their charges.

After electrophoresis, the gel is stained with Coomassie blue (Imperial Protein Stain)
in order to quantify only the proteins in the samples. The protein identity of the rAAV is
30 confirmed by the presence of the three capsids proteins of AAV called VP1, VP2 and VP3
relating to their molecular weight (87, 72 and 62 KDa). The protein purity of rAAV is calculated
as a relative amount of capsid proteins compared to the total amount of proteins present in the
sample.

The electrophoresis gel is analyzed after Coomassie blue staining by a luminescent image analyzer with a CCD camera. Each band of protein is detected as a peak and the signal is integrated by the analyzer. The area of each peak is then calculated; a 100% pure product contains only the three VP1, VP2 and VP3 protein bands.

5

A11. Vector particles quantification by SDS-PAGE and Silver staining

The silver staining, based on the methodology of Heukeshoven and Dernick (Heukeshoven and Dernick Electrophoresis, 1985), allows the detection of proteins and nucleic acids separated by polyacrylamide gel electrophoresis.

10 The sample is loaded on the same gel as the rAAV used for the standard curve. The standard curve is generated using a referent rAAV lot, produced and characterized in the same way as the rAAV8RSM (Ayuso *et al*, Hum Gene Ther. 2014), and containing 100% full particles.

After electrophoresis, the gel is analyzed using an image analyzer with a CCD camera, and the signal of the band corresponding to VP3 is integrated for each lane. The quantity
15 of total capsids present in the sample is extrapolated from the standard curve, and reported in particles per mL.

The ratio full/total particles is based on the following calculation: **Titer in vector genome per mL/titer in particles per mL.**

For reference: Simplified method for silver staining of proteins in polyacrylamide
20 gels and the mechanism of silver staining. (Electrophoresis 6 (1985) 103-112, Heukeshoven, J. and Dernick, R.) & Manufacturing and Characterization of a recombinant associated virus type 8 reference standard material, Ayuso *et al*, (Hum Gene Ther. 2014 Oct 2).

A12. Residual host cell DNA by qPCR (albumin and E1A from HEK 293 cells)

The residual Human Host Cell DNA is determined using a real-time quantitative PCR
25 method targeting the human albumin or the adenovirus type 5 E1A genes.

A plasmid containing the specific amplicons (albumin and E1A) is used to generate the standard curve for the qPCR. This plasmid is linearized by restriction endonuclease digestion, filled and stored at $\leq -18^{\circ}\text{C}$ to ensure stability and reproducibility of the standard curve in each assay. The calculation of $1^{\circ}10$ copies of albumin gene sequence is based on the following
30 calculation:

$$\text{Quantity of DNA (g)} = (\text{660} \times \text{plasmid size (bp)} \times \text{1e10 copies}) / \text{6.02} \times \text{10e23}.$$

The relative quantity of albumin or E1A amplicon DNA is assigned by extrapolation of copy number per reaction from the standard curve containing a known amount of albumin or E1A amplicon DNA.

A second standard curve is performed with genomic DNA from HEK293 cell line, from 50 pg to 5 ng per well. This standard curve indicates the correlation between the amount of albumin or E1A copy and the corresponding amount in ng of HEK293 DNA.

B. RESULTS

Figures 3A and 3B illustrate the elution profile of a clarified supernatant containing AAV4 particles, from a step of Immunoaffinity chromatography (see figure 3A) on an AVB column. Close-up of the elution profile peak is further disclosed in figure 3B.

The purification method is achieved at a 1L scale Titration of the vector genome at each step and characterization of the final product are established according to the above-mentioned procols and summarized in the following tables:

Harvest	1.1. 10¹⁴
Depth filtration	9.2. 10 ¹³
AVB column	8.7. 10¹³
Tangential Flow Filtration	7.2. 10 ¹³
Total Yield	65%

Table 1 : titration of the vector genome from a 1L scale purification

Titer (vg/mL)	3.8 10¹²
resDNA (Alb) ng/dose*	1.2
resDNA (E1A) ng/dose*	15
Purity (Proteins)	100%

* where a dose is 1 10¹² vector genome (vg).

20

Table 2 : characterization of the final product

Conclusion : this protocol is efficient for purifying clinical-grade AAV4 preparation from clarified supernatant at a 1L scale, and in very good yields. Advantageously, the AVB column immunochromatography step only impacts moderately the amount of the vector genome.

EXAMPLE 2: Two-step chromatography for purifying AAV4 particles using a first step of Immunoaffinity chromatography, followed by a second step of anion-exchange chromatography (Alternative Protocol)

5

A. MATERIAL & METHODS

The Material & Methods section of Example 1 is also applicable to Example 2 apart from:

10 A3. Chromatographic immuno-affinity purification step (Alternative Protocol)

This step is performed with an Akta Pilot (GE Healthcare) controlled by a Unicorn software. The clarified lysate is injected at 150 cm/h linear flow rate through an AVB column (185 mL of packed matrix) equilibrated with PBS. During this step the AAV binds specifically to the column. Impurities do not bind to the column and are discarded in the flowthrough. To wash
15 the unspecific bound material, the column is then rinsed with PBS.

