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(54) **Title:** METHOD FOR DETERMINING AAV GENOMES

(57) **Abstract:** Herein is reported a method for the determination of viral genome DNA copy number in a sample, wherein the method comprises the steps of incubating the sample with proteinase K and determining the viral genome DNA copy number by digital droplet polymerase chain reaction, wherein the sample is free of DNA, which is not encapsidated within a viral particle, wherein the incubation with proteinase K is in the presence of 0.05 (w/v) % to 1.5 (w/v) % sodium dodecyl sulfate.

# Method for determining AAV genomes

The current invention is in the field of gene therapy. In more detail, herein is reported a method for determining viral genome copy numbers in process and purified samples with ddPCR, wherein the sample is incubated prior to the PCR with proteinase K in the presence of a detergent.

## Background of the Invention

With their good safety profile, high therapeutic efficacy and the possibility of target specific engineering, adeno-associated virus (AAV) particles are commonly used as gene transfer vehicles for research and in clinical approaches. For recombinant production, accurate and robust analytical methods for viral vector characterization are required. Commonly, vector genome titration is performed by droplet digital PCR (ddPCR), which is a method for absolute quantification of nucleic acids.

In literature, several pretreatment methods are reported for AAV vector genome titration, for both, real time and droplet digital PCR. Prior to absolute quantification of target sequences within the capsid, free transient plasmids and other unpackaged nucleic acids, like host cell DNA, need to be degraded with nucleases like DNase I (Zolotukhin, S., et al. 1999; Furuta-Hanawa, B., et al. 2019; Fripont, S., et al. 2019; Dobnik, D., et al. 2019; Sanmiguel J., et al. 2019). Furthermore, the capsid needs to be degraded for better vector genome accessibility. Heat inactivation of the AAV particles and the resulting denaturation of its capsid proteins are sufficient for that purpose (Wang, Y., et al. 2019). However, in most publications an additional protein digest with Proteinase K is described (Zolotukhin, S., et al. 1999; Fripont, S., et al .2019; Dobnik, D., et al. 2019; Sanmiguel J., et al. 2019). Moreover, there are various recommendations for dilution buffers for subsequent dilution series of the treated samples: Besides of nuclease-free water (Zolotukhin, S., et al. 1999), TE buffer (Furuta-Hanawa, B., et al. 2019; Wang, Y., et al. 2019) or PCR buffers (Sanmiguel J., et al. 2019), additives like the anti-surfactant Pluronic F-68 (Furuta-Hanawa, B., et al. 2019; Sanmiguel J., et al. 2019) and sheared salmon sperm (sss)DNA (Lock, M., et al. 2014; Sanmiguel J., et al. 2019) are described in literature.

Suoranta, T., et al. (Hum. Gen. Ther. 32 (2020) 1270-1279) compared extraction of AAV1, AAV2, AAV5, AAV6, AAV8, and AAV9 genomes after iodixanol gradient ultracentrifugation in phosphate buffered saline solution by heat denaturation, proteinase K treatment, and kit extraction using qPCR and ddPCR. Kit extraction, which contained proteinase K treatment in the presence of additional carrier RNA in denaturing buffer before spin-column purification, significantly increased the titers acquired for all the serotypes in both qPCR and ddPCR. Importantly, Suoranta et al. found that no study has presented conclusive data on genome availability in ddPCR.

CN 109957561 discloses a method for extracting nucleic acids from a sample, wherein the method comprises the steps of adding a lysis solution to the sample to be extracted to release nucleic acid molecules; further adding a lauroyl sarcosine sodium salt solution; further adding a mixed solution containing sodium iodide, glycogen and isopropanol, whereby a precipitate containing the nucleic acid is formed which is recovered by centrifuging the sample.

US 8,652,821 discloses a reagent mixture for purifying RNA-free DNA, which comprises a protease, an RNase, and a detergent.

US 11,028,372 discloses a scalable purification method for AAV particles of the serotype rh.10.

WO 03/104413 discloses a not further specified dot blot analysis of pseudotyped recombinant AAV virions comprising proteinase K incubation followed by phenol extraction and ethanol precipitation.

WO 2007/084773 discloses a not further specified dot blot analysis of infectious parvovirus vectors produced in insect cells.

Binny, C.J. and Nathwani, A.C. disclose an agarose gel analysis method for the determination whether the genome packaged into a scAAV vector is, as intended, a short double-stranded hairpin structure or a short ssDNA or a long, non-folded ssDNA (Meth. Mol. Biol. 891 (2012) 109-131).

US 2021/0284699 discloses a qPCR method for analyzing rAAV particles that have been purified using a method comprising the steps of (a) generating a viral particle extract comprising a plurality of rAAV provided herein, wherein the viral particle extract comprises the supernatant of lysed producer cells, or a derivative thereof; (b) contacting the viral particle extract with an ionic detergent to generate a first mixture; (c) contacting the first mixture with an acid to generate a second mixture; (d) centrifuging the second mixture to generate a supernatant; (e) filtering the supernatant with one or more filters to generate a filtrate; (f) performing one or more cycles of buffer exchange of the filtrate to a final storage buffer.

## Summary of the Invention

Herein is reported a method for the determination of viral genome DNA copy number in a sample, wherein the method comprises the steps of incubating the sample with proteinase K and determining the viral genome DNA copy number by digital droplet polymerase chain reaction, wherein the sample is free of DNA, which is not encapsidated within a viral particle, wherein the incubation with proteinase K is in the presence of a 0.05 (w/v) % to 1.5 (w/v) % sodium dodecyl sulfate.

A non-limiting number of aspects (independent subject matter) and embodiments (dependent subject matter) is

1. A method for the determination of viral genome DNA copy number in a sample, wherein the method comprises the step of

- determining the viral genome DNA copy number by digital droplet polymerase chain reaction,

wherein the sample is free of DNA, which is not encapsidated within a viral particle,

wherein the sample has not been incubated with a nuclease, and

wherein the sample has not been incubated with a protease.

2. The method according to aspect 1, wherein the nuclease is a restriction enzyme.

3. The method according to any one of aspect 1 or embodiment 2, wherein the nuclease is DNase I.

4. The method according to any one of aspect 1 or embodiments 2 to 3, wherein the protease is proteinase K.

5. A method for the determination of viral genome DNA copy number in a sample, wherein the method comprises the steps of

- incubating the sample with proteinase K,
- determining the viral genome DNA copy number by digital droplet polymerase chain reaction.

6. The method according to aspect 5, wherein the sample is a cell lysate.

7. The method according to any one of aspect 5 or embodiment 6, wherein the sample is a lysed cell sample.

8. The method according to embodiment 7, wherein the lysed cell sample has been obtained by lysing cells producing the virus with a detergent or by chemical means; optionally cell debris has been removed from the lysed cell sample.

9. The method according to any one of aspect 5 or embodiments 6 to 8, wherein the sample comprises viral particles, wherein the viral DNA genome is encapsidated, and free DNA, which is not encapsidated within a viral particle.

10. A method for the determination of viral genome DNA copy number in a sample, wherein the method comprises the steps of

- incubating the sample with proteinase K,
- determining the viral genome DNA copy number by digital droplet polymerase chain reaction,

wherein the sample is free of DNA, which is not encapsidated within a viral particle,

wherein the incubation with proteinase K is in the presence of a detergent.

11. The method according to aspect 10, wherein the detergent is sodium dodecyl sulfate.

12. The method according to any one of aspect 10 or embodiment 11, wherein the final concentration of the detergent during the incubation with proteinase K is 0.05 (w/v) % to 1.5 (w/v) %

13. The method according to any one of aspect 10 or embodiments 11 to 12, wherein the final concentration of the detergent during the incubation with proteinase K is about 0.1 (w/v) %.

14. The method according to any one of aspect 10 or embodiments 11 to 12, wherein the final concentration of the detergent during the incubation with proteinase K is about 1 (w/v) %.

15. The method according to any one of aspects 5 or aspect 10 or embodiments 6 to 9 or embodiments 11 to 14, wherein the method comprises the following steps

- incubating the sample with a nuclease to obtain a digested sample,
- incubating the digested sample with proteinase K to obtain a proteinase K incubated sample,
- determining the viral genome DNA copy number in the proteinase K incubated sample by digital droplet polymerase chain reaction.

16. The method according to embodiment 15, wherein the digested sample is diluted most 2.5-times for the incubation with proteinase K.

17. The method according to any one of embodiments 15 to 16, wherein the complete digested sample is incubated with proteinase K.

18. The method according to any one of aspects 1 or aspect 5 or aspect 10 or embodiments 2 to 4 or embodiments 6 to 9 or embodiments 11 to 17, wherein the digital droplet polymerase chain reaction comprises the following steps

a) incubating the sample at about 95 °C for 10 minutes,

b) performing a thermal cycle of incubating the sample at about 94 °C for 30 seconds followed by incubating the sample at about 60 °C for 1 minute,

c) repeating step b) for 15 to 60 times,

d) performing a final elongation step at 98 °C for 10 minutes.

19. The method according to embodiment 18, wherein in steps a) and d) are performed with a temperature ramping of 2 °C.

20. The method according to any one of aspects 1 or aspect 5 or aspect 10 or embodiments 2 to 4 or embodiments 6 to 9 or embodiments 11 to 19, wherein the sample is maintained at temperatures of 95 °C or lower.

21. The method according to any one of aspects 1 or aspect 5 or aspect 10 or embodiments 2 to 4 or embodiments 6 to 9 or embodiments 11 to 19, wherein the sample is not exposed to a temperature above 95 °C.

22. The method according to any one of aspects 1 or aspect 5 or aspect 10 or embodiments 2 to 4 or embodiments 6 to 9 or embodiments 11 to 19, wherein the method is performed at a temperature of at most 95 °C except for the final elongation step of the digital droplet polymerase chain reaction.

23. The method according to any one of aspect 5 or aspect 10 or embodiments 2 to 4 or embodiments 6 to 9 or embodiments 11 to 22, wherein the total amount of proteinase K employed in the incubation is 1 mU to 50 mU.

24. The method according to any one of aspect 5 or aspect 10 or embodiments 2 to 4 or embodiments 6 to 9 or embodiments 11 to 23, wherein the total amount of proteinase K employed in the incubation is 10 mU to 40 mU.

25. The method according to any one of aspect 5 or aspect 10 or embodiments 2 to 4 or embodiments 6 to 9 or embodiments 11 to 24, wherein the total amount of proteinase K employed in the incubation is 15 mU to 35 mU.

26. The method according to any one of aspect 5 or aspect 10 or embodiments 2 to 4 or embodiments 6 to 9 or embodiments 11 to 25, wherein the total amount of proteinase K employed in the incubation is 20 mU to 32 mU.

27. The method according to any one of aspect 5 or aspect 10 or embodiments 2 to 4 or embodiments 6 to 9 or embodiments 11 to 26, wherein the total amount of proteinase K employed in the incubation is about 20 mU.

28. The method according to any one of aspect 5 or aspect 10 or embodiments 2 to 4 or embodiments 6 to 9 or embodiments 11 to 26, wherein the total amount of proteinase K employed in the incubation is about 32 mU.

29. The method according to any one of aspect 1 or aspect 5 or aspect 10 or embodiments 2 to 4 or embodiments 6 to 9 or embodiments 11 to 28, wherein the determining of the viral genome DNA copy number is a quantifying of the viral genome DNA copy number.

30. The method according to any one of aspect 1 or aspect 5 or aspect 10 or embodiments 2 to 4 or embodiments 6 to 9 or embodiments 11 to 29, wherein the volume of the sample is about 10 µL.

31. The method according to any one of aspect 5 or aspect 10 or embodiments 2 to 4 or embodiments 6 to 9 or embodiments 11 to 30, wherein the incubating with proteinase K is in a total volume of 100 µL.

32. The method according to any one of embodiments 15 to 31, wherein the nuclease is DNase I.

33. The method according to any one of embodiments 15 to 32, wherein the total amount of nuclease employed in the incubation is about 5 U.

34. The method according to any one of embodiments 15 to 33, wherein the incubating with the nuclease is in a total volume of 50 µL.

35. The method according to any one of embodiments 15 to 34, wherein the incubating with the nuclease is at final concentrations of 40 mM Tris*HCl, 10 mM $MgSO_4$, 1 mM $CaCl_2$ at a pH value of about 8.

36. The method according to any one of embodiments 15 to 35, wherein the incubating with the nuclease is at 37 °C for 30 minutes.

37. The method according to embodiment 36, wherein the incubating is followed by an inactivating of the nuclease at 95 °C for 15 minutes.

38. The method according to any one of embodiments 15 to 37, wherein the incubating with proteinase K is at final concentrations of 20 mM Tris*HCl, 1 mM EDTA, 100 mM NaCl at a pH value of about 8.

39. The method according to any one of embodiments 15 to 37, wherein the incubating with proteinase K is at final concentrations of 20 mM Tris*HCl, 1 mM EDTA, 100 mM NaCl at a pH value of about 8.

40. The method according to any one of embodiments 15 to 37, wherein the incubating with proteinase K is at final concentrations of 20 mM Tris*HCl, 1 mM EDTA, 100 mM NaCl, 1 (w/v) % sodium dodecyl sulfate at a pH value of about 8.

41. The method according to any one of embodiments 15 to 37, wherein the incubating with proteinase K is at final concentrations of 30 mM Tris*HCl, 5 mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.5 mM EDTA, 50 mM NaCl, 0.1 (w/v) % sodium dodecyl sulfate at a pH value of about 8.

42. The method according to any one of embodiments 15 to 41, wherein the incubating with proteinase K is at 50 °C for 60 minutes.

43. The method according to embodiment 42, wherein the incubating is followed by an inactivating of the protease at 95 °C for 15 minutes.

44. The method according to any one of aspect 1 or aspect 5 or aspect 10 or embodiments 2 to 4 or embodiments 7 to 9 or embodiments 11 to 43, wherein the volume of the sample is an affinity chromatography purified cell lysate.

45. The method according to any one of aspect 1 or aspect 5 or aspect 10 or embodiments 2 to 4 or embodiments 6 to 9 or embodiments 11 to 43, wherein the method is performed in the absence of a precipitation step or/and glycogen.

**Detailed Description of the Invention**

The current invention is based, at least in part, on the finding that for AAV genome copy number determination the sample has to be incubated prior to the PCR with proteinase K in case of crude cell lysate samples comprising AAV particles and additionally comprising not virally encapsidated DNA. The presence of a detergent can further improve the determination.

The current invention is based, at least in part, on the finding that for AAV genome copy number determination the sample has to be incubated prior to the PCR with proteinase K in the presence of a detergent in case of purified samples comprising AAV particles and which are essentially free of not virally encapsidated DNA. Without being bound to this theory, it is assumed that the detergent prevents the formation of aggregates of protein fragments and viral genomic DNA, which prevent the later amplification by polymerase chain reaction, and thereby results in an underestimation of the viral genome copy number in the sample.

The current invention is based, at least in part, on the finding that for AAV genome copy number determination after sequential treatment with a nuclease and a protease the protease treatment has to be carried out in the presence of a detergent. Without being bound to this theory it is assumed that the detergent prevents the formation of aggregates of protein fragments and viral genomic DNA which prevent the later amplification by polymerase chain reaction and thereby underestimation of the viral genome copy number in the sample.

The current invention is further based, at least in part, on the finding that for purified samples comprising AAV particles and which are essentially free of not virally

encapsidated DNA the determination of the AAV genome copy number has to be done either without nuclease and proteinase pre-treatment or with proteinase K incubation in the presence of a detergent prior to the polymerase chain reaction. Without being bound by this theory, it is assumed that the incubation of a highly purified sample, i.e. not comprising essential amounts of not virally encapsidated DNA, with a nuclease alone or in combination with a protease in the absence of a detergent results in aggregation and thereby artificially lowered AAV genome copy numbers.

The current invention is further based, at least in part, on the finding that for purified samples comprising AAV particles and which are essentially free of not virally encapsidated DNA the determination of the AAV genome copy number has to be done without a thermal denaturation step at a temperature higher than 95 °C. Without being bound by this theory, it is assumed that the thermal treatment of a highly purified sample, i.e. not comprising essential amounts of not virally encapsidated DNA, at temperatures above 95 °C results in aggregation and thereby artificially lowered AAV genome copy numbers.

The invention is further based, at least in part, that low amounts of proteinase K in the presence of a detergent are sufficient to make substantially all AAV genomes in a sample accessible for polymerase chain reaction and thereby determination/quantification. Without being bound by this theory, it is assumed that high concentrations of proteinase K interfere with the polymerase chain reaction and that by using substantially reduced, i.e. low, amounts of proteinase K results in an improved polymerase chain reaction by reducing PCR inhibition. Especially the inhibition of the polymerase chain reaction by proteinaceous substances in cell lysates of AAV producing cells can be reduced or even eliminated by the proteinase K incubation in the presence of a detergent.

The invention is further based, at least in part, on the finding, that for the sequential incubation with a nuclease and a protease of crude cell lysate samples the sample shall not be diluted after the nuclease incubation and the total volume of the incubation mixture shall be used in the protease incubation step.

In general, the more pre-treatment steps are required prior to the determination of the copy number of AAV genomes in a ddPCR method the higher is the likelihood of contamination.

**DEFINITIONS**

Useful methods and techniques for carrying out the current invention are described in e.g. Ausubel, F.M. (ed.), Current Protocols in Molecular Biology, Volumes I to III (1997); Glover, N.D., and Hames, B.D., ed., DNA Cloning: A Practical Approach, Volumes I and II (1985), Oxford University Press; Freshney, R.I. (ed.), Animal Cell Culture – a practical approach, IRL Press Limited (1986); Watson, J.D., et al., Recombinant DNA, Second Edition, CHSL Press (1992); Winnacker, E.L., From Genes to Clones; N.Y., VCH Publishers (1987); Celis, J., ed., Cell Biology, Second Edition, Academic Press (1998); Freshney, R.I., Culture of Animal Cells: A Manual of Basic Technique, second edition, Alan R. Liss, Inc., N.Y. (1987).

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and equivalents thereof known to those skilled in the art, and so forth. As well, the terms "a" (or "an"), "one or more" and "at least one" can be used interchangeably herein. It is also to be noted that the terms "comprising", "including", and "having" can be used interchangeably.

The term "AAV helper functions" denotes AAV-derived coding sequences (proteins) which can be expressed to provide AAV gene products and AAV particles that, in turn, function in trans for productive AAV replication and packaging. Thus, AAV helper functions include AAV open reading frames (ORFs), including rep and cap and others such as AAP for certain AAV serotypes. The rep gene 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 gene expression products (capsids) supply necessary packaging functions. AAV

helper functions are used to complement AAV functions in trans that are missing from AAV vector genomes.

The term “about” denotes a range of +/- 20 % of the thereafter following numerical value. In certain embodiments, the term about denotes a range of +/- 10 % of the thereafter following numerical value. In certain embodiments, the term about denotes a range of +/- 5 % of the thereafter following numerical value.

The term “comprising” also encompasses the term “consisting of”.

The terms "empty capsid" and "empty particle", refer to an AAV particle that has an AAV protein shell but that lacks in whole or part a nucleic acid that encodes a protein or is transcribed into a transcript of interest flanked by AAV ITRs, i.e. a vector. Accordingly, the empty capsid does not function to transfer a nucleic acid that encodes a protein or is transcribed into a transcript of interest into the host cell.

The term “endogenous” denotes that something is naturally occurring within a cell; naturally produced by a cell; likewise, an “endogenous gene locus/cell-endogenous gene locus” is a naturally occurring locus in a cell.

As used herein, the term “exogenous” indicates that a nucleotide sequence does not originate from a specific cell and is introduced into said cell by DNA delivery methods, e.g., by transfection, electroporation, or transduction by viral vectors. Thus, an exogenous nucleotide sequence is an artificial sequence wherein the artificiality can originate, e.g., from the combination of subsequences of different origin (e.g. a combination of a recombinase recognition sequence with an SV40 promoter and a coding sequence of green fluorescent protein is an artificial nucleic acid) or from the deletion of parts of a sequence (e.g. a sequence coding only the extracellular domain of a membrane-bound receptor or a cDNA) or the mutation of nucleobases. The term “endogenous” refers to a nucleotide sequence originating from a cell. An “exogenous” nucleotide sequence can have an “endogenous” counterpart that is identical in base compositions, but where the sequence is becoming an “exogenous” sequence by its introduction into the cell, e.g., via recombinant DNA technology.

An "isolated" composition is one, which has been separated from one or more component(s) of its natural environment. In some embodiments, a composition is purified to greater than 95 % or 99 % purity as determined by, for example, electrophoretic (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis, CE-SDS) or chromatographic (e.g., size exclusion chromatography or ion exchange or reverse phase HPLC). For review of methods for assessment of e.g. antibody purity, see, e.g., Flatman, S. et al., J. Chrom. B 848 (2007) 79-87.

An "isolated" nucleic acid refers to a nucleic acid molecule that has been separated from one or more component(s) of its natural environment. An isolated nucleic acid includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

An "isolated" polypeptide or antibody refers to a polypeptide molecule or antibody molecule that has been separated from one or more component(s) of its natural environment.

The term "mammalian cell comprising an exogenous nucleotide sequence" encompasses cells into which one or more exogenous nucleic acid(s) have been introduced, including the progeny of such cells. These can be the starting point for further genetic modification. Thus, the term “a mammalian cell comprising an exogenous nucleotide sequence” encompasses a cell comprising an exogenous nucleotide sequence integrated at a single site within a locus of the genome of said mammalian cell, wherein the exogenous nucleotide sequence comprises at least a first and a second recombination recognition site (these recombination recognition sites are different) flanking at least one first selection marker. In certain embodiments, the mammalian cell comprising an exogenous nucleotide sequence is a cell comprising an exogenous nucleotide sequence integrated at a single site within a locus of the genome of said cell, wherein the exogenous nucleotide sequence comprises a first and a second recombination recognition sequence flanking at least one first selection marker, and a third recombination recognition sequence located

between the first and the second recombination recognition sequence, and all the recombination recognition sequences are different.

A “mammalian cell comprising an exogenous nucleotide sequence” and a “recombinant cell” are both "transfected cells". This term includes the primary transfected cell as well as progeny derived therefrom without regard to the number of passages. Progeny may, e.g., not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that has the same function or biological activity as in the originally transfected cell are encompassed.

The "nucleic acids encoding AAV packaging proteins" refer generally to one or more nucleic acid molecule(s) that includes nucleotide sequences providing AAV functions deleted from an AAV vector, which is(are) to be used to produce a transduction competent recombinant AAV particle. The nucleic acids encoding AAV packaging proteins are commonly used to provide expression of AAV rep and/or cap genes to complement missing AAV functions that are necessary for AAV replication; however, the nucleic acid constructs lack AAV ITRs and can neither replicate nor package themselves. Nucleic acids encoding AAV packaging proteins can be in the form of a plasmid, phage, transposon, cosmid, virus, or particle. A number of nucleic acid constructs have been described, such as the commonly used plasmids pAAV/Ad and pIM29+45, which encode both rep and cap gene expression products. See, e.g., Samulski et al., J. Virol. 63 (1989) 3822-3828; and McCarty et al., J. Virol. 65 (1991) 2936-2945. A number of plasmids have been described which encode rep and/or cap gene expression products (e.g., US 5,139,941 and US 6,376,237). Any one of these nucleic acids encoding AAV packaging proteins can comprise the DNA element or nucleic acid according to the invention.

The term "nucleic acids encoding helper proteins" refers generally to one or more nucleic acid molecule(s) that include nucleotide sequences encoding proteins and/or RNA molecules that provide adenoviral helper function(s). A plasmid with nucleic acid(s) encoding helper protein(s) can be transfected into a suitable cell, wherein the plasmid is then capable of supporting AAV particle production in said cell. Any one of these nucleic acids encoding helper proteins can comprise the DNA element or

nucleic acid according to the invention. Expressly excluded from the term are infectious viral particles, as they exist in nature, such as adenovirus, herpesvirus or vaccinia virus particles.

As used herein, the term "operably linked" refers to a juxtaposition of two or more components, wherein the components are in a relationship permitting them to function in their intended manner. For example, a promoter and/or an enhancer is operably linked to a coding sequence/open reading frame/gene if the promoter and/or enhancer acts to modulate the transcription of the coding sequence/open reading frame/gene. In certain embodiments, DNA sequences that are "operably linked" are contiguous. In certain embodiments, e.g., when it is necessary to join two protein encoding regions, such as a secretory leader and a polypeptide, the sequences are contiguous and in the same reading frame. In certain embodiments, an operably linked promoter is located upstream of the coding sequence/open reading frame/gene and can be adjacent to it. In certain embodiments, e.g., with respect to enhancer sequences modulating the expression of a coding sequence/open reading frame/gene, the two components can be operably linked although not adjacent. An enhancer is operably linked to a coding sequence/open reading frame/gene if the enhancer increases transcription of the coding sequence/open reading frame/gene. Operably linked enhancers can be located upstream, within, or downstream of coding sequences/open reading frames/genes and can be located at a considerable distance from the promoter of the coding sequence/open reading frame/gene.

The term "packaging proteins" refers to non-AAV derived viral and/or cellular functions upon which AAV is dependent for its replication. Thus, the term captures proteins and RNAs that are required in AAV replication, including those moieties involved in activation of AAV gene transcription, stage specific AAV mRNA splicing, AAV DNA replication, synthesis of Cap expression products and AAV capsid assembly. Viral-based accessory functions can be derived from any of the known helper viruses such as adenovirus, herpesvirus (other than herpes simplex virus type-I) and vaccinia virus.

As used herein, "AAV packaging proteins" refer to AAV-derived sequences, which function in trans for productive AAV replication. Thus, AAV packaging proteins are encoded by the major AAV open reading frames (ORFs), rep and cap. The rep proteins 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 (capsid) proteins supply necessary packaging functions. AAV packaging proteins are used herein to complement AAV functions in trans that are missing from AAV vectors.

A "plasmid" is a form of nucleic acid or polynucleotide that typically has additional elements for expression (e.g., transcription, replication, etc.) or propagation (replication) of the plasmid. A plasmid as used herein also can be used to reference such nucleic acid or polynucleotide sequences. Accordingly, in all aspects the inventive compositions and methods are applicable to nucleic acids, polynucleotides, as well as plasmids, e.g., for producing cells that produce viral (e.g., AAV) vectors, to produce viral (e.g., AAV) particles, to produce cell culture medium that comprises viral (e.g., AAV) particles, etc.

The term “recombinant cell” as used herein denotes a cell after final genetic modification, such as, e.g., a cell expressing a polypeptide of interest or producing a rAAV particle of interest and that can be used for the production of said polypeptide of interest or rAAV particle of interest at any scale. For example, “a mammalian cell comprising an exogenous nucleotide sequence” that has been subjected to recombinase mediated cassette exchange (RMCE) whereby the coding sequences for a polypeptide of interest have been introduced into the genome of the host cell is a “recombinant cell”. Although the cell is still capable of performing further RMCE reactions, it is not intended to do so.

A “recombinant AAV vector" is derived from the wild-type genome of a virus, such as AAV by using molecular biological methods to remove the wild type genome from the virus (e.g., AAV), and replacing it with a non-native nucleic acid, such as a nucleic acid transcribed into a transcript or that encodes a protein. Typically, for

AAV one or both inverted terminal repeat (ITR) sequences of the wild-type AAV genome are retained in the recombinant AAV vector. A "recombinant" AAV vector is distinguished from a wild-type viral AAV genome, since all or a part of the viral genome has been replaced with a non-native (i.e., heterologous) sequence with respect to the viral genomic nucleic acid. Incorporation of a non-native sequence therefore defines the viral vector (e.g., AAV) as a "recombinant" vector, which in the case of AAV can be referred to as a "rAAV vector."

A recombinant vector (e.g., AAV) sequence can be packaged - referred to herein as a "particle" - for subsequent infection (transduction) of a cell, ex vivo, in vitro or in vivo. Where a recombinant vector sequence is encapsulated or packaged into an AAV particle, the particle can also be referred to as a "rAAV". Such particles include proteins that encapsulate or package the vector genome. Particular examples include viral envelope proteins, and in the case of AAV, capsid proteins, such as AAV VP1, VP2 and VP3.

As used herein, the term “selection marker” denotes a gene that allows cells carrying the gene to be specifically selected for or against, in the presence of a corresponding selection agent. For example, but not by way of limitation, a selection marker can allow the host cell transformed with the selection marker gene to be positively selected for in the presence of the respective selection agent (selective cultivation conditions); a non-transformed host cell would not be capable of growing or surviving under the selective cultivation conditions. Selection markers can be positive, negative or bi-functional. Positive selection markers can allow selection for cells carrying the marker, whereas negative selection markers can allow cells carrying the marker to be selectively eliminated. A selection marker can confer resistance to a drug or compensate for a metabolic or catabolic defect in the host cell. In prokaryotic cells, amongst others, genes conferring resistance against ampicillin, tetracycline, kanamycin or chloramphenicol can be used. Resistance genes useful as selection markers in eukaryotic cells include, but are not limited to, genes for aminoglycoside phosphotransferase (APH) (e.g., hygromycin phosphotransferase (HYG), neomycin and G418 APH), dihydrofolate reductase (DHFR), thymidine kinase (TK), glutamine synthetase (GS), asparagine synthetase, tryptophan

synthetase (indole), histidinol dehydrogenase (histidinol D), and genes encoding resistance to puromycin, blasticidin, bleomycin, phleomycin, chloramphenicol, Zeocin, and mycophenolic acid. Further marker genes are described in WO 92/08796 and WO 94/28143.

Beyond facilitating a selection in the presence of a corresponding selection agent, a selection marker can alternatively be a molecule normally not present in the cell, e.g., green fluorescent protein (GFP), enhanced GFP (eGFP), synthetic GFP, yellow fluorescent protein (YFP), enhanced YFP (eYFP), cyan fluorescent protein (CFP), mPlum, mCherry, tdTomato, mStrawberry, J-red, DsRed-monomer, mOrange, mKO, mCitrine, Venus, YPet, Emerald, CyPet, mCFPm, Cerulean, and T-Sapphire. Cells expressing such a molecule can be distinguished from cells not harboring this gene, e.g., by the detection or absence, respectively, of the fluorescence emitted by the encoded polypeptide.