AAV is eluted with a PBS pH 2.0 buffer. Eluted fractions are simultaneously neutralized with 10% of Buffer Tris 1M, MgCl₂ 20 mM, CaCl 10 mM, pH 8.

Eluted fractions neutralized with Tris buffer are supplemented with 0.001% of Pluronic F68 and frozen at <-70°C, or even <-80°C, until titration of AAV by QPCR to detect the
20 amount of AAV in each fraction.

A4. 1st Alternative: CsCl gradient after immuno-affinity purification step (Alternative Protocol)

Fractions eluted from the AVB column and containing the AAV are pooled. To
25 concentrate AAV and to obtain a suitable volume for gradient, the pool of AAV fractions is submitted to a Tangential Flow Filtration against Saline Ocular Solution with 0.001% of Pluronic F68.

The diafiltered AAV is resuspended in 12 mL of 1,376 g/cm³ density CsCl, transferred to an UltraClear tube for SW41 rotor. The gradient is centrifuged at 38,000 rpm for 48
30 hours at 15°C to separate full particles from “empty” one. The enriched-full particles band is collected with a 5 or 10 mL syringe. The viral suspension is then subjected to Tangential Flow Filtration (TFF).

**A5. 2nd Alternative: CIMmultus anion-exchange chromatography after immuno-
35 affinity purification step (Alternative Protocol).**

This step is realised with an Akta Pilot (GE Healthcare) controlled by a Unicorn software.

Fractions eluted from the AVB column and containing the AAV are pooled and submitted to a TFF step for diafiltration. The goal of this TFF step is to change the buffer of
5 neutralized eluted fractions from AVB to be able to bind AAV to the CIMmultus QA column.

All the buffers used during the IEX chromatography step contain 0.001% of Pluronic F68.

The diafiltered AAV is injected through a Monolith CIMmultus QA column equilibrated with buffer 1 : Tris 20 mM, NaCl 10 mM, MgCl₂ 2 mM, CaCl₂ 1 mM, pH 9 to
10 promote the binding of AAV to the column.

At the end of the injection of the AAV containing suspension, the column is washed with buffer 1 to discard everything that could be bound unspecifically.

The elution step is a NaCl salt gradient with a very shallow slope which is the result of mixing buffer 1 (Tris 20 mM, NaCl 10 mM, MgCl₂ 2 mM, CaCl₂ 1 mM, pH 8) with buffer 2
15 (Tris 20 mM, NaCl 500 mM, MgCl₂ 2 mM, CaCl₂ 1 mM, pH 8). The slow increase of salt allows to elute gradually AAV and to separate sub populations as full and empty particles if sub populations are present.

Eluted fractions are collected in polypropylene bottles and stored at <-70°C, or even <-80°C until the TFF.

20

A6. Tangential Flow Filtration (TFF) (Alternative Protocol).

Selected fractions are pooled (around 0.5L in volume). TFF is done using a mPES hollow fiber with a cut off of 100 KDa. AAV is diafiltered and concentrated against Saline Ocular Solution (SS0) containing Pluronic F68 0.001%.

25

The pooled fractions are first slightly concentrated, diafiltered against 10 volumes of buffer and finally concentrated to reach the expected concentration. TFF allows to discard the remaining small size impurities as well as to formulate the viral vector in the right buffer for injection and to concentrate to the right level. The filtered purified and concentrated viral vector is stored at <-70°C, or even <-80°C, until titration.

30

A7. Sterile Filtration (Alternative Protocol).

AAV is diluted with SOS (Saline Ocular Solution) containing Pluronic F68 to obtain the precise concentration necessary for the injection and sterile filtered with a 0.2 µm PES filter before to be aliquoted in final container according to the expected volume. Doses are stored at <-
35 70°C, or even <-80 °C until injection.

B. RESULTS

B1 AAV4 results at 1L scale

The purification method is achieved at a 1L scale. The second step is either an IEX chromatography step or a CsCl gradient, as indicated in Table 3. Titration of the vector genome at each step is summarized in the following table :

	Affinity + CsCl gradient	Affinity + IEX
Harvest	1.1 10¹⁴	7.0 10¹³
Depth filtration	9.8 10 ¹³	6.9 10 ¹³
AVB column	1.3 10¹⁴	6.9 10¹³
Tangential Flow Filtration 1	7.8 10 ¹³	4.8 10 ¹³
2nd step (CsCl or IEX)	5.3 10 ¹³	3.4 10 ¹³
Tangential Flow Filtration 2	4.4 10 ¹³	2.7 10 ¹³
Final Product (0.2µm filtered)	3.9 10 ¹³	3.0 10 ¹³
Total Yield	35%	43%
Purity	> 90%	> 90%

Table 3 : titration of the vector genome from a 1L scale purification (Alternative protocol)

10 B2 AAV4 results at 3L scale

This is an example of a 3L scale, where AAV4 is purified with the process described above comprising a Triton X-100 extraction, a depth filtration, an affinity chromatography step, a TFF, a IEX chromatography step, a TFF and a 0.2µm sterile filtration.