As used herein, the term "serotype" is a distinction based on AAV capsids being serologically distinct. Serologic distinctiveness is determined on the basis of the lack of cross-reactivity between antibodies to one AAV as compared to another AAV. Such cross-reactivity differences are usually due to differences in capsid protein sequences/antigenic determinants (e.g., due to VP1, VP2, and/or VP3 sequence differences of AAV serotypes). Despite the possibility that AAV variants including capsid variants may not be serologically distinct from a reference AAV or other AAV serotype, they differ by at least one nucleotide or amino acid residue compared to the reference or other AAV serotype.

Under the traditional definition, a serotype means that the virus of interest has been tested against serum specific for all existing and characterized serotypes for neutralizing activity and no antibodies have been found that neutralize the virus of interest. As more naturally occurring virus isolates are discovered and/or capsid mutants generated, there may or may not be serological differences with any of the currently existing serotypes. Thus, in cases where the new virus (e.g., AAV) has no serological difference, this new virus (e.g., AAV) would be a subgroup or variant of the corresponding serotype. In many cases, serology testing for neutralizing activity

has yet to be performed on mutant viruses with capsid sequence modifications to determine if they are of another serotype according to the traditional definition of serotype. Accordingly, for the sake of convenience and to avoid repetition, the term "serotype" broadly refers to both serologically distinct viruses (e.g., AAV) as well as viruses (e.g., AAV) that are not serologically distinct that may be within a subgroup or a variant of a given serotype.

The terms "transduce" and "transfect" refer to introduction of a molecule such as a nucleic acid (viral vector, plasmid) into a cell. A cell has been "transduced" or "transfected" when exogenous nucleic acid has been introduced inside the cell membrane. Accordingly, a "transduced cell" is a cell into which a "nucleic acid" or "polynucleotide" has been introduced, or a progeny thereof in which an exogenous nucleic acid has been introduced. In particular embodiments, a "transduced" cell (e.g., in a mammal, such as a cell or tissue or organ cell) has a genetic change following incorporation of an exogenous molecule, for example, a nucleic acid (e.g., a transgene). A "transduced" cell(s) can be propagated and the introduced nucleic acid transcribed and/or protein expressed.

In a "transduced" or "transfected" cell, the nucleic acid (viral vector, plasmid) may or may not be integrated into genomic nucleic acid. If an introduced nucleic acid becomes integrated into the nucleic acid (genomic DNA) of the recipient cell or organism, it can be stably maintained in that cell or organism and further passed on to or inherited by progeny cells or organisms of the recipient cell or organism. Finally, the introduced nucleic acid may exist in the recipient cell or host organism extrachromosomally, or only transiently. A number of techniques are known, see, e.g., Graham et al., Virology 52 (1973) 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., Gene 13 (1981) 197. Such techniques can be used to introduce one or more exogenous DNA moieties into suitable host cells.

The term "transgene" is used herein to conveniently refer to a nucleic acid that is intended or has been introduced into a cell or organism. Transgenes include any

nucleic acid, such as a gene that is transcribed into a transcript or that encodes a polypeptide or protein.

A “vector" refers to the portion of the recombinant plasmid sequence ultimately packaged or encapsulated, either directly or in form of a single strand or RNA, to form a viral (e.g., AAV) particle. In cases recombinant plasmids are used to construct or manufacture recombinant viral particles, the viral particle does not include the portion of the "plasmid" that does not correspond to the vector sequence of the recombinant plasmid. This non-vector portion of the recombinant plasmid is referred to as the "plasmid backbone", which is important for cloning and amplification of the plasmid, a process that is needed for propagation and recombinant virus production, but is not itself packaged or encapsulated into virus (e.g., AAV) particles. Thus, a “vector" refers to the nucleic acid that is packaged or encapsulated by a virus particle (e.g., AAV).

**RECOMBINANT CELL**

Generally, for efficient as well as large-scale production of a proteinaceous compound of interest, such as e.g. a rAAV particle or a therapeutic polypeptide, a cell expressing and, if possible, also secreting said proteinaceous compound is required. Such a cell is termed “recombinant cell” or “recombinant production cell”.

For the generation of a “recombinant production cell” a suitable mammalian cell is transfected with the required nucleic acid sequences encoding said proteinaceous compound of interest. Transfection of additional helper polypeptides may be necessary.

For the generation of stable recombinant production cells, a second step follows, wherein a single cell stably expressing the proteinaceous compound of interest is selected. This can be done, e.g., based on the co-expression of a selection marker, which had been co-transfected with the nucleic acid sequences encoding the proteinaceous compound of interest, or be the expression of the proteinaceous compound itself.

For expression of a coding sequence, i.e. of an open reading frame, additional regulatory elements, such as a promoter and polyadenylation signal (sequence), are necessary. Thus, an open reading frame is operably linked to said additional regulatory elements for transcription. This can be achieved by integrating it into a so-called expression cassette. The minimal regulatory elements required for an expression cassette to be functional in a mammalian cell are a promoter functional in said mammalian cell, which is located upstream, i.e. 5', to the open reading frame, and a polyadenylation signal (sequence) functional in said mammalian cell, which is located downstream, i.e. 3', to the open reading frame. Additionally a terminator sequence may be present 3' to the polyadenylation signal (sequence). For expression, the promoter, the open reading frame/coding region and the polyadenylation signal sequence have to be arranged in an operably linked form.

Likewise, a nucleic acid that is transcribed into a non-protein coding RNA is called "RNA gene". Also for expression of an RNA gene, additional regulatory elements, such as a promoter and a transcription termination signal or polyadenylation signal (sequence), are necessary. The nature and localization of such elements depends on the RNA polymerase that is intended to drive the expression of the RNA gene. Thus, an RNA gene is normally also integrated into an expression cassette.

In case the proteinaceous compound of interest is an AAV particle, which is composed of different (monomeric) capsid polypeptides and a single stranded DNA molecule and which in addition requires other adenoviral helper functions for production and encapsulation, a multitude of expression cassettes differing in the contained open reading frames/coding sequences are required. In this case, at least an expression cassette for each of the transgene, the different polypeptides forming the capsid of the AAV vector, for the required helper functions as well as the VA RNA are required. Thus, individual expression cassettes for each of the helper E1A, E1B, E2A, E4orf6, the VA RNA, the rep and cap genes are required.

As outlined in the previous paragraphs, the more complex the proteinaceous compound of interest or the higher the number of additional required helper polypeptides and/or RNAs, respectively, the higher is the number of required,

different expression cassettes. Inherently with the number of expression cassettes, also the total size of the nucleic acid. However, there is a practical upper limit to the size of a nucleic acid that can be transferred, which is in the range of about 15 kbps (kilo-base-pairs). Above this limit handling and processing efficiency profoundly drops. This issue can be addressed by using two or more separate plasmids. Thereby the different expression cassettes are allocated to different plasmids, whereby each plasmid comprises only some of the expression cassettes.

In certain embodiments of all aspects and embodiments, each of the expression cassettes comprise in 5'-to-3' direction a promoter, an open reading frame/coding sequence or an RNA gene and a polyadenylation signal sequence, and/or a terminator sequence. In certain embodiments, the open reading frame encodes a polypeptide and the expression cassette comprises a polyadenylation signal sequence with or without additional terminator sequence. In certain embodiments, the expression cassette comprises a RNA gene, the promoter is a type 2 Pol III promoter and a polyadenylation signal sequence or a polyU terminator is present. See, e.g., Song et al. Biochemical and Biophysical Research Communications 323 (2004) 573–578. In certain embodiments, the expression cassette comprises a RNA gene, the promoter is a type 2 Pol III promoter and a polyU terminator sequence.

In certain embodiments of all aspects and embodiments, the open reading frame encodes a polypeptide, the promoter is the human CMV promoter with or without intron A, the polyadenylation signal sequence is the bGH (bovine growth hormone) polyA signal sequence and the terminator is the hGT (human gastrin terminator).

In certain embodiments of all aspects and embodiments the promoter is the human CMV promoter with intron A, the polyadenylation signal sequence is the bGH polyadenylation signal sequence and the terminator is the hGT, except for the expression cassette of the RNA gene and the expression cassette of the selection marker, wherein for the selection marker the promoter is the SV40 promoter and the polyadenylation signal sequence is the SV40 polyadenylation signal sequence and a terminator is absent, and wherein for the RNA gene the promoter is a wild-type type 2 polymerase III promoter and the terminator is a polymerase II or III terminator.

## ADENO-ASSOCIATED VIRUS (AAV)

For a general review of AAVs and of the adenovirus or herpes helper functions see, Berns and Bohensky, Advances in Virus Research, Academic Press., 32 (1987) 243-306. The genome of AAV is described in Srivastava et al., J. Virol., 45 (1983) 555-564. In US 4,797,368 design considerations for constructing recombinant AAV vectors are described (see also WO 93/24641). Additional references describing AAV vectors are West et al., Virol. 160 (1987) 38-47; Kotin, Hum. Gene Ther. 5 (1994) 793-801; and Muzyczka J. Clin. Invest. 94 (1994) 1351. Construction of recombinant AAV vectors described in US 5,173,414; Lebkowski et al., Mol. Cell. Biol. 8 (1988) 3988-3996; Tratschin et al., Mol. Cell. Biol. 5 (1985) 3251-3260; Tratschin et al., Mol. Cell. Biol., 4 (1994) 2072-2081; Hermonat and Muzyczka Proc. Natl. Acad. Sci. USA 81 (1984) 6466-6470; Samulski et al. J. Virol. 63 (1989) 3822-3828.

An adeno-associated virus (AAV) is a replication-deficient parvovirus. It can replicate only in cells, in which certain viral functions are provided by a co-infecting helper virus, such as adenoviruses, herpesviruses and, in some cases, poxviruses such as vaccinia. Nevertheless, an AAV can replicate in virtually any cell line of human, simian or rodent origin provided that the appropriate helper viral functions are present.

Without helper viral genes being present, an AAV establishes latency in its host cell. Its genome integrates into a specific site in chromosome 19 [(Chr) 19 (q13.4)], which is termed the adeno-associated virus integration site 1 (AAVS1). For specific serotypes, such as AAV-2 other integration sites have been found, such as, e.g., on chromosome 5 [(Chr) 5 (p13.3)], termed AAVS2, and on chromosome 3 [(Chr) 3 (p24.3)], termed AAVS3.

AAVs are categorized into different serotypes. These have been allocated based on parameters, such as hemagglutination, tumorigenicity and DNA sequence homology. Up to now, more than 10 different serotypes and more than a hundred sequences corresponding to different clades of AAV have been identified.

The capsid protein type and symmetry determines the tissue tropism of the respective AAV. For example, AAV-2, AAV-4 and AAV-5 are specific to retina, AAV-2, AAV-5, AAV-8, AAV-9 and AAVrh-10 are specific for brain, AAV-1, AAV-2, AAV-6, AAV-8 and AAV-9 are specific for cardiac tissue, AAV-1, AAV-2, AAV-5, AAV-6, AAV-7, AAV-8, AAV-9 and AAV-10 are specific for liver, AAV-1, AAV-2, AAV-5 and AAV-9 are specific for lung.

Pseudotyping denotes a process comprising the cross packaging of the AAV genome between various serotypes, i.e. the genome is packaged with differently originating capsid proteins.

The wild-type AAV genome has a size of about 4.7 kb. The AAV genome further comprises two overlapping genes named rep and cap, which comprise multiple open reading frames (see, e.g., Srivastava et al., J. Viral., 45 (1983) 555-564; Hermonat et al., J. Viral. 51 (1984) 329-339; Tratschin et al., J. Virol., 51 (1984) 611-619). The Rep protein encoding open reading frame provides for four proteins of different size, which are termed Rep78, Rep68, Rep52 and Rep40. These are involved in replication, rescue and integration of the AAV. The Cap protein encoding open reading frame provides four proteins, which are termed VP1, VP2, VP3, and AAP. VP1, VP2 and VP3 are part of the proteinaceous capsid of the AAV particles. The combined rep and cap open reading frames are flanked at their 5'- and 3'-ends by so-called inverted terminal repeats (ITRs). For replication, an AAV requires in addition to the Rep and Cap proteins the products of the genes E1A, E1B, E4orf6, E2A and VA of an adenovirus or corresponding factors of another helper virus.

In the case of an AAV of the serotype 2 (AAV-2), for example, the ITRs each have a length of 145 nucleotides and flank a coding sequence region of about 4470 nucleotides. Of the ITR's 145 nucleotides 125 nucleotides have a palindromic sequence and can form a T-shaped hairpin structure. This structure has the function of a primer during viral replication. The remaining 20, non-paired, nucleotides are denoted as D-sequence.

The AAV genome harbors three transcription promoters P5, P19, and P40 (Laughlin et al., Proc. Natl. Acad. Sci. USA 76 (1979) 5567-5571) for the expression of the rep and cap genes.

The ITR sequences have to be present in cis to the coding region. The ITRs provide a functional origin of replication (ori), signals required for integration into the target cell's genome, and efficient excision and rescue from host cell chromosomes or recombinant plasmids. The ITRs further comprise origin of replication like-elements, such as a Rep-protein binding site (RBS) and a terminal resolution site (TRS). It has been found that the ITRs themselves can have the function of a transcription promoter in an AAV vector (Flotte et al., J. Biol. Chem. 268 (1993) 3781-3790; Flotte et al., Proc. Natl. Acad. Sci. USA 93 (1993) 10163-10167).

For replication and encapsidation, respectively, of the viral single-stranded DNA genome an in trans organization of the rep and cap gene products are required.

The rep gene locus comprises two internal promoters, termed P5 and P19. It comprises open reading frames for four proteins. Promoter P5 is operably linked to a nucleic acid sequence providing for non-spliced 4.2 kb mRNA encoding the Rep protein Rep78 (chromatin nickase to arrest cell cycle), and a spliced 3.9 kb mRNA encoding the Rep protein Rep68 (site-specific endonuclease). Promoter P19 is operably linked to a nucleic acid sequence providing for a non-spliced mRNA encoding the Rep protein Rep52 and a spliced 3.3 kb mRNA encoding the Rep protein Rep40 (DNA helicases for accumulation and packaging).

The two larger Rep proteins, Rep78 and Rep68, are essential for AAV duplex DNA replication, whereas the smaller Rep proteins, Rep52 and Rep40, seem to be essential for progeny, single-strand DNA accumulation (Chejanovsky & Carter, Virology 173 (1989) 120-128).

The larger Rep proteins, Rep68 and Rep78, can specifically bind to the hairpin conformation of the AAV ITR. They exhibit defined enzyme activities, which are required for resolving replication at the AAV termini. Expression of Rep78 or Rep68

could be sufficient for infectious particle formation (Holscher, C., et al. J. Virol. 68 (1994) 7169-7177 and 69 (1995) 6880-6885).

It is deemed that all Rep proteins, primarily Rep78 and Rep68, exhibit regulatory activities, such as induction and suppression of AAV genes as well as inhibitory effects on cell growth (Tratschin et al., Mol. Cell. Biol. 6 (1986) 2884-2894; Labow et al., Mol. Cell. Biol., 7 (1987) 1320-1325; Khleif et al., Virology, 181 (1991) 738-741).

Recombinant overexpression of Rep78 results in phenotype with reduced cell growth due to the induction of DNA damage. Thereby the host cell is arrested in the S phase, whereby latent infection by the virus is facilitated (Berthet, C., et al., Proc. Natl. Acad. Sci. USA 102 (2005) 13634-13639).

Tratschin et al. reported that the P5 promoter is negatively auto-regulated by Rep78 or Rep68 (Tratschin et al., Mol. Cell. Biol. 6 (1986) 2884-2894). Due to the toxic effects of expression of the Rep protein, only very low expression has been reported for certain cell lines after stable integration of AAV (see, e.g., Mendelson et al., Virol. 166 (1988) 154-165).

The cap gene locus comprises one promoter, termed P40. Promoter P40 is operably linked to a nucleic acid sequence providing for 2.6 kb mRNA, which, by alternative splicing and use of alternative start codons, encodes the Cap proteins VP1 (87 kDa, non-spliced mRNA transcript), VP2 (72 kDa, from the spliced mRNA transcript), and VP3 (61 kDa, from alternative start codon). VP1 to VP3 constitute the building blocks of the viral capsid. The capsid has the function to bind to a cell surface receptor and allow for intracellular trafficking of the virus. VP3 accounts for about 90 % of total viral particle protein. Nevertheless, all three proteins are essential for effective capsid production.

It has been reported that inactivation of all three capsid proteins VP1 to VP3 prevents accumulation of single-strand progeny AAV DNA. Mutations in the VP1 amino-terminus ("Lip-negative" or "Inf-negative") still allows for assembly of single-stranded DNA into viral particles whereby the infectious titer is greatly reduced.

The AAP open reading frame is encoding the assembly activating protein (AAP). It has a size of about 22 kDa and transports the native VP proteins into the nucleolar region for capsid assembly. This open reading frame is located upstream of the VP3 protein encoding sequence.

In individual AAV particles, only one single-stranded DNA molecule is contained. This may be either the "plus" or "minus" strand. AAV viral particles containing a DNA molecule are infectious. Inside the infected cell, the parental infecting single strand is converted into a double strand, which is subsequently amplified. The amplification results in a large pool of double stranded DNA molecules from which single strands are displaced and packaged into capsids.

Adeno-associated viral (AAV) vectors can transduce dividing cells as well as resting cells. It can be assumed that a transgene introduced using an AAV vector into a target cell will be expressed for a long period. One drawback of using an AAV vector is the limitation of the size of the transgene that can be introduced into cells.

Viral vectors such as parvo-virus particles, including AAV serotypes and variants thereof, provide a means for delivery of nucleic acid into cells ex vivo, in vitro and in vivo, which encode proteins such that the cells express the encoded protein. AAVs are viruses useful as gene therapy vectors as they can penetrate cells and introduce nucleic acid/genetic material so that the nucleic acid/genetic material may be stably maintained in cells. In addition, these viruses can introduce nucleic acid/genetic material into specific sites, for example. Because AAV are not associated with pathogenic disease in humans, AAV vectors are able to deliver heterologous polynucleotide sequences (e.g., therapeutic proteins and agents) to human patients without causing substantial AAV pathogenesis or disease.

Viral vectors, which may be used, include, but are not limited to, adeno-associated virus (AAV) particles of multiple serotypes (e.g., AAV-1 to AAV-12, and others) and hybrid/chimeric AAV particles.

AAV particles may be used to advantage as vehicles for effective gene delivery. Such particles possess a number of desirable features for such applications, including

tropism for dividing and non-dividing cells. Early clinical experience with these vectors also demonstrated no sustained toxicity and immune responses were minimal or undetectable. AAV are known to infect a wide variety of cell types in vivo and in vitro by receptor-mediated endocytosis or by transcytosis. These vector systems have been tested in humans targeting retinal epithelium, liver, skeletal muscle, airways, brain, joints and hematopoietic stem cells.

Recombinant AAV particles do not typically include viral genes associated with pathogenesis. Such vectors typically have one or more of the wild-type AAV genes deleted in whole or in part, for example, rep and/or cap genes, but retain at least one functional flanking ITR sequence, as necessary for the rescue, replication, and packaging of the recombinant vector into an AAV particle. For example, only the essential parts of the vector e.g., the ITR and LTR elements, respectively, are included. An AAV vector genome would therefore include sequences required in cis for replication and packaging (e.g., functional ITR sequences).

Recombinant AAV vectors, as well as methods and uses thereof, include any viral strain or serotype. As a non-limiting example, a recombinant AAV vector can be based upon any AAV genome, such as AAV-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11, -12, 2i8, AAV rh74 or AAV 7m8 for example. Such vectors can be based on the same strain or serotype (or subgroup or variant), or be different from each other. As a non-limiting example, a recombinant AAV vector based upon one serotype genome can be identical to one or more of the capsid proteins that package the vector. In addition, a recombinant AAV vector genome can be based upon an AAV (e.g., AAV2) serotype genome distinct from one or more of the AAV capsid proteins that package the vector. For example, the AAV vector genome can be based upon AAV2, whereas at least one of the three capsid proteins could be an AAV1, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV-2i8, AAV rh74, AAV 7m8 or a variant thereof, for example. AAV variants include variants and chimeras of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV-2i8, AAV rh74 and AAV 7m8 capsids.

In certain embodiments of all aspects and embodiments, adeno-associated virus (AAV) vectors include AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV-2i8, AAV rh74 and AAV 7m8, as well as variants (e.g., capsid variants, such as amino acid insertions, additions, substitutions and deletions) thereof, for example, as set forth in WO 2013/158879, WO 2015/013313 and US 2013/0059732 (disclosing LK01, LK02, LK03, etc.).

AAV and AAV variants (e.g., capsid variants) serotypes (e.g., VP1, VP2, and/or VP3 sequences) may or may not be distinct from other AAV serotypes, including, for example, AAV1-AAV12 (e.g., distinct from VP1, VP2, and/or VP3 sequences of any of AAV1-AAV12 serotypes).

In certain embodiments of all aspects and embodiments, an AAV particle related to a reference serotype has a polynucleotide, polypeptide or subsequence thereof that includes or consists of a sequence at least 80% or more (e.g., 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.1 %, 99.2%, 99.3%, 99.4%, 99.5%, etc.) identical to one or more AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV-2i8, AAV rh74 or AAV 7m8 (e.g., such as an ITR, or a VP1, VP2, and/or VP3 sequences).

Compositions, methods and uses of the invention include AAV sequences (polypeptides and nucleotides), and subsequences thereof that exhibit less than 100% sequence identity to a reference AAV serotype such as AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV-2i8, AAV rh74, or AAV 7m8, but are distinct from and not identical to known AAV genes or proteins, such as AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV-2i8, AAV rh74, or AAV 7m8, genes or proteins, etc. In certain embodiments of all aspects and embodiments, an AAV polypeptide or subsequence thereof includes or consists of a sequence at least 75% or more identical, e.g., 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, etc., up to 100% identical to any reference AAV sequence or subsequence thereof, such as AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10,

AAV11, AAV12, AAV-2i8, AAV rh74, or AAV 7m8 (e.g., VP1, VP2 and/or VP3 capsid or ITR). In certain embodiments, an AAV variant has 1, 2, 3, 4, 5, 5-10, 10-15, 15-20 or more amino acid substitutions.

Recombinant AAV particles, including AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV-2i8, AAV rh74 or AAV 7m8, and variant, related, hybrid and chimeric sequences, can be constructed using recombinant techniques that are known to the skilled artisan, to include one or more nucleic acid sequences (transgenes) flanked with one or more functional AAV ITR sequences.

Recombinant particles (e.g., rAAV particles) can be incorporated into pharmaceutical compositions. Such pharmaceutical compositions are useful for, among other things, administration and delivery to a subject in vivo or ex vivo. In certain embodiments, the pharmaceutical composition contains a pharmaceutically acceptable carrier or excipient. Such excipients include any pharmaceutical agent that does not itself induce an immune response harmful to the individual receiving the composition, and which may be administered without undue toxicity.

Protocols for the generation of adenoviral vectors have been described in US 5,998,205; US 6,228,646; US 6,093,699; US 6,100,242; WO 94/17810 and WO 94/23744, which are incorporated herein by reference in their entirety.

## SPECIFIC EMBODIMENTS OF THE METHOD ACCORDING TO THE INVENTION

In order to allow viral genome copy determination, the viral genomes have to be made accessible, i.e. the shielding capsid must be opened. For this, heat denaturation is convenient and commonly used. However, it has been found that heat denaturation results in artificially lowered viral genome copy numbers.

The current inventors have shown that heat denaturation at temperatures above 95 °C, such as e.g. 98 °C, results in a reduction of the determined viral copy genome number. This has been exemplified using the ATCC AAV2 standard VR-1616. The

lot used in the experiments has a nominal viral genome copy number (vgcn) of 3.28*10E10 vg/mL. The results are shown in the following Table 1.

**Table 1:**

| condition | absolute vgcn | relative vgcn |
|---|---|---|
| no pre-treatment | 2.735-3.306*10E10/mL | 83-101 % |
| heat denaturation @ 98 °C, 10 min. | 1.012*10E10/mL | 31 % |

Likewise, the current inventors have shown that incubation with proteinase K (PK) in aqueous or buffered solution in the absence of a detergent also results in a reduction of the determined viral copy genome number. This has been exemplified using the ATCC AAV2 standard VR-1616. The lot used in the experiments has a nominal viral genome copy number (vgcn) of 3.28*10E10 vg/mL. The results are shown in the following Table 2.

**Table 2:**

| condition | absolute vgcn | relative vgcn |
|---|---|---|
| no pre-treatment | 2.735-3.306*10E10/mL | 83-101 % |
| 2 mU PK in water, 50 °C, 60 min.; 95 °C, 15 min.; 4 °C | 1.202*10E9/mL | 4 % |
| 2 mU PK in 20 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 50 °C, 60 min.; 95 °C, 15 min.; 4 °C | 1.327*10E10/mL | 40 % |

In combination, the current inventors have shown that heat denaturation at temperatures above 95 °C, such as e.g. 98 °C, even in combination with an incubation with proteinase K (PK) in buffer in the absence of a detergent results in a reduction of the determined viral copy genome number. This has been exemplified using the ATCC AAV2 standard VR-1616. The lot used in the experiments has a nominal viral

genome copy number (vgcn) of 3.28*10E10 vg/mL. The results are shown in the following Table 3.

**Table 3:**

| condition | absolute vgcn | relative vgcn |
|---|---|---|
| no pre-treatment | 2.735-3.306*10E10/mL | 83-101 % |
| heat denaturation @ 98 °C, 10 min.; 1 mU PK in 20 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 50 °C, 60 min.; 95 °C, 15 min.; 4 °C | 1.814*10E10/mL | 55 % |

The current inventors have now found that the reduction of the determined viral genome copy genome number can be overcome by performing the incubation with proteinase K in the presence of sodium dodecyl sulfate (SDS). This is shown in this example using the ATCC AAV2 standard VR-1616. The lot used in the experiments has a nominal viral genome copy number (vgcn) of 3.28*10E10 vg/mL. The results are shown in the following Table 4.

**Table 4:**

| condition | absolute vgcn | relative vgcn |
|---|---|---|
| no pre-treatment | 2.735-3.306*10E10/mL | 83-101 % |
| 2 mU PK in 20 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 1% SDS, 50 °C, 60 min.; 95 °C, 15 min.; 4 °C | 3.520*10E10/mL | 107 % |

The recovery values obtained with the conditions of Table 4 are fulfilling the acceptance criteria for the validation of assay procedures according to EMA and FDA guidelines, i.e. are within +/- 15 % of the nominal value.

The current inventors have further shown that treatment with DNase I prior to viral genome number determination results in a reduction of the determined viral copy

genome number. This has been exemplified using the ATCC AAV2 standard VR-1616. The lot used in the experiments has a nominal viral genome copy number (vgcn) of 3.28*10E10 vg/mL. The results are shown in the following Table 5.

**Table 5:**

| condition | absolute vgcn | relative vgcn |
|---|---|---|
| no pre-treatment | 2.735-3.306*10E10/mL | 83-101 % |
| 5 U DNase I, 40 mM Tris-HCl, pH 8.0, 10 mM MgSO4, 1 mM CaCl2, 37 °C, 30 min.; 95 °C, 15 min. | 1.795*10E10/mL | 55 % |

The reduction by the DNase I treatment is independent from the further processing of the sample, i.e. of heat denaturation or proteinase K incubation. The different tested conditions, which all show a reduction of the determined viral copy number, are summarized in Table 6. The ATCC AAV2 standard VR-1616 has been used. The lot used in the experiments has a nominal viral genome copy number (vgcn) of 3.28*10E10 vg/mL.

**Table 6:**

| condition | absolute vgcn | relative vgcn |
|---|---|---|
| no pre-treatment | 2.735-3.306*10E10/mL | 83-101 % |
| 5 U DNase I, 40 mM Tris-HCl, pH 8.0, 10 mM MgSO4, 1 mM CaCl2, 37 °C, 30 min.; 95 °C, 15 min. | 1.795*10E10/mL | 55 % |
| 5 U DNase I, 40 mM Tris-HCl, pH 8.0, 10 mM MgSO4, 1 mM CaCl2, 37 °C, 30 min.; 95 °C, 15 min.; 2 mU PK in water, 50 °C, 60 min.; 95 °C, 15 min.; 4 °C | 4.947*10E9/mL | 15 % |

| condition | absolute vgcn | relative vgcn |
|---|---|---|
| 5 U DNase I, 40 mM Tris-HCl, pH 8.0, 10 mM MgSO4, 1 mM CaCl2, 37 °C, 30 min.; 95 °C, 15 min.; 2 mU PK in 20 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 50 °C, 60 min.; 95 °C, 15 min.; 4 °C | 9.590*10E9/mL | 29 % |
| 5 U DNase I, 40 mM Tris-HCl, pH 8.0, 10 mM MgSO4, 1 mM CaCl2, 37 °C, 30 min.; 95 °C, 15 min.; 2 mU PK in 20 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 1% SDS, 50 °C, 60 min.; 95 °C, 15 min.; 4 °C | 2.347*10E10/mL | 72 % |

The finding by the current inventors was confirmed using lysates of an AAV2 producing HEK293 cell cultivation. The established viral genome copy number (vgcn) in the lysate was 1.746*10E10 vg/mL (lysate 18). The results are shown in the following Table 7.

It can be seen that incubation with proteinase K allows for a recovery of 96 % of the viral genomes. This can further be increased by incubating the sample with proteinase K in the presence of SDS to 100 %.

**Table 7:**

| condition | absolute vgcn | relative vgcn |
|---|---|---|
| no pre-treatment | 3.594*10E9/mL | 21 % |
| heat denaturation @ 98 °C, 10 min. | 3.514*10E9/mL | 20 % |
| 2 mU PK in water, 50 °C, 60 min.; 95 °C, 15 min.; 4 °C | 7.636*10E9/mL | 44 % |

| condition | absolute vgcn | relative vgcn |
|---|---|---|
| heat denaturation @ 98 °C, 10 min.; 2 mU PK in 20 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 50 °C, 60 min.; 95 °C, 15 min.; 4 °C | 7.020*10E9/mL | 40 % |
| 2 mU PK in 20 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 50 °C, 60 min.; 95 °C, 15 min.; 4 °C | 1.671*10E10/mL | 96 % |
| 2 mU PK in 20 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 1% SDS, 50 °C, 60 min.; 95 °C, 15 min.; 4 °C | 1.746*10E10/mL | 100 % |

It has to be pointed out that no further purification of the proteinase K incubated lysate had been performed, i.e., for example, no column-based or extractive purification.

The determined viral genome copy number with additional DNase I treatment were in the range of 4% to 16 % only as shown in the following Table 8.