In the Table 4 are summarized the results of each step, by titration of the vector genome.

The elution profile is obtained from the IEX chromatography step. Each fraction is analyzed on a silver stained SDS Polyacrylamide gel. All the fractions were loaded at the same vector genome amount. The signal of capsid proteins, more important in the first fractions, and the ratio of absorbance curves at 280 and 254 nm, show that the first fractions eluted are mostly contaminated by empty capsids.

The selected fractions mostly comprise full capsids.

The intensity of capsid proteins of each fraction was compared to a reference AAV4 preparation obtained by purification on a two-CsCl gradient process and containing > 90% of full particles.

	Affinity + IEX
Harvest	2.9 10¹⁴
Depth filtration	4.3 10 ¹⁴
Affinity AVB column	5.9 10¹⁴
Tangential Flow Filtration 1	3.7 10 ¹⁴
IEX CIM QA column	1.9 10 ¹⁴
Tangential Flow Filtration 2	1.6 10 ¹⁴
Final Product (0.2µm filtered)	1.3 10 ¹⁴
Total Yield	45%

Table 4 : titration of the vector genome from a 3L scale purification

	Affinity + IEX
Titer (vg/mL)	3.4 10¹²
Infectious Titer (pi/mL)	3.7 10 ⁶
Ratio vg/pi	9.3 10 ⁵
Purity (Proteins)	> 99%
resDNA (Alb) ng/dose*	< 0.25
resDNA (E1A) ng/dose*	21.4

* where a dose is 1*10¹² vector genome (vg)

5

Table 5 : characterization of the final product

It is to note that the ratio vg/pi is very high due to the poor permissivity of HeLa cells for AAV4. Nevertheless this ratio is comparable to the ratio of the AAV4 reference preparation.

10

Conclusion : this protocol is efficient for purifying clinical-grade AAV4 preparation from clarified supernatant at a 3L scale, and in very good yields. Advantageously, the IEX column chromatography step impacts the amount of the full particles.

EXAMPLE 3: Two-step chromatography for purifying AAVrh10 particles using
15 a first step of Immunoaffinity chromatography, followed by a second step of CsCl gradient or anion-exchange chromatography (Alternative Protocol)

A. MATERIAL & METHODS

The Material & Methods section of Example 1 is also applicable to Example 3 apart
5 from:

A1. Harvest of the cell lysate (Alternative Protocol)

Insect Sf9 cells infected with baculoviruses in a 2L bioreactor are collected at 96 +/- 5
hours post infection. The entire suspension is treated with Triton X-100 (0.5% v/v final, 27°C,
10 during 2.5 hours). A major part of the viral particle is released in the supernatant.

A3. Chromatographic immuno-affinity purification step (Alternative Protocol)

This step is performed with an Akta Pilot (GE Healthcare) controlled by a Unicorn
software. The clarified cell lysate is injected at 60 cm/h linear flow rate through an AVB column
15 (185 mL of packed matrix) equilibrated with DPBS supplemented with Calcium and Magnesium.
During this step the AAV binds specifically to the column. Impurities do not bind to the column
and are discarded in the flowthrough. To wash the unspecific bound material, the column is then
rinsed with DPBS.

AAV is eluted with a citric acid buffer 50 mM containing 300 mM NaCl, pH 3.4.
20 Eluted fractions are simultaneously neutralized with 10% of Buffer Tris 1M, MgCl₂ 20 mM, CaCl
10 mM, pH 8.5.

A4. 1st Alternative: CsCl gradient after immuno-affinity purification step (Alternative Protocol)

25 Fractions eluted from the AVB column and containing the peak of AAV (detected by
the measure of absorbance at 280 and 254 nm) are pooled. To concentrate AAV and to obtain a
suitable volume for gradient, the pool of AAV fractions is submitted to a PEG 8 000 precipitation
overnight at 4°C followed by a centrifugation at 4 000 rpm during 1 hour at 4°C. The pellet is
resuspended in 12 mL of 1,376 g/cm³ density CsCl, transferred to an UltraClear tube for SW41
30 rotor. The gradient is centrifuged at 38,000 rpm for 48 hours at 15°C to separate full particles
from “empty” one. The enriched-full particles band is collected with a 5 or 10 mL syringe. The
viral suspension is then subjected to a dialysis against DPBS with Calcium and Magnesium. The
dialysed viral suspension is supplemented with 0.001% of Pluronic F68 and frozen at <-80°C.