**Table 8:**

| condition | absolute vgcn | relative vgcn |
|---|---|---|
| no pre-treatment | 3.594*10E9/mL | 21 % |
| 5 U DNase I, 40 mM Tris-HCl, pH 8.0, 10 mM MgSO4, 1 mM CaCl2, 37 °C, 30 min.; 95 °C, 15 min. | 6.960*10E8/mL | 4 % |

| condition | absolute vgcn | relative vgcn |
|---|---|---|
| 5 U DNase I, 40 mM Tris-HCl, pH 8.0, 10 mM MgSO4, 1 mM CaCl2, 37 °C, 30 min.; 95 °C, 15 min.; 2 mU PK in water, 50 °C, 60 min.; 95 °C, 15 min.; 4 °C | 1.179*10E9/mL | 7 % |
| 5 U DNase I, 40 mM Tris-HCl, pH 8.0, 10 mM MgSO4, 1 mM CaCl2, 37 °C, 30 min.; 95 °C, 15 min.; 2 mU PK in 20 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 50 °C, 60 min.; 95 °C, 15 min.; 4 °C | 1.746*10E9/mL | 10 % |
| 5 U DNase I, 40 mM Tris-HCl, pH 8.0, 10 mM MgSO4, 1 mM CaCl2, 37 °C, 30 min.; 95 °C, 15 min.; 2 mU PK in 20 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 1% SDS, 50 °C, 60 min.; 95 °C, 15 min.; 4 °C | 2.716*10E9/mL | 16 % |

The current inventors have found that for the sequential incubation with DNase I and proteinase K it is advantageous to use the entire incubation mixture of the DNase I incubation for the proteinase K incubation. Thereby the determined viral genome copy number could be increased to 80 % of the established number. The established viral genome copy number (vgcn) in the lysate was 1.746*10E10 vg/mL (lysate 18) and 2.340*10E9 vg/mL (lysate 31). Such an effect cannot be seen with purified samples, such as the ATCC standard (nominal viral genome copy number (vgcn) of 3.28*10E10 vg/mL). The results are shown in Table 9.

**Table 9:**

| condition | absolute vgcn | relative vgcn |
|---|---|---|
| 10 µL lysate 18 for DNase I incubation; 1:10 diluted for PK incubation | 2.716*10E9/mL | 16 % |
| 10 µL lysate 31 for DNase I incubation; no dilution for PK incubation | 1.872*10E9/mL | 80 % |
| 10 µL ATCC standard for DNase I incubation; 1:10 diluted for PK incubation | 2.347*10E10/mL | 72 % |
| 10 µL ATCC standard for DNase I incubation; no dilution for PK incubation | 2.468*10E10/mL | 75 % |

The current inventors have found that a recovery of more than 80 % of viral genomes can be achieved using the combination of DNase I and proteinase K incubation without dilution using affinity chromatography purified cell lysates. The established viral genome copy number (vgcn) in the affinity purified lysate was 5.110*10E10 vg/mL (affinity purified lysate 31) and 7.320*10E10 vg/mL (affinity purified lysate 33). The results are shown in Table 10.

**Table 10:**

| condition | absolute vgcn | relative vgcn |
|---|---|---|
| 10 µL affinity purified lysate 31 for DNase I incubation; no dilution for PK incubation | 4.430*10E10/mL | 87 % |
| 10 µL affinity purified lysate 33 for DNase I incubation; no dilution for PK incubation | 7.320*10E10/mL | 100 % |

## VIRAL GENOME QUANTIFICATION

In order to allow correct viral genome quantification the sample must be free of plasmid DNA as well as unpackaged vector genomes, both containing at least parts of the viral genome. Further, the packaged AAV genomes must be available from the first PCR cycle.

To remove unpackaged DNA, which might interfere in the ddPCR process, DNase I digest is commonly used.

To make the encapsulated DNA accessible for ddPCR, capsid opening is required. This can be done either by incubation at high temperature or proteinase K digest. The requirement for proteinase K digestion at all, is heavily discussed in the art.

### Droplet digital polymerase chain reaction

Droplet digital PCR (ddPCR) allows for the absolute quantification of viral genomes without the need for the generation of a standard curve.

In more detail, droplet digital polymerase chain reaction (ddPCR) enables absolute quantification of nucleic acids by randomly distributing a PCR reaction mixture into discrete partitions, where some have no nucleic target sequences and others have one or more template copies present (Hindson, B., et al., Anal. Chem. 83 (2011) 8604-8610). Due to partitioning, thousands of independent PCR reactions are performed during thermal cycling. At the endpoint, the fraction of target-positive partitions is read out and used for the calculation of initial template DNA concentration (Pinheiro, L., et al., Anal. Chem. 84 (2011) 1003-1011).

First, up to 20,000 droplets with a volume of about 1 nL are formed in a water-oil emulsion. Thereby the PCR reaction mix, comprising of the nucleic acid template, forward (fwd) and reverse (rev) primer, a TaqMan probe and a ddPCR supermix, which contains a Thermus aquaticus (Taq) DNA polymerase, dNTPs and PCR buffer, is partitioned (see, e.g., Hindson, B., et al. 2011; Taylor, S, et al., Sci. Rep. 7 (2017) 2409). In each droplet, an individual PCR reaction is carried out during thermal cycling, depending on presence or absence of the DNA target.

In droplets containing template DNA, target sequences are amplified. During amplification, the 5'-to-3' exonuclease activity of Taq polymerase hydrolyses the TaqMan probe, which is bound to the template strand. Due to degradation of the probe into smaller fragments, the 5'-located fluorophore is no longer in close proximity to its 3'-located quencher. Thereby signal quenching is abolished and a fluorescence signal is generated. Partitions lacking template sequences show no amplification and therefore no hydrolysis of TaqMan probes and fluorescence generation, respectively, as the fluorescence of the 5'-located fluorophore remains quenched (see, e.g., Holland, P., et al., Proc. Natl. Acad. Sci. USA 88(1991) 7276-7580). Since probes with distinct fluorophores are available, that have different excitation and emission wavelengths, ddPCR reactions can be performed as multiplex reactions within one droplet. Commonly used fluorophores for two-dimensional ddPCR are 6-carboxyfluorescein (FAM) and hexachloro-6-carboxyfluorescein (HEX), both quenched by black hole quencher 1 (BHQ1) (see, e.g., Furuta-Hanawa, B., et al. Hum. Gen. Therap. Meth. 30 (2019) 127-136).

As an endpoint analysis, the fluorescence signal of each droplet after thermal cycling is read out. Using Poisson statistics, the copy number of target sequences ($\lambda$) can be calculated from the ratio of positive to total readouts (p), according to equation 1 (see, e.g., Hindson, B., et al. (2011)).

$$\lambda = - \ln (1 - p) \qquad (1)$$

Because ddPCR relies on an endpoint measurement, target sequence quantification is to a certain extent independent of the PCR reaction efficiency. This is in contrast to real time PCR (qPCR), which is commonly used for viral genome titration (see, e.g., Taylor, S, et al. (2017)). Further, no standards or calibration samples need to be used (see, e.g., Dorange, F., Bec, C., Cell Gen. Therap. Ins. 4 (2018) 119-129).

**RECOMBINANT AAV PARTICLES**

Different methods that are known in the art for generating rAAV particles. For example, transfection using AAV plasmid and AAV helper sequences in conjunction with co-infection with one AAV helper virus (e.g., adenovirus, herpesvirus, or

vaccinia virus) or transfection with a recombinant AAV plasmid, an AAV helper plasmid, and an helper function plasmid. Non-limiting methods for generating rAAV particles are described, for example, in US 6,001,650, US 6,004,797, WO 2017/096039, and WO 2018/226887. Following recombinant rAAV particle production (i.e. particle generation in cell culture systems), rAAV particles can be obtained from the host cells and cell culture supernatant and purified.

For the generation of recombinant AAV particles, expression of the Rep and Cap proteins, the helper proteins E1A, E1B, E2A and E4orf6 as well as the adenoviral VA RNA in a single mammalian cell is required. The helper proteins E1A, E1B, E2A and E4orf6 can be expressed using any promoter as shown by Matsushita et al. (Gene Ther. 5 (1998) 938-945), especially the CMV IE promoter. Thus, any promoter can be used.

Generally, to produce recombinant AAV particles, different, complementing plasmids are co-transfected into a host cell. One of the plasmids comprises the transgene sandwiched between the two cis acting AAV ITRs. The missing AAV elements required for replication and subsequent packaging of progeny recombinant genomes, i.e. the open reading frames for the Rep and Cap proteins, are contained in trans on a second plasmid. The overexpression of the Rep proteins results in inhibitory effects on cell growth (Li, J., et al., J. Virol. 71 (1997) 5236-5243). Additionally, a third plasmid comprising the genes of a helper virus, i.e. E1, E4orf6, E2A and VA from adenovirus, is required for AAV replication.

To reduce the number of required plasmids, Rep, Cap and the adenovirus helper genes may be combined on a single plasmid.

Alternatively, the host cell may already stably express the E1 gene products. Such a cell is a HEK293 cell. The human embryonic kidney clone denoted as 293 was generated back in 1977 by integrating adenoviral DNA into human embryonic kidney cells (HEK cells) (Graham, F.L., et al., J. Gen. Virol. 36 (1977) 59-74). The HEK293 cell line comprises base pair 1 to 4344 of the adenovirus serotype 5 genome. This encompasses the E1A and E1B genes as well as the adenoviral packaging signals (Louis, N., et al., Virology 233 (1997) 423-429).

When using HEK293 cells the missing E2A, E4orf6 and VA genes can be introduced either by co-infection with an adenovirus or by co-transfection with an E2A-, E4orf6- and VA-expressing plasmid (see, e.g., Samulski, R.J., et al., J. Virol. 63 (1989) 3822-3828; Allen, J.M., et al., J. Virol. 71 (1997) 6816-6822; Tamayose, K., et al., Hum. Gene Ther. 7 (1996) 507-513; Flotte, T.R., et al., Gene Ther. 2 (1995) 29-37; Conway, J.E., et al., J. Virol. 71 (1997) 8780-8789; Chiorini, J.A., et al., Hum. Gene Ther. 6 (1995) 1531-1541; Ferrari, F.K., et al., J. Virol. 70 (1996) 3227-3234; Salvetti, A., et al., Hum. Gene Ther. 9 (1998) 695-706; Xiao, X., et al., J. Virol. 72 (1998) 2224-2232; Grimm, D., et al., Hum. Gene Ther. 9 (1998) 2745-2760; Zhang, X., et al., Hum. Gene Ther. 10 (1999) 2527-2537). Alternatively, adenovirus/AAV or herpes simplex virus/AAV hybrid vectors can be used (see, e.g., Conway, J.E., et al., J. Virol. 71 (1997) 8780-8789; Johnston, K.M., et al., Hum. Gene Ther. 8 (1997) 359-370; Thrasher, A.J., et al., Gene Ther. 2 (1995) 481-485; Fisher, J.K., et al., Hum. Gene Ther. 7 (1996) 2079-2087; Johnston, K.M., et al., Hum. Gene Ther. 8 (1997) 359-370).

Thus, cell lines in which the rep gene is integrated and expressed tend to grow slowly or express Rep proteins at very low levels.

In order to limit the transgene activity to specific tissues, i.e. to limit the site of integration the transgene can be operably linked to an inducible or tissue specific promoter (see, e.g., Yang, Y., et al. Hum. Gene. Ther. 6 (1995) 1203-1213).

One difficulty in the production of rAAV particles is the inefficient packaging of the rAAV vector, resulting in low titers. Packaging has been difficult for several reasons including

- preferred encapsidation of wild-type AAV genomes if they are present;
- difficulty in generating sufficient complementing functions such as those provided by the wild-type rep and cap genes due to the inhibitory effect associated with the rep gene products;
- the limited efficiency of the co-transfection of the plasmid constructs.

All this is based on the biological properties of the Rep proteins. Especially the inhibitory (cytostatic and cytotoxic) properties of the Rep proteins as well as the

ability to reverse the immortalized phenotype of cultured cells is problematic. Additionally, Rep proteins down-regulate their own expression when the widely used AAV P5 promoter is employed (see, e.g., Tratschin et al., Mol. Cell. Biol. 6 (1986) 2884-2894).

In certain embodiments of all aspects and embodiments, the rAAV particles are derived from an AAV selected from the group consisting of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, Rh.10, Rh74 and 7m8.

In certain embodiments of all aspects and embodiments, the rAAV particles comprise a capsid sequence having 70 % or more sequence identity to an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, Rh.10, Rh74, or 7m8 capsid sequence.

In certain embodiments of all aspects and embodiments, the rAAV particles comprise an ITR sequence having 70 % or more sequence identity to an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, or AAV10 ITR sequence.

**E1A, E1B, E2 and E4**

The coding sequences of E1A and E1B (open reading frames) can be derived from a human adenovirus, such as, e.g., in particular of human adenovirus serotype 2 or serotype 5. An exemplary sequence of human Ad5 (adenovirus serotype 5) is found in GenBank entries X02996, AC_000008 and that of an exemplary human Ad2 in GenBank entry AC_000007. Nucleotides 505 to 3522 comprise the nucleic acid sequences encoding E1A and E1B of human adenovirus serotype 5. Plasmid pSTK146 as reported in EP 1 230 354, as well as plasmids pGS119 and pGS122 as reported in WO 2007/056994, can also be used a source for the E1A and E1B open reading frames.

E1A is the first viral helper gene that is expressed after adenoviral DNA enters the cell nucleus. The E1A gene encodes the 12S and 13S proteins, which are based on the same E1A mRNA by alternative splicing. Expression of the 12S and 13S proteins results in the activation of the other viral functions E1B, E2, E3 and E4. Additionally,

expression of the 12S and 13S proteins force the cell into the S phase of the cell cycle. If only the E1A-derived proteins are expressed, the cell will dye (apoptosis).

E1B is the second viral helper gene that is expressed. It is activated by the E1A-derived proteins 12S and 13S. The E1B gene derived mRNA can be spliced in two different ways resulting in a first 55 kDa transcript and a second 19 kDa transcript. The E1B 55 kDa protein is involved in the modulation of the cell cycle, the prevention of the transport of cellular mRNA in the late phase of the infection, and the prevention of E1A-induced apoptosis. The E1B 19 kDa protein is involved in the prevention of E1A-induced apoptosis of cells.

The E2 gene encodes different proteins. The E2A transcript codes for the single strand-binding protein (SSBP), which is essential for AAV replication

Also the E4 gene encodes several proteins. The E4 gene derived 34 kDa protein (E4orf6) prevents the accumulation of cellular mRNAs in the cytoplasm together with the E1B 55 kDa protein, but also promotes the transport of viral RNAs from the cell nucleus into the cytoplasm.

**Adenoviral VA RNA gen**

The viral associated RNA (VA RNA) is a non-coding RNA of adenovirus (Ad), regulating translation. The adenoviral genome comprises two independent copies: VAI (VA RNAI) and VAII (VA RNAII). Both are transcribed by RNA polymerase III (see, e.g., Machitani, M., et al., J. Contr. Rel. 154 (2011) 285-289) from a type 2 polymerases III promoter. For recombinant production the adenoviral VA RNA gene can be driven by any promoter.

The structure, function, and evolution of adenovirus-associated RNA using a phylogenetic approach was investigated by Ma, Y. and Mathews, M.B. (J. Virol. 70 (1996) 5083-5099). They provided alignments as well as consensus VA RNA sequences based on 47 known human adenovirus serotypes. Said disclosure is herewith incorporated by reference in its entirety into the current application.

VA RNAs, VAI and VAII, are consisting of 157-160 nucleotides (nt).

Depending on the serotype, adenoviruses contain one or two VA RNA genes. VA RNAI is believed to play the dominant pro-viral role, while VA RNAII can partially compensate for the absence of VA RNAI (Vachon, V.K. and Conn, G.L., Virus Res. 212 (2016) 39-52).

The VA RNAs are not essential, but play an important role in efficient viral growth by overcoming cellular antiviral machinery. That is, although VA RNAs are not essential for viral growth, VA RNA-deleted adenovirus cannot grow during the initial step of vector generation, where only a few copies of the viral genome are present per cell, possibly because viral genes other than VA RNAs that block the cellular antiviral machinery may not be sufficiently expressed (see Maekawa, A., et al. Nature Sci. Rep. 3 (2013) 1136).

Maekawa, A., et al. (Nature Sci. Rep. 3 (2013) 1136) reported efficient production of adenovirus vector lacking genes of virus-associated RNAs that disturb cellular RNAi machinery, wherein HEK293 cells that constitutively and highly express flippase recombinase were infected to obtain VA RNA-deleted adenovirus by FLP recombinase-mediated excision of the VA RNA locus.

The human adenovirus 2 VA RNAI corresponds to nucleotides 10586-10810 of GenBank entry AC_000007 sequence. The human adenovirus 5 VA RNAI corresponds to nucleotides 10579-10820 of GenBank entry AC_000008 sequence.

**Methods for producing rAAV particles**

Carter et al. have shown that the entire rep and cap open reading frames in the wild-type AAV genome can be deleted and replaced with a transgene (Carter, B. J., in "Handbook of Parvoviruses", ed. by P. Tijssen, CRC Press, pp. 155-168 (1990)). Further, it has been reported that the ITRs have to be maintained to retain the function of replication, rescue, packaging, and integration of the transgene into the genome of the target cell.

When cells comprising the respective viral helper genes are transduced by an AAV vector, or, vice versa, when cells comprising an integrated AAV provirus are transduced by a suitable helper virus, then the AAV provirus is activated and enters

a lytic infection cycle again (Clark, K.R., et al., Hum. Gene Ther. 6 (1995) 1329-1341; Samulski, R.J., Curr. Opin. Genet. Dev. 3 (1993) 74-80).

Aspects of the current invention are methods of transducing cells with nucleic acids (e.g., plasmids) comprising all required elements for the production of recombinant AAV particles, wherein the viral genome copy number is determined with a method according to the current invention. Thus, as the plasmids encode viral packaging proteins and/or helper proteins the cells can produce recombinant viral particles that include a nucleic acid that encodes a protein of interest or comprises a sequence that is transcribed into a transcript of interest.

The invention provides a viral (e.g., AAV) particle production platform that includes features that distinguish it from current 'industry-standard' viral (e.g., AAV) particle production processes by using the method according to the current invention.

More generally, cells transfected or transduced with DNA for the recombinant production of AAV particles can be referred to as "recombinant cell". Such a cell can be, for example, a yeast cell, an insect cell, or a mammalian cell, and has been used as recipient of a nucleic acid (plasmid) encoding packaging proteins, such as AAV packaging proteins, a nucleic acid (plasmid) encoding helper proteins, and a nucleic acid (plasmid) that encodes a protein or is transcribed into a transcript of interest, i.e. a transgene placed between two AAV ITRs. The term includes the progeny of the original cell, which has been transduced or transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total nucleic acid complement as the original parent, due to natural, accidental, or deliberate mutation.

Numerous cell growth media appropriate for sustaining cell viability or providing cell growth and/or proliferation are commercially available. Examples of such medium include serum free eukaryotic growth mediums, such as medium for sustaining viability or providing for the growth of mammalian (e.g., human) cells. Non-limiting examples include Ham's F12 or F12K medium (Sigma-Aldrich), FreeStyle (FS) F17 medium (Thermo-Fisher Scientific), MEM, DMEM, RPMI-1640 (Thermo-Fisher Scientific) and mixtures thereof. Such media can be supplemented

with vitamins and/or trace minerals and/or salts and/or amino acids, such as essential amino acids for mammalian (e.g., human) cells.

Helper protein plasmids can be in the form of a plasmid, phage, transposon or cosmid. In particular, it has been demonstrated that the full-complement of adenovirus genes are not required for helper functions. For example, adenovirus mutants incapable of DNA replication and late gene synthesis have been shown to be permissive for AAV replication. Ito et al., J. Gen. Virol. 9 (1970) 243; Ishibashi et al., Virology 45 (1971) 317.

Mutants within the E2B and E3 regions have been shown to support AAV replication, indicating that the E2B and E3 regions are probably not involved in providing helper function. Carter et al., Virology 126 (1983) 505. However, adenoviruses defective in the E1 region, or having a deleted E4 region, are unable to support AAV replication. Thus, for adenoviral helper proteins, E1A and E4 regions are likely required for AAV replication, either directly or indirectly (see, e.g., Laughlin et al., J. Virol. 41 (1982) 868; Janik et al., Proc. Natl. Acad. Sci. USA 78 (1981) 1925; Carter et al., Virology 126 (1983) 505). Other characterized adenoviral mutants include: E1B (Laughlin et al. (1982), supra; Janik et al. (1981 ), supra; Ostrove et al., Virology 104 (1980) 502); E2A (Handa et al., J. Gen. Virol. 29 (1975) 239; Strauss et al., J. Virol. 17 (1976) 140; Myers et al., J. Virol. 35 (1980) 665; Jay et al., Proc. Natl. Acad. Sci. USA 78 (1981) 2927; Myers et al., J. Biol. Chem. 256 (1981) 567); E2B (Carter, Adeno-Associated Virus Helper Functions, in I CRC Handbook of Parvoviruses (P. Tijssen ed., 1990)); E3 (Carter et al. (1983), supra); and E4 (Carter et al.(1983), supra; Carter (1995)).

Studies of the helper proteins provided by adenoviruses having mutations in the E1B have reported that the E1B 55 kDa protein is required for AAV particle production, while E1B 19 kDa is not. In addition, WO 97/17458 and Matshushita et al. (Gene Therapy 5 (1998) 938-945) described helper function plasmids encoding various adenoviral genes. An example of a helper plasmid comprise an adenovirus VA RNA coding region, an adenovirus E4orf6 coding region, an adenovirus E2A 72 kDa

coding region, an adenovirus E1A coding region, and an adenovirus E1B region lacking an intact E1B 55 kDa coding region (see, e.g., WO 01/83797).

Thus, herein is provided a method for producing recombinant AAV vectors or AAV particles comprising said recombinant AAV vectors, which comprise a nucleic acid that encodes a protein or is transcribed into a transcript of interest, using the method according to the current invention for viral genome number determination.

One aspect of the current invention is a method for producing recombinant AAV vectors or AAV particles comprising said recombinant AAV vectors, which comprise a nucleic acid that encodes a protein or is transcribed into a transcript of interest, comprises the steps of

(i) providing one or more plasmids comprising nucleic acids encoding AAV packaging proteins and/or nucleic acids encoding helper proteins;

(ii) providing a plasmid comprising a nucleic acid that encodes a protein of interest or is transcribed into a transcript of interest;

(iii) contacting one or more mammalian or insect cells with the provided plasmids;

(iv) either further adding a transfection reagent and optionally incubating the plasmid/transfection reagent/cell mixture; or providing a physical means, such as an electric current, to introduce the nucleic acid into the cells;

(v) cultivating the transfected cells;

(vi) harvesting the cultivated cells and/or culture medium from the cultivated cells to produce a cell and/or culture medium harvest; and

(vii) lysing the cells and optionally isolating recombinant AAV vector or AAV particle from the cell and/or culture medium harvest lysate;

(viii) determining the viral genome copy number in or after steps (vi) or/and step (vii) with a method according to the current invention;

thereby producing a recombinant AAV vector or AAV particle comprising a nucleic acid that encodes a protein of interest or is transcribed into a transcript of interest.

One aspect of the current invention is a method for producing recombinant AAV vectors or AAV particles comprising said recombinant AAV vectors, which comprise a nucleic acid that encodes a protein or is transcribed into a transcript of interest, comprises the steps of

(i) providing one or more plasmids comprising nucleic acids encoding AAV packaging proteins and/or nucleic acids encoding helper proteins;

(ii) providing a plasmid comprising a nucleic acid that encodes a protein of interest or is transcribed into a transcript of interest;

(iii)

(a) either generating a stable transfected cell by

- contacting one or more mammalian or insect cells with the provided plasmids of (i);

- either further adding a transfection reagent and optionally incubating the plasmid/transfection reagent/cell mixture; or providing a physical means, such as an electric current, to introduce the nucleic acid into the cells;

- selecting a first stably transfected cell;

- contacting the selected first stably transfected cell with the provided plasmid of (ii); and

- either further adding a transfection reagent and optionally incubating the plasmid/transfection reagent/cell mixture; or providing a physical means, such as an electric current, to introduce the nucleic acid into the cells;

(b) or generating a transient transfected cell by

- contacting one or more mammalian or insect cells with the provided plasmids of (i) and (ii); and

- either further adding a transfection reagent and optionally incubating the plasmid/transfection reagent/cell mixture; or providing a physical means, such as an electric current, to introduce the nucleic acid into the cells;

(iv) cultivating the transfected cell of (iii);

(v) harvesting the cultivated cells and/or culture medium from the cultivated cells to produce a cell and/or culture medium harvest;

(vi) lysing the cells and optionally isolating recombinant AAV vector or AAV particle from the cell and/or culture medium harvest lysate;

(vii) determining the viral genome copy number in or after step (v) or/and step (vi) with the method according to the current invention;

thereby producing recombinant AAV vector or AAV particle comprising a nucleic acid that encodes a protein of interest or is transcribed into a transcript of interest.

One aspect of the current invention is a method for producing recombinant AAV vectors or AAV particles comprising said recombinant AAV vectors, which comprise a nucleic acid that encodes a protein or is transcribed into a transcript of interest, comprises the steps of

(i) providing a mammalian or insect cell comprising nucleic acids encoding AAV packaging proteins and/or nucleic acids encoding helper proteins,;

(ii) providing a plasmid comprising a nucleic acid that encodes a protein of interest or is transcribed into a transcript of interest;

(iii)

(a) either generating a stable transfected cell by

- contacting one or more mammalian or insect cells with the provided plasmids of (i);

- either further adding a transfection reagent and optionally incubating the plasmid/transfection reagent/cell mixture; or providing a physical means, such as an electric current, to introduce the nucleic acid into the cells;

- selecting a first stably transfected cell;

- contacting the selected first stably transfected cell with the provided plasmid of (ii); and

- either further adding a transfection reagent and optionally incubating the plasmid/transfection reagent/cell mixture; or providing a physical means, such as an electric current, to introduce the nucleic acid into the cells;

(b) or generating a transient transfected cell by

- contacting one or more mammalian or insect cells with the provided plasmids of (i) and (ii); and

- either further adding a transfection reagent and optionally incubating the plasmid/transfection reagent/cell mixture; or providing a physical means, such as an electric current, to introduce the nucleic acid into the cells;

(iv) cultivating the transfected cell of (iii);

(v) harvesting the cultivated cells and/or culture medium from the cultivated cells to produce a cell and/or culture medium harvest;

(vi) lysing the cells and optionally isolating and/or purifying recombinant AAV vector or AAV particle from the cell and/or culture medium harvest; and

(vii) determining the viral genome copy number in or after step (v) or/and step (vi) with the method according to the current invention;

thereby producing recombinant AAV vector or AAV particle comprising a nucleic acid that encodes a protein of interest or is transcribed into a transcript of interest.

The introduction of the nucleic acid (plasmids) into cells can be done in multiple ways.

Diverse methods for the DNA transfer into mammalian cells have been reported in the art. These are all useful in the methods according to the current invention. In certain embodiments of all aspects and embodiments, electroporation, nucleofection, or microinjection for nucleic acid transfer/transfection is used. In certain embodiments of all aspects and embodiments, an inorganic substance (such as, e.g., calcium phosphate/DNA co-precipitation), a cationic polymer (such as, e.g., polyethylenimine, DEAE-dextran), or a cationic lipid (lipofection) is used for nucleic acid transfer/transfection is used. Calcium phosphate and polyethylenimine are the most commonly used reagents for transfection for nucleic acid transfer in larger scales (see, e.g., Baldi et al., Biotechnol. Lett. 29 (2007) 677-684), whereof polyethylenimine is preferred.

In certain embodiments of all aspects and embodiments, the nucleic acid (plasmid) is provided in a composition in combination with polyethylenimine (PEI), optionally in combination with cells. In certain embodiments, the composition includes a plasmid/PEI mixture, which has a plurality of components: (a) one or more plasmids comprising nucleic acids encoding AAV packaging proteins and/or nucleic acids encoding helper proteins; (b) a plasmid comprising a nucleic acid that encodes a protein or is transcribed into a transcript of interest; and (c) a polyethylenimine (PEI) solution. In certain embodiments, the plasmids are in a molar ratio range of about

1:0.01 to about 1:100, or are in a molar ratio range of about 100: 1 to about 1:0.01, and the mixture of components (a), (b) and (c) has optionally been incubated for a period of time from about 10 seconds to about 4 hours.

In certain embodiments of all aspects and embodiments, the compositions further comprise cells. In certain embodiments, the cells are in contact with the plasmid/PEI mixture of components (a), (b) and/or (c).

In certain embodiments of all aspects and embodiments, the composition, optionally in combination with cells, further comprise free PEI. In certain embodiments, the cells are in contact with the free PEI.

In certain embodiments of all aspects and embodiments, the cells have been in contact with the mixture of components (a), (b) and/or (c) for at least about 4 hours, or about 4 hours to about 140 hours, or for about 4 hours to about 96 hours. In one preferred embodiment, the cells have been in contact with the mixture of components (a), (b) and/or (c) and optionally free PEI, for at least about 4 hours.

The composition may comprise further plasmids or/and cells. Such plasmids and cells may be in contact with free PEI. In certain embodiments, the plasmids and/or cells have been in contact with the free PEI for at least about 4 hours, or about 4 hours to about 140 hours, or for about 4 hours to about 96 hours.

The invention also provides methods for producing transfected cells. The method includes the steps of providing one or more plasmids; providing a solution comprising polyethylenimine (PEI); and mixing the plasmid(s) with the PEI solution to produce a plasmid/PEI mixture. In certain embodiments, such mixtures are incubated for a period in the range of about 10 seconds to about 4 hours. In such methods, cells are then contacted with the plasmid/PEI mixture to produce a plasmid/PEI cell culture; then free PEI is added to the plasmid/PEI cell culture produced to produce a free PEI/plasmid/PEI cell culture; and then the free PEI/plasmid/PEI cell culture produced is incubated for at least about 4 hours, thereby producing transfected cells. In certain embodiments, the plasmids comprise one or more or all of a rep open reading frame, a cap open reading frame, E1A, E1B, E2

and E4orf6 open reading frames and a nucleic acid that encodes a protein or is transcribed into a transcript of interest.