A5. 2nd Alternative: POROS HQ anion-exchange chromatography after immuno-affinity purification step (Alternative Protocol).

This step is realised with an Akta Explorer (GE Healthcare) controlled by a Unicorn software.

5 Fractions eluted from the AVB column and containing the AAV are pooled, supplemented with 0.001% of Pluronic F68 and stored at 4°C overnight. The day after, the AAV pool is submitted to a TFF step for diafiltration. The goal of this TFF step is to change the buffer of neutralized eluted fractions from AVB to be able to bind AAV to the POROS HQ column.

The diafiltered AAV is injected through a POROS HQ column equilibrated with
10 buffer 1 : Tris 20 mM, MgCl₂ 2 mM, CaCl₂ 1 mM, pH 8.5 to promote the binding of AAV to the column.

At the end of the injection of the AAV containing suspension, the column is washed with buffer 1 to discard everything that could be bound unspecifically.

The elution step is a Na Citrate salt gradient with a very shallow slope which is the
15 result of mixing buffer 2 (Tris 10 mM, Na Citrate 10 mM MgCl₂ 2 mM, CaCl₂ 1 mM, pH 8 with 0.001% Pluronic F68) with buffer 3 (Tris 10 mM, Na Citrate 500 mM, MgCl₂ 2 mM, CaCl₂ 1 mM, pH 8, with 0.001% Pluronic F68). The slow increase of salt (gradient to obtain 70% of buffer 3 in 40 column volumes) allows to elute gradually AAV and to separate sub populations as full and empty particles if sub populations are present.

20 Eluted fractions are collected in polypropylene tubes and stored at < -80°C until the TFF.

A6. Tangential Flow Filtration (TFF) (Alternative Protocol).

Selected fractions are pooled. TFF is done using a mPES hollow fiber with a cut off
25 of 100 KDa. AAV is diafiltered and concentrated against DPBS with Calcium and Magnesium containing Pluronic F68 0.001%.

The pooled fractions are first slightly concentrated, diafiltered against 10 volumes of buffer and finally concentrated to reach the expected concentration. The filtered purified and concentrated viral vector is stored at < -80°C until titration.

30

B. RESULTS

B1 AAVrh10 results at 1L scale

The purification method is achieved at a 1L scale. The second step is either a CsCl gradient or an IEX chromatography step, as indicated in Table 6. Titration of the vector genome at each step is summarized in the following table :

	Affinity + CsCl gradient	Affinity + IEX
Clarified cell lysate	5.1 10¹³	6.2 10¹³
AVB column	2.9 10¹³	2.5 10¹³
Tangential Flow Filtration 1	Non Applicable	1.6 10 ¹³
2nd step (CsCl or IEX)	3.1 10¹³	1.5 10¹³
Formulated Purified Product	2.5 10 ¹³	1.5 10 ¹³
Total Yield	49%	24%

5 **Table 6 : titration of the vector genome equivalent to 1L scale purification (Alternative protocol)**

10

EXAMPLE 4: Two-step chromatography for purifying AAV2 particles using a first step of Immunoaffinity chromatography, followed by a second step of CsCl gradient (Alternative Protocol)

15

A. MATERIAL & METHODS

The Material & Methods section of Example 1 is also applicable to Example 4 apart from:

20

A1. Harvest of the cell lysate (Alternative Protocol)

Insect Sf9 cells infected with baculoviruses in a 2L bioreactor are collected at 96 +/- 5 hours post infection. The entire suspension is treated with Triton X-100 (0.5% v/v final, 27°C, during 2,5 hours). An additional step of nuclease digestion is realised by adding Benzonase 5U/mL during 2 additional hours at 37°C.

25

A3. Chromatographic immuno-affinity purification step (Alternative Protocol)

This step is performed with an Akta Pilot (GE Healthcare) controlled by a Unicorn software. The clarified lysate is injected at 115 cm/h linear flow rate through an AVB column (185 mL of packed matrix) equilibrated with DPBS supplemented with Calcium and Magnesium. During this step the AAV binds specifically to the column. Impurities do not bind to the column and are discarded in the flowthrough. To wash the unspecific bound material, the column is then rinsed with DPBS.

AAV is eluted with a citric acid buffer 50 mM containing 300 mM NaCl, pH 3.4. Eluted fractions are simultaneously neutralized with 10% of Buffer Tris 1M, MgCl₂ 20 mM, CaCl 10 mM, pH 8.5.

10

A4. 1st Alternative: CsCl gradient after immuno-affinity purification step (Alternative Protocol)

Fractions eluted from the AVB column and containing the peak of AAV (detected by the measure of absorbance at 280 and 254 nm) are pooled. To concentrate AAV and to obtain a suitable volume for gradient, the pool of AAV fractions is submitted to a PEG 8 000 precipitation overnight at 4°C followed by a centrifugation at 4 000 rpm during 1 hour at 4°C. The pellet is resuspended in 12 mL of 1,376 g/cm³ density CsCl, transferred to an UltraClear tube for SW41 rotor. The gradient is centrifuged at 38,000 rpm for 48 hours at 15°C to separate full particles from “empty” one. The enriched-full particles band is collected with a 5 or 10 mL syringe. The viral suspension is then subjected to a dialysis against DPBS with Calcium and Magnesium. The dialysed viral suspension is supplemented with 0.001% of Pluronic F68 and frozen at <-80°C.