Further provided are methods for producing transfected cells that produce recombinant AAV vector or AAV particle, which include providing one or more plasmids comprising nucleic acids encoding AAV packaging proteins and/or nucleic acids encoding helper proteins; providing a plasmid comprising a nucleic acid that encodes a protein or is transcribed into a transcript of interest; providing a solution comprising polyethylenimine (PEI); mixing the aforementioned plasmids with the PEI solution, wherein the plasmids are in a molar ratio range of about 1:0.01 to about 1:100, or are in a molar ratio range of about 100:1 to about 1:0.01, to produce a plasmid/PEI mixture (and optionally incubating the plasmid/PEI mixture for a period in the range of about 10 seconds to about 4 hours); contacting cells with the plasmid/PEI mixture, to produce a plasmid/PEI cell culture; adding free PEI to the plasmid/PEI cell culture produced to produce a free PEI/plasmid/PEI cell culture; and incubating the free PEI/plasmid/PEI cell culture for at least about 4 hours, thereby producing transfected cells that produce recombinant AAV vector or particle comprising a nucleic acid that encodes a protein or is transcribed into a transcript of interest, whereby the viral genome copy number is determined with a method according to the current invention.

Additionally provided are methods for producing recombinant AAV vector or AAV particle comprising a nucleic acid that encodes a protein or is transcribed into a transcript of interest, which includes providing one or more plasmids comprising nucleic acids encoding AAV packaging proteins and/or nucleic acids encoding helper proteins; providing a plasmid comprising a nucleic acid that encodes a protein of interest or is transcribed into a transcript of interest; providing a solution comprising polyethylenimine (PEI); mixing the aforementioned plasmids with the PEI solution, wherein the plasmids are in a molar ratio range of about 1:0.01 to about 1:100, or are in a molar ratio range of about 100:1 to about 1:0.01, to produce a plasmid/PEI mixture (and optionally incubating the plasmid/PEI mixture for a period of time in the range of about 10 seconds to about 4 hours); contacting cells with the plasmid/PEI mixture produced as described to produce a plasmid/PEI cell culture;

adding free PEI to the plasmid/PEI cell culture produced as described to produce a free PEI/plasmid/PEI cell culture; incubating the plasmid/PEI cell culture or the free PEI/plasmid/PEI cell culture produced for at least about 4 hours to produce transfected cells; harvesting the transfected cells produced and/or culture medium from the transfected cells produced to produce a cell and/or culture medium harvest; lysing the cells and optionally isolating the recombinant AAV vector or particle from the cell and/or culture medium harvest lysate, whereby the viral genome copy number is determined in the lysate or the isolated AAV particle with a method according to the current invention; and thereby producing recombinant AAV vector or particle comprising a nucleic acid that encodes a protein or is transcribed into a transcript of interest.

Methods for producing recombinant AAV vectors or AAV particles using the method according to the current invention can include one or more further steps or features. An exemplary step or feature includes, but is not limited to, a step of harvesting the cultivated cells produced and/or harvesting the culture medium from the cultivated cells produced to produce a cell and/or culture medium harvest. An additional exemplary step or feature includes, but is not limited to lysing the harvested cells and optionally isolating the recombinant AAV vector or AAV particle from the cell and/or culture medium harvest lysate; whereby the viral genome copy number is determined using a method according to the current invention; and thereby producing recombinant AAV vector or AAV particle comprising a nucleic acid that encodes a protein or is transcribed into a transcript of interest.

In certain embodiments of all aspects and embodiments, PEI is added to the plasmids and/or cells at various time points. In certain embodiments, free PEI is added to the cells before, at the same time as, or after the plasmid/PEI mixture is contacted with the cells.

In certain embodiments of all aspects and embodiments, the cells are at particular densities and/or cell growth phases and/or viability when contacted with the plasmid/PEI mixture and/or when contacted with the free PEI. In one preferred

embodiment, cells are at a density in the range of about 1x10E5 cells/mL to about 1x10E8 cells/mL when contacted with the plasmid/PEI mixture and/or when contacted with the free PEI. In certain embodiments, viability of the cells when contacted with the plasmid/PEI mixture or with the free PEI is about 60 % or greater than 60 %, or wherein the cells are in log phase growth when contacted with the plasmid/PEI mixture, or viability of the cells when contacted with the plasmid/PEI mixture or with the free PEI is about 90 % or greater than 90 %, or wherein the cells are in log phase growth when contacted with the plasmid/PEI mixture or with the free PEI.

Encoded AAV packaging proteins include, in certain embodiments of all aspects and embodiments, AAV rep and/or AAV cap. Such AAV packaging proteins include, in certain embodiments of all aspects and embodiments, AAV rep and/or AAV cap proteins of any AAV serotype.

Encoded helper proteins include, in certain embodiments of all aspects and embodiments, adenovirus E1A and E1B, adenovirus E2 and/or E4, VA RNA, and/or non-AAV helper proteins.

In certain embodiments of all aspects and embodiments, the nucleic acids (plasmids) are used at particular amounts or ratios. In certain embodiments, the total amount of plasmid comprising the nucleic acid that encodes a protein or is transcribed into a transcript of interest and the one or more plasmids comprising nucleic acids encoding AAV packaging proteins and/or nucleic acids encoding helper proteins is in the range of about 0.1 μg to about 15 μg per mL of cells. In certain embodiments, the molar ratio of the plasmid comprising the nucleic acid that encodes a protein or is transcribed into a transcript of interest to the one or more plasmids comprising nucleic acids encoding AAV packaging proteins and/or nucleic acids encoding helper proteins is in the range of about 1:5 to about 1:1, or is in the range of about 1:1 to about 5:1.

In certain embodiments of all aspects and embodiments, a first plasmid comprises the nucleic acids encoding AAV packaging proteins and a second plasmid comprises the nucleic acids encoding helper proteins.

In certain embodiments of all aspects and embodiments, the molar ratio of the plasmid comprising the nucleic acid that encodes a protein or is transcribed into a transcript of interest to a first plasmid comprising the nucleic acids encoding AAV packaging proteins to a second plasmid comprising the nucleic acids encoding helper proteins is in the range of about 1-5: 1: 1, or 1: 1-5: 1, or 1: 1: 1-5 in co-transfection.

In certain embodiments of all aspects and embodiments, the cell is a eukaryotic cell. In certain embodiments, the eukaryotic cell is a mammalian cell. In one preferred embodiment, the cell is a HEK293 cell or a CHO cell.

The cultivation can be performed using the generally used conditions for the cultivation of eukaryotic cells of about 37 °C, 95 % humidity and 8 vol.-% $CO_2$. The cultivation can be performed in serum containing or serum free medium, in adherent culture or in suspension culture. The suspension cultivation can be performed in any fermentation vessel, such as, e.g., in stirred tank reactors, wave reactors, rocking bioreactors, shaker vessels or spinner vessels or so called roller bottles. Transfection can be performed in high throughput format and screening, respectively, e.g. in a 96 or 384 well format.

Methods according to the current invention include AAV particles of any serotype, or a variant thereof. In certain embodiments of all aspects and embodiments, a recombinant AAV particle comprises any of AAV serotypes 1-12, an AAV VP1, VP2 and/or VP3 capsid protein, or a modified or variant AAV VP1, VP2 and/or VP3 capsid protein, or wild-type AAV VP1, VP2 and/or VP3 capsid protein. In certain embodiments of all aspects and embodiments, an AAV particle comprises an AAV serotype or an AAV pseudotype, where the AAV pseudotype comprises an AAV capsid serotype different from an ITR serotype.

Methods according to the invention that provide or include AAV vectors or particles can also include other elements. Examples of such elements include but are not limited to: an intron, an expression control element, one or more adeno-associated virus (AAV) inverted terminal repeats (ITRs) and/or a filler/stuffer polynucleotide sequence. Such elements can be within or flank the nucleic acid that encodes a protein or is transcribed into a transcript of interest, or the expression control element

can be operably linked to nucleic acid that encodes a protein or is transcribed into a transcript of interest, or the AAV ITR(s) can flank the 5'- or 3'-terminus of nucleic acid that encodes a protein or is transcribed into a transcript of interest, or the filler polynucleotide sequence can flank the 5'- or 3'-terminus of nucleic acid that encodes a protein or is transcribed into a transcript of interest.

Expression control elements include constitutive or regulatable control elements, such as a tissue-specific expression control element or promoter.

ITRs can be any of AAV2 or AAV6 or AAV8 or AAV9 serotypes, or a combination thereof. AAV particles can include any VP1, VP2 and/or VP3 capsid protein having 75 % or more sequence identity to any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV10, AAV11, AAV-2i8, AAV rh74 or AAV 7m8 VP1, VP2 and/or VP3 capsid proteins, or comprises a modified or variant VP1, VP2 and/or VP3 capsid protein selected from any of: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV10, AAV11, AAV-2i8, AAV rh74 and AAV 7m8 AAV serotypes.

Following production of recombinant viral (e.g., AAV) particles as set forth herein, if desired, the viral (e.g., rAAV) particles can be purified and/or isolated from host cells using a variety of conventional methods. Such methods include column chromatography, CsCl gradients, iodixanol gradient and the like.

For example, a plurality of column purification steps such as purification over an anion exchange column, an affinity column and/or a cation exchange column can be used. (See, e.g., WO 02/12455 and US 2003/0207439). Alternatively, or in addition, a iodixanol or CsCl gradient steps can be used (see, e.g., US 2012/0135515; and US 2013/0072548). Further, if the use of infectious virus is employed to express the packaging and/or helper proteins, residual virus can be inactivated, using various methods. For example, adenovirus can be inactivated by heating to temperatures of approximately 60 °C for, e.g., 20 minutes or more. This treatment effectively inactivates the helper virus since AAV is heat stable while the helper adenovirus is heat labile.

An objective in the rAAV vector production and purification systems is to implement strategies to minimize/control the generation of production related impurities such as proteins, nucleic acids, and vector-related impurities, including wild-type/pseudo wild-type AAV species (wtAAV) and AAV-encapsulated residual DNA impurities.

Considering that the rAAV particle represents only a minor fraction of the biomass, rAAV particles need to be purified to a level of purity, which can be used as a clinical human gene therapy product (see, e.g., Smith P.H., et al., Mo. Therapy 7 (2003) 8348; Chadeuf G., et al, Mo. Therapy 12 (2005) 744; report from the CHMP gene therapy expert group meeting, European Medicines Agency EMEA/CHMP 2005, 183989/2004).

As an initial step, typically the cultivated cells that produce the rAAV particles are harvested, optionally in combination with harvesting cell culture supernatant (medium) in which the cells (suspension or adherent) producing rAAV particles have been cultured. The harvested cells and optionally cell culture supernatant may be used as is, as appropriate, lysed or concentrated. Further, if infection is employed to express helper functions, residual helper virus can be inactivated. For example, adenovirus can be inactivated by heating to temperatures of approximately 60 °C for, e.g., 20 minutes or more, which inactivates only the helper virus since AAV is heat stable while the helper adenovirus is heat labile.

Cells and/or supernatant of the harvest are lysed by disrupting the cells, for example, by chemical or physical means, such as detergent, microfluidization and/or homogenization, to release the rAAV particles. Concurrently during cell lysis or subsequently after cell lysis, a nuclease, such as, e.g., benzonase, is added to degrade contaminating DNA. Typically, the resulting lysate is clarified to remove cell debris, e.g. by filtering or centrifuging, to render a clarified cell lysate. In a particular example, lysate is filtered with a micron diameter pore size filter (such as a 0.1-10.0 μm pore size filter, for example, a 0.45 μm and/or pore size 0.2 μm filter), to produce a clarified lysate.

The lysate (optionally clarified) contains AAV particles (comprising rAAV vectors as well as empty capsids) and production/process related impurities, such as soluble

cellular components from the host cells that can include, inter alia, cellular proteins, lipids, and/or nucleic acids, and cell culture medium components. The optionally clarified lysate is then subjected to purification steps to purify AAV particles (comprising rAAV vectors) from impurities using chromatography. The clarified lysate may be diluted or concentrated with an appropriate buffer prior to the first chromatography step.

After cell lysis, optional clarifying, and optional dilution or concentration, a plurality of subsequent and sequential chromatography steps can be used to purify rAAV particles.

A first chromatography step may be cation exchange chromatography or anion exchange chromatography. If the first chromatography step is cation exchange chromatography the second chromatography step can be anion exchange chromatography or size exclusion chromatography (SEC). Thus, in certain embodiments of all aspects and embodiments, rAAV particle purification is via cation exchange chromatography, followed by purification via anion exchange chromatography.

Alternatively, if the first chromatography step is cation exchange chromatography the second chromatography step can be size exclusion chromatography (SEC). Thus, in certain embodiments of all aspects and embodiments, rAAV particle purification is via cation exchange chromatography, followed by purification via size exclusion chromatography (SEC).

Still alternatively, a first chromatography step may be affinity chromatography. If the first chromatography step is affinity chromatography the second chromatography step can be anion exchange chromatography. Thus, in certain embodiments of all aspects and embodiments, rAAV particle purification is via affinity chromatography, followed by purification via anion exchange chromatography.

Optionally, a third chromatography can be added to the foregoing chromatography steps. Typically, the optional third chromatography step follows cation exchange, anion exchange, size exclusion or affinity chromatography.

Thus, in certain embodiments of all aspects and embodiments, rAAV particle purification is via cation exchange chromatography, followed by purification via anion exchange chromatography, followed by purification via size exclusion chromatography (SEC).

In addition, in certain embodiments of all aspects and embodiments, further rAAV particle purification is via cation exchange chromatography, followed by purification via size exclusion chromatography (SEC), followed by purification via anion exchange chromatography.

In yet further embodiments of all aspects and embodiments, rAAV particle purification is via affinity chromatography, followed by purification via anion exchange chromatography, followed by purification via size exclusion chromatography (SEC).

In yet further embodiments of all aspects and embodiments, rAAV particle purification is via affinity chromatography, followed by purification via size exclusion chromatography (SEC), followed by purification via anion exchange chromatography.

Cation exchange chromatography functions to separate the AAV particles from cellular and other components present in the clarified lysate and/or column eluate from an affinity or size exclusion chromatography. Examples of strong cation exchange resins capable of binding rAAV particles over a wide pH range include, without limitation, any sulfonic acid based resin as indicated by the presence of the sulfonate functional group, including aryl and alkyl substituted sulfonates, such as sulfopropyl or sulfoethyl resins. Representative matrices include but are not limited to POROS HS, POROS HS 50, POROS XS, POROS SP, and POROS S (strong cation exchangers available from Thermo Fisher Scientific, Inc., Waltham, MA, USA). Additional examples include Capto S, Capto S ImpAct, Capto S ImpRes (strong cation exchangers available from GE Healthcare, Marlborough, MA, USA), and commercial DOWEX®, AMBERLITE®, and AMBERLYST® families of resins available from Aldrich Chemical Company (Milliwaukee, WI, USA). Weak cation exchange resins include, without limitation, any carboxylic acid based resin.

Exemplary cation exchange resins include carboxymethyl (CM), phospho (based on the phosphate functional group), methyl sulfonate (S) and sulfopropyl (SP) resins.