20

A7. Sterile Filtration.

AAV is filtered with a 0,2 µm PES filter before to be aliquoted in final container according to the expected volume. Doses are stored at <-80 °C.

25

B. RESULTS

B1 AAV2 results at 1L scale

The purification method is achieved at a 1L scale. The second step is a Cscl gradient, as indicated in Table 7. Titration of the vector genome at each step is summarized in the following table :

30

	Affinity + CsCl gradient
Clarified cell lysate	3.2 10¹³

	Affinity + CsCl gradient
AVB column	1.7 10¹³
2nd step (CsCl or IEX)	9.2 10¹²
Formulated Purified Product	6.6 10 ¹²
Total Yield	21%

Table 7 : titration of the vector genome equivalent to 1L scale purification (Alternative protocol)

EXAMPLE 5: Comparison between the method of the invention and a method for purifying rAAV particles comprising cation- and anion- exchange chromatographies

A. MATERIAL & METHODS

Description of the method comprising cation- and anion-exchange chromatographies

Transfected cells were harvested between 48 and 72 hours post transfection by rapping the CellStacks 5 (CS5) on all sides. The entire suspension of AAV4 was lysed by homogenization in a high pressure homogenizer (Emulsiflex C55, Avestin) at 900 +/- 50 bars. The pressure drop in this system leads to the disruption of cells, allowing the extraction of AAV particles in the supernatant and the reduction of cell debris size.

The homogenized cell lysate is depth filtered through a Millistack COHC unit (Merck Millipore) and a PES 0.5/0.2 µm filter to discard the cell debris.

The clarified cell lysate was pre-purified and concentrated with a Mustang Q (Pall Life Science) step : this anionic exchange membrane allows to bind AAV to the membrane at high flow rate and then to elute selectively AAV with NaCl discarding around 80% DNA and 50% protein impurities. The low volume of elution allows to concentrate 100 times the AAV product.

Pre-purified AAV was stored at <-80°C.

Pre-purified AAV from Mustang Q was further purified through a Capto MMC column (GE Healthcare), which is a multimodal chromatography matrix involving cationic exchange and hydrophobic interactions properties. AAV4 was eluted with a buffer containing 600 mM NH4Cl and stored at <-80°C.

The eluted AAV from Capto MMC was diluted to adjust the conductivity and pH parameters to bind on the last chromatography support, a Capto Q column (GE Healthcare). This anion exchange step is a polishing step where AAV was eluted with a Tris buffer containing 180 mM NaCl.

Eluted AAV from Capto Q was diafiltered with a 100 KDa hollow fiber (GE Healthcare) allowing to formulate and to concentrate the AAV. The diafiltered AAV was stored at <-80°C and finally 0.2 µm sterile filtered.

5 Description of the method of the invention

rAAV4 particles were purified according to the method of the invention as described in Example 1.

B. Results

10 Total Yield obtained with the method comprising cation- and anion-exchange chromatography is about 1-2%, whereas total Yield obtained with the method of the invention is at least 20%.

15 SEQUENCE LISTING

SEQ ID N°1	Expression cassette encoding human PDE6β
SEQ ID N°2	Nucleic acid coding for human PDE6-β
SEQ ID N°3	[5'ITR] inverted terminal repeat derived from AAV-2
SEQ ID N°4	[3'ITR] inverted terminal repeat derived from AAV-2
SEQ ID N°5	Promoter for Rhodopsine Kinase
SEQ ID N°6	[PolyA] signal derived from the Bovine Growth Hormone (bGH)
SEQ ID N°7	Expression cassette of human RPE65
SEQ ID N°8	Human RPE65 gene promoter
SEQ ID N°9	Nucleic acid coding for human RPE65
SEQ ID N°10	AAV-4 VP1 coding sequence (2205 nt)
SEQ ID N°11	AAV-4 VP2 coding sequence (409-2205 of VP1)
SEQ ID N°12	AAV-4 VP3 coding sequence (589-2205 of VP1)
SEQ ID N°13	AAV-5 VP1 coding sequence (2175 nt)
SEQ ID N°14	AAV-5 VP2 coding sequence (409-2175 of VP1)
SEQ ID N°15	AAV-5 VP3 coding sequence (577-2175 of VP1)

CLAIMS

1. A method for obtaining purified recombinant Adeno-Associated Virus particles (rAAV), comprising the steps of :

a) performing a depth filtration of a starting material previously obtained from cells producing rAAV particles, the said starting material being selected in a group comprising a cell lysate and a culture supernatant, whereby a rAAV-containing clarified composition is provided;

b) submitting the rAAV-containing clarified composition to an immunoaffinity purification step, whereby a first rAAV enriched composition is provided;

c) submitting the first rAAV enriched composition at least once to:

c1) a step of anion-exchange chromatography on a chromatographic support wherein elution is performed by using a salt gradient, preferably a linear salt gradient, and wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided; or

c2) a step of density gradient centrifugation, wherein the rAAV-containing fraction is collected, whereby a second rAAV enriched composition is provided;

d) submitting the second rAAV enriched composition to a step of tangential flow filtration, whereby purified recombinant Adeno-Associated Virus particles (rAAV) are provided.

2. The method according to any one of the preceding claims, wherein step b) is performed by using a chromatography support onto which antibodies or fragments thereof directed to the said rAAV particles, are immobilized.

3. The method according to the preceding claim, wherein the antibodies are monoclonal.

4. The method according to claims 2 or 3, wherein the antibodies are camelid antibodies.

5. The method according to claim 1, wherein step b) is performed using an AVB SepharoseTM chromatography (GE Healthcare) support.

6. The method according to any one of the preceding claims, wherein the at least one chromatographic support at step c1) is a monolithic chromatographic support.

7. The method according to any one of the preceding claims, wherein step d) is performed by using a filter membrane having a molecular weight cut-off value ranging from 20 kDa to 150 kDa.

8. The method according to any one of the preceding claims, wherein the rAAV particles belong to a AAV serotype selected in a group comprising AAV1, AAV2, AAV3, AAV4,

AAV5, AAV8, AAV9, AAV10, and *rhesus macaque*-derived serotypes, including AAVrh10, and mixtures thereof; in particular AAV4.

9. The method according to any one of the preceding claims, wherein the rAAV particles consist of rAAV5 particles containing DNA comprising an expression cassette encoding human PDE6-beta.

10. The method according to any one of claims 1 to 8, wherein the rAAV particles consist of rAAV4 particles containing DNA comprising an expression cassette encoding human RPE65.

11. The method according to any one of the preceding claims, wherein said purified rAAV particles are suitable for use for gene therapy.

12. A rAAV particle obtained by the method according to any one of claims 1 to 11.
13. An AAV plasmid comprising an expression cassette encoding human PDE6-beta.
14. A host cell transfected with an AAV plasmid according to the preceding claim.
15. A rAAV particle produced by the host cell according to the preceding claim.
16. The rAAV particle according to the preceding claim, for use for gene therapy.

AAV2-RK-hPDE6 β -bGHpA

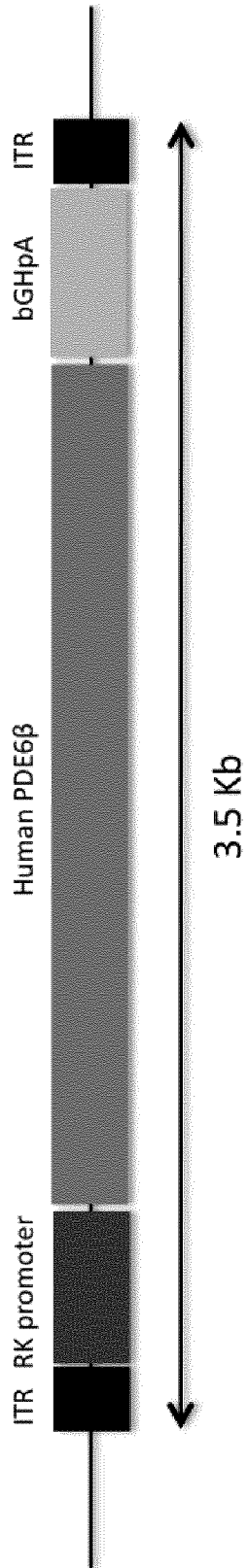


Figure 1A

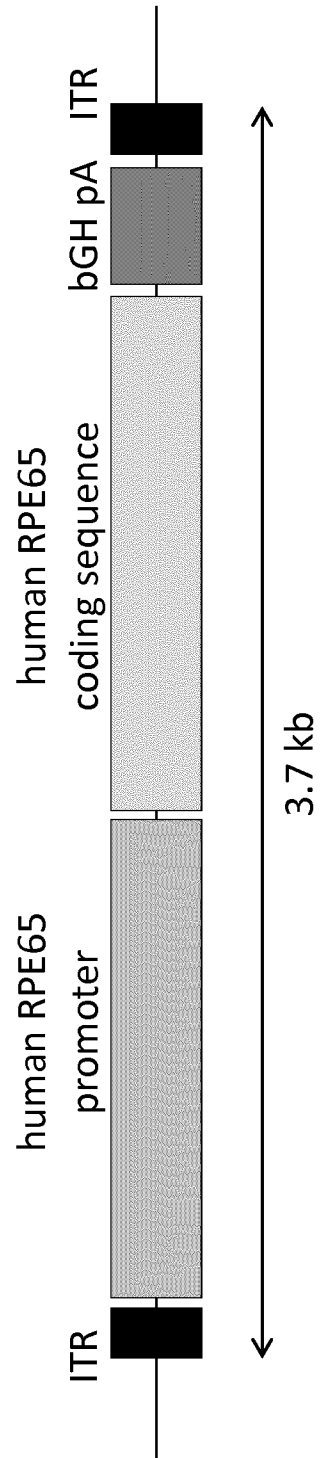


Figure 1B

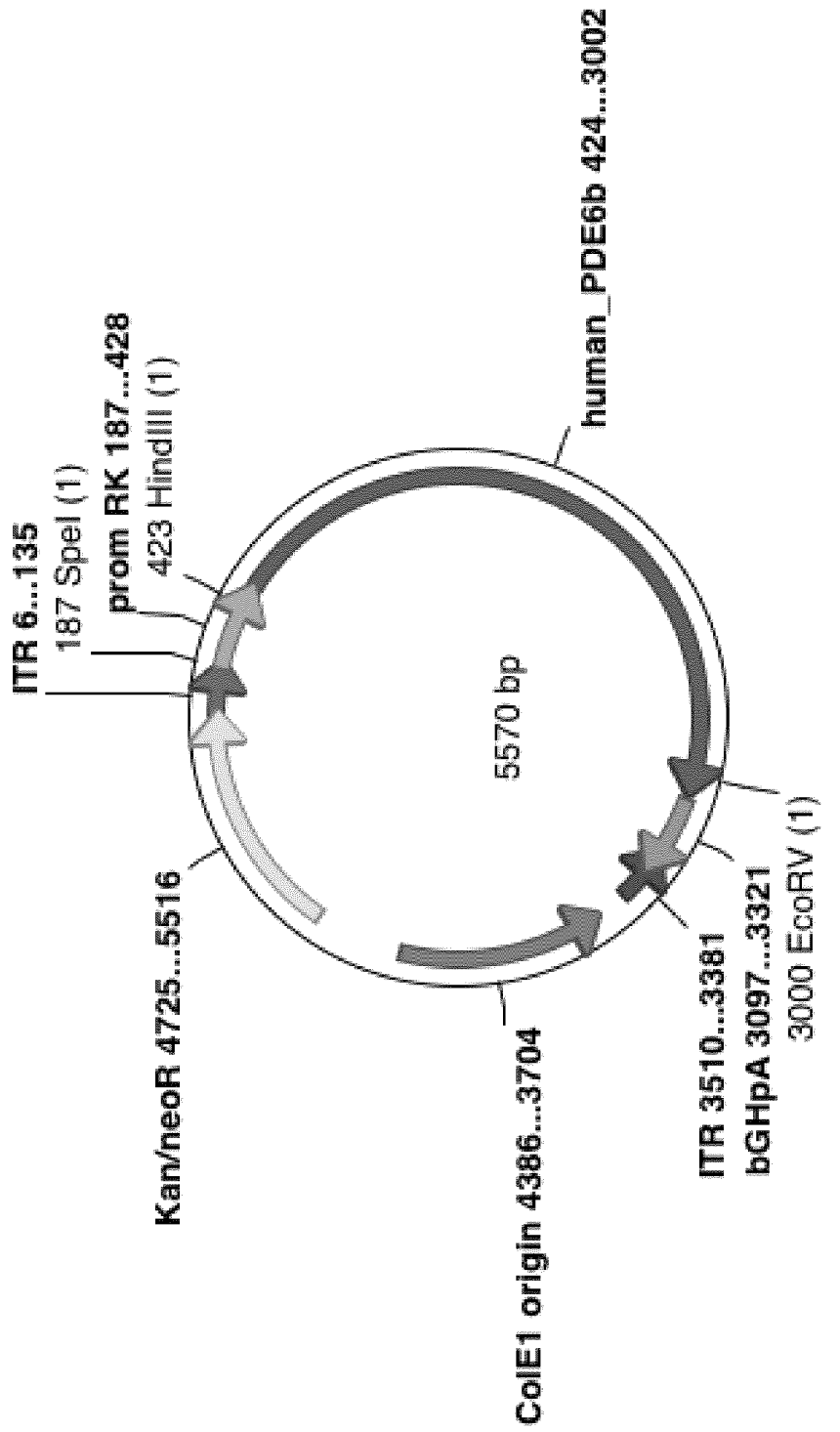


Figure 2

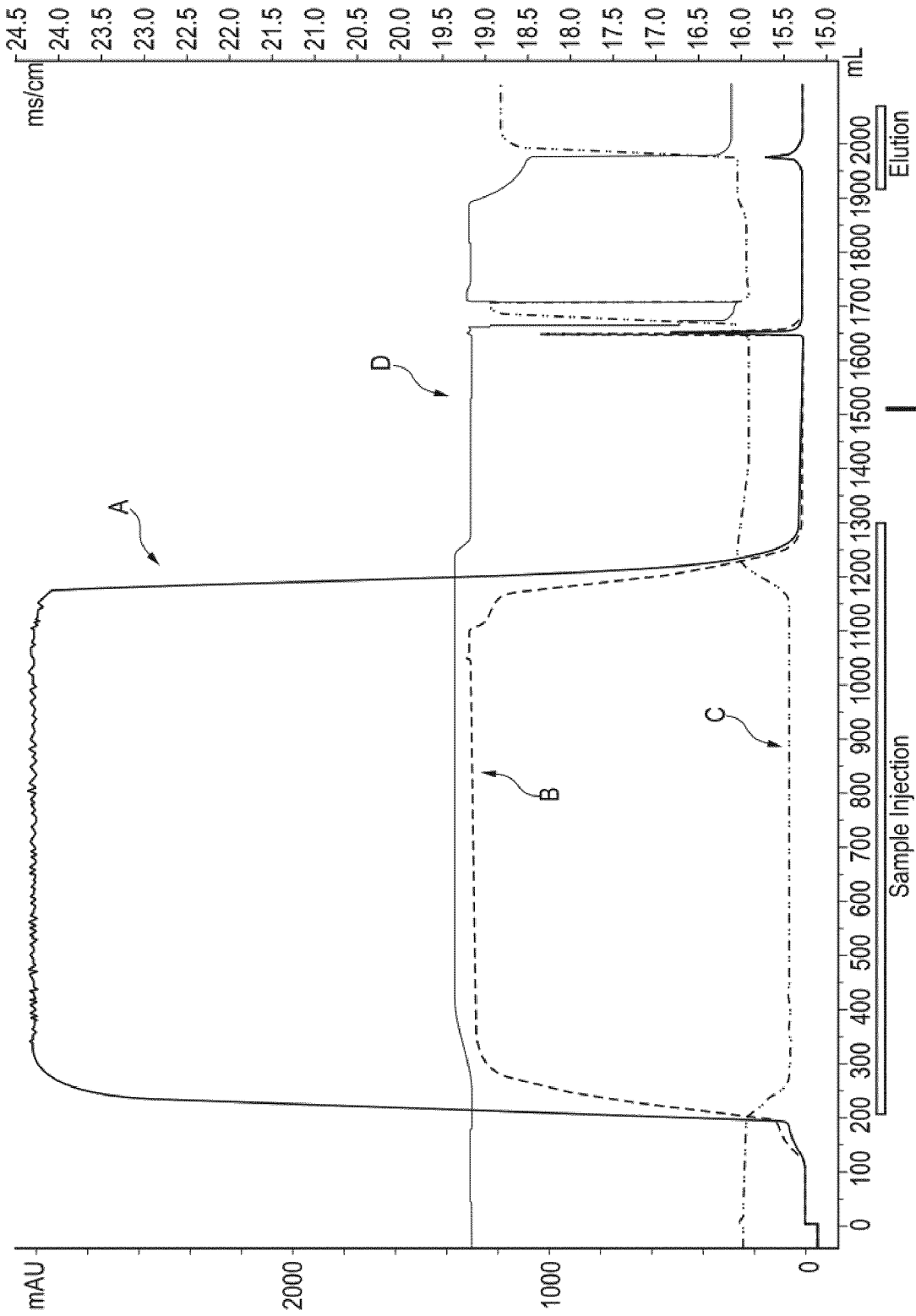


Figure 3A

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/052740

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N7/02 A61K48/00
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
C12N
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/148143 A1 (GENZYME CORP [US]; SHELDON PAULENE MCLEAN QUIGLEY [US]; GAGNON PETER S) 23 December 2010 (2010-12-23) page 1, paragraph 2 page 20, paragraph 59 - page 21, paragraph 61 page 32, paragraph 92 - page 34, paragraph 96 -----	1-12
X	WO 2011/094198 A1 (PHILADELPHIA CHILDREN HOSPITAL [US]; WRIGHT JOHN FRASER [US]; QU GUANG) 4 August 2011 (2011-08-04) cited in the application	12
A	page 1, line 15 - line 17 page 3, line 1 - line 15 figure 2 -----	1

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search 13 April 2016	Date of mailing of the international search report 04/07/2016
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Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Sitch, David
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/052740

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-12

Remark on Protest

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-12

A method for purification of AAV particles from a cell lysate or supernatant comprising: a) depth filtration, b) immunoaffinity purification, c) anion exchange chromatography, or density gradient centrifugation, followed by d) tangential flow filtration; recombinant AAV obtained by said method.

2. claims: 13-16

AAV plasmid encoding human PDE6 beta, and related subject matter

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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