Anion exchange chromatography functions to separate AAV particles from proteins, cellular and other components present in the clarified lysate and/or column eluate from an affinity or cation exchange or size exclusion chromatography. Anion exchange chromatography can also be used to reduce and thereby control the amount of empty capsids in the eluate. For example, the anion exchange column having rAAV particle bound thereto can be washed with a solution comprising NaCl at a modest concentration (e.g., about 100-125 mM, such as 110-115 mM) and a portion of the empty capsids can be eluted in the flow through without substantial elution of the rAAV particles. Subsequently, rAAV particles bound to the anion exchange column can be eluted using a solution comprising NaCl at a higher concentration (e.g., about 130-300 mM NaCl), thereby producing a column eluate with reduced or depleted amounts of empty capsids and proportionally increased amounts of rAAV particles comprising an rAAV vector.

Exemplary anion exchange resins include, without limitation, those based on polyamine resins and other resins. Examples of strong anion exchange resins include those based generally on the quaternized nitrogen atom including, without limitation, quaternary ammonium salt resins such as trialkylbenzyl ammonium resins. Suitable exchange chromatography materials include, without limitation, MACRO PREP Q (strong anion-exchanger available from BioRad, Hercules, CA, USA); UNOSPHERE Q (strong anion-exchanger available from BioRad, Hercules, CA, USA); POROS 50HQ (strong anion-exchanger available from Applied Biosystems, Foster City, CA, USA); POROS XQ (strong anion-exchanger available from Applied Biosystems, Foster City, CA, USA); POROS SOD (weak anion-exchanger available from Applied Biosystems, Foster City, CA, USA); POROS 50PI (weak anion-exchanger available from Applied Biosystems, Foster City, CA, USA); Capto Q, Capto XQ, Capto Q ImpRes, and SOURCE 30Q (strong anion-exchanger available from GE healthcare, Marlborough, MA, USA); DEAE SEPHAROSE (weak anion-exchanger available from Amersham Biosciences, Piscataway, NJ, USA); Q SEPHAROSE (strong anion-exchanger available from Amersham Biosciences,

Piscataway, NJ, USA). Additional exemplary anion exchange resins include aminoethyl (AE), diethylaminoethyl (DEAE), diethylaminopropyl (DEPE) and quaternary amino ethyl (QAE).

A manufacturing process to purify recombinant AAV particles intended as a product to treat human disease should achieve the following objectives: 1) consistent particle purity, potency and safety; 2) manufacturing process scalability; and 3) acceptable cost of manufacturing.

Exemplary processes for recombinant AAV particle purification are reported in WO 2019/006390.

The below outlined recombinant adeno-associated virus particle (rAAV particle) purification and production methods are scalable up to large scale. For example, to a suspension culture of 5, 10, 10-20, 20-50, 50-100, 100-200 or more liters volume. The recombinant adeno-associated virus particle purification and production methods are applicable to a wide variety of AAV serotypes/capsid variants.

In certain embodiments of all aspects and embodiments, the purification of rAAV particles comprises the steps of:

(a) harvesting cells and/or cell culture supernatant comprising rAAV particles to produce a harvest;

(b) optionally concentrating the harvest produced in step (a) to produce a concentrated harvest;

(c) lysing the harvest produced in step (a) or the concentrated harvest produced in step (b) to produce a lysate;

(d) treating the lysate produced in step (c) to reduce contaminating nucleic acid in the lysate thereby producing a nucleic acid reduced lysate;

(e) optionally filtering the nucleic acid reduced lysate produced in step (d) to produce a clarified lysate, and optionally diluting the clarified lysate to produce a diluted clarified lysate;

(f) subjecting the nucleic acid reduced lysate of step (d), the clarified lysate of step (e), or the diluted clarified lysate produced in step (e) to a cation exchange column chromatography to produce a column eluate comprising rAAV particles, thereby separating rAAV particles from protein impurities or other production/process related impurities, and optionally diluting the column eluate to produce a diluted column eluate;

(g) subjecting the column eluate or the diluted column eluate produced in step (f) to an anion exchange chromatography to produce a second column eluate comprising rAAV particles, thereby separating rAAV particles from protein impurities or production/process related impurities, and optionally concentrating the second column eluate to produce a concentrated second column eluate;

(h) subjecting the second column eluate or the concentrated second column eluate produced in step (g) to a size exclusion column chromatography (SEC) to produce a third column eluate comprising rAAV particles, thereby separating rAAV particles from protein impurities or production/process related impurities, and optionally concentrating the third column eluate to produce a concentrated third column eluate; and

(i) filtering the third column eluate or the concentrated third column eluate produced in step (h), thereby producing purified rAAV particles;

whereby the viral genome copy number is determined with the method according to the invention in or after one or more of steps (a) to (i).

In certain embodiments, steps (a) to (f) are maintained and combined with the following steps:

(g) subjecting the column eluate or the concentrated column eluate produced in step (f) to a size exclusion column chromatography (SEC) to produce a second column eluate comprising rAAV particles, thereby separating rAAV particles from protein impurities or other production/process related

impurities, and optionally diluting the second column eluate to produce a concentrated second column eluate;

(h) subjecting the second column eluate or the diluted second column eluate produced in step (g) to an anion exchange chromatography to produce a third column eluate comprising rAAV particles thereby separating rAAV particles from protein impurities production/process related impurities and optionally diluting the third column eluate to produce a diluted third column eluate; and

(i) filtering the third column eluate or the concentrated third column eluate produced in step (h), thereby producing purified rAAV particles;

whereby the viral genome copy number is determined with the method according to the invention in or after one or more of steps (a) to (i).

In certain embodiments, steps (a) to (g) are maintained and combined with the following step:

(h) filtering the second column eluate or the concentrated second column eluate produced in step (g), thereby producing purified rAAV particles;

whereby the viral genome copy number is determined with the method according to the invention in or after one or more of steps (a) to (h).

In embodiment, steps (a) to (e) are maintained and combined with the following steps:

(f) subjecting the nucleic acid reduced lysate in step (d), or clarified lysate or diluted clarified lysate produced in step (e) to an AAV affinity column chromatography to produce a column eluate comprising rAAV particles, thereby separating rAAV particles from protein impurities or other production/process related impurities, and optionally concentrating the column eluate to produce a concentrated column eluate;

(g) subjecting the column eluate or the concentrated column eluate produced in step (f) to a size exclusion column chromatography (SEC) to produce a second column eluate comprising rAAV particles, thereby separating rAAV particles from protein impurities or other production/process related impurities, and optionally diluting the second column eluate to produce a diluted second column eluate;

(h) optionally subjecting the second column eluate or the diluted second column eluate produced in step (g) to an anion exchange chromatography to produce a third column eluate comprising rAAV particles, thereby separating rAAV particles from protein impurities or other production/process related impurities, and optionally diluting the third column eluate to produce a diluted third column eluate; and

(i) filtering the second column eluate or the diluted second column eluate produced in step (g), or filtering the third column eluate or the concentrated third column eluate produced in step (h), thereby producing purified rAAV particles;

whereby the viral genome copy number is determined with the method according to the invention in or after one or more of steps (a) to (i).

In certain embodiments of all aspects and embodiments, concentrating of step (b) and/or step (f) and/or step (g) and/or step (h) is via ultrafiltration/diafiltration, such as by tangential flow filtration (TFF).

In certain embodiments of all aspects and embodiments, concentrating of step (b) reduces the volume of the harvested cells and cell culture supernatant by about 2-20 fold.

In certain embodiments of all aspects and embodiments, concentrating of step (f) and/or step (g) and/or step (h) reduces the volume of the column eluate by about 5-20 fold.

In certain embodiments of all aspects and embodiments, lysing of the harvest produced in step (a) or the concentrated harvest produced in step (b) is by physical or chemical means. Non-limiting examples of physical means include microfluidization and homogenization. Non-limiting examples of chemical means include detergents. Detergents include non-ionic and ionic detergents. Non-limiting examples of non-ionic detergents include Triton X-100. Non-limiting examples of detergent concentration is between about 0.1 and 1.0 % (v/v) or (w/v), inclusive.

In certain embodiments of all aspects and embodiments, step (d) comprises treating with a nuclease thereby reducing contaminating nucleic acid. Non-limiting examples of a nuclease include benzonase.

In certain embodiments of all aspects and embodiments, filtering of the clarified lysate or the diluted clarified lysate of step (e) is via a filter. Non-limiting examples of filters are those having a pore diameter of between about 0.1 and 10.0 microns, inclusive.

In certain embodiments of all aspects and embodiments, diluting of the clarified lysate of step (e) is with an aqueous buffered phosphate, acetate or Tris solution. Non-limiting examples of solution pH are between about pH 4.0 and pH 7.4, inclusive. Non-limiting examples of Tris solution pH are greater than pH 7.5, such as between about pH 8.0 and pH 9.0, inclusive.

In certain embodiments of all aspects and embodiments, diluting of the column eluate of step (f) or the second column eluate of step (g) is with an aqueous buffered phosphate, acetate or Tris solution. Non-limiting examples of solution pH are between about pH 4.0 and pH 7.4, inclusive. Non-limiting examples of Tris solution pH are greater than pH 7.5, such as between about pH 8.0 and pH 9.0, inclusive.

In certain embodiments of all aspects and embodiments, the rAAV particles resulting from step (i) are formulated with a surfactant to produce a rAAV particle formulation.

In certain embodiments of all aspects and embodiments, the anion exchange column chromatography of step (f), (g) and/or (h) comprises polyethylene glycol (PEG) modulated column chromatography.

In certain embodiments of all aspects and embodiments, the anion exchange column chromatography of step (g) and/or (h) is washed with a PEG solution prior to elution of the rAAV particles from the column.

In certain embodiments of all aspects and embodiments, the PEG has an average molecular weight in a range of about 1,000 g/mol to 80,000 g/mol, inclusive.

In certain embodiments of all aspects and embodiments, the PEG is at a concentration of about 4 % to about 10 % (w/v), inclusive.

In certain embodiments of all aspects and embodiments, the anion exchange column of step (g) and/or (h) is washed with an aqueous surfactant solution prior to elution of the rAAV particles from the column.

In certain embodiments of all aspects and embodiments, the cation exchange column of step (f) is washed with a surfactant solution prior to elution of the rAAV particles from the column.

In certain embodiments of all aspects and embodiments, the PEG solution and/or the surfactant solution comprises an aqueous Tris-HCl/NaCl buffer, an aqueous phosphate/NaCl buffer, or an aqueous acetate/NaCl buffer.

In certain embodiments of all aspects and embodiments, NaCl concentration in the buffer or solution is in a range of between about 20-300 mM NaCl, inclusive, or between about 50-250 mM NaCl, inclusive.

In certain embodiments of all aspects and embodiments, the surfactant comprises a cationic or anionic surfactant.

In certain embodiments of all aspects and embodiments, the surfactant comprises a twelve carbon chained surfactant.

In certain embodiments of all aspects and embodiments, the surfactant comprises Dodecyltrimethylammonium chloride (DTAC) or Sarkosyl.

In certain embodiments of all aspects and embodiments, the rAAV particles are eluted from the anion exchange column of step (f), (g) and/or (h) with an aqueous Tris-HCl/NaCl buffer.

In certain embodiments of all aspects and embodiments, the Tris-HCl/NaCl buffer comprises 100-400 mM NaCl, inclusive, optionally at a pH in a range of about pH 7.5 to about pH 9.0, inclusive.

In certain embodiments of all aspects and embodiments, the anion exchange column of step (f), (g) and/or (h) is washed with an aqueous Tris-HCl/NaCl buffer.

In certain embodiments of all aspects and embodiments, the NaCl concentration in the aqueous Tris-HCl/NaCl buffer is in a range of about 75-125 mM, inclusive.

In certain embodiments of all aspects and embodiments, the aqueous Tris-HCl/NaCl buffer has a pH from about pH 7.5 to about pH 9.0, inclusive.

In certain embodiments of all aspects and embodiments, the anion exchange column of step (f), (g) and/or (h) is washed one or more times to reduce the amount of empty capsids in the second or third column eluate.

In certain embodiments of all aspects and embodiments, the anion exchange column wash removes empty capsids from the column prior to rAAV particle elution and/or instead of rAAV particle elution, thereby reducing the amount of empty capsids in the second or third column eluate.

In certain embodiments of all aspects and embodiments, the anion exchange column wash removes at least about 50 % of the total empty capsids from the column prior to rAAV particle elution and/or instead of rAAV particle elution, thereby reducing the amount of empty capsids in the second or third column eluate by about 50 %.

In certain embodiments of all aspects and embodiments, the NaCl concentration in the aqueous Tris-HCl/NaCl buffer is in a range of about 110-120 mM, inclusive.

In certain embodiments of all aspects and embodiments, ratios and/or amounts of the rAAV particles and empty capsids eluted are controlled by a wash buffer.

In certain embodiments of all aspects and embodiments, the rAAV particles are eluted from the cation exchange column of step (f) in an aqueous phosphate/NaCl buffer, or an aqueous acetate/NaCl buffer. Non-limiting NaCl concentration in a buffer is in a range of about 125-500 mM NaCl, inclusive. Non-limiting examples of buffer pH are between about pH 5.5 to about pH 7.5, inclusive.

In certain embodiments of all aspects and embodiments, the anion exchange column of step (f), (g) and/or (h) comprises a quaternary ammonium functional group such as quaternized polyethylenimine.

In certain embodiments of all aspects and embodiments, the size exclusion column (SEC) of step (g) and/or (h) has a separation/fractionation range (molecular weight) from about 10,000 g/mol to about 600,000 g/mol, inclusive.

In certain embodiments of all aspects and embodiments, the cation exchange column of step (f) comprises a sulfonic acid or functional group such as sulphopropyl.

In certain embodiments of all aspects and embodiments, the AAV affinity column comprises a protein or ligand that binds to AAV capsid protein. Non-limiting examples of a protein include an antibody that binds to AAV capsid protein. More specific non-limiting examples include a single-chain Llama antibody (Camelid) that binds to AAV capsid protein.

In certain embodiments of all aspects and embodiments, the method excludes a step of cesium chloride gradient ultracentrifugation.

In certain embodiments of all aspects and embodiments, the method recovers approximately 50-90 % of the total rAAV particles from the harvest produced in step (a) or the concentrated harvest produced in step (b).

In certain embodiments of all aspects and embodiments, the method produces rAAV particles having a greater purity than rAAV particles produced or purified by a single AAV affinity column purification.

In certain embodiments of all aspects and embodiments, steps (c) and (d) are performed substantially concurrently.

In certain embodiments of all aspects and embodiments, the NaCl concentration is adjusted to be in a range of about 100-400 mM NaCl, inclusive, or in a range of about 140-300 mM NaCl, inclusive, after step (c) but prior to step (f).

In certain embodiments of all aspects and embodiments, the cells are suspension growing or adherent growing cells.

In certain embodiments of all aspects and embodiments, the cells are mammalian cells. Non-limiting examples include HEK cells, such as HEK-293 cells, and CHO cells, such as CHO-K1 cells.

Methods to determine infectious titer of rAAV particles containing a transgene are known in the art (see, e.g., Zhen et al., Hum. Gene Ther. 15 (2004) 709). Methods for assaying for empty capsids and rAAV particles with packaged transgenes are known (see, e.g., Grimm et al., Gene Therapy 6 (1999) 1322-1330; Sommer et al., Malec. Ther. 7 (2003) 122-128).

To determine the presence or amount of degraded/denatured capsid, purified rAAV particle can be subjected to SDS-polyacrylamide gel electrophoresis, consisting of any gel capable of separating the three capsid proteins, for example, a gradient gel, then running the gel until sample is separated, and blotting the gel onto nylon or nitrocellulose membranes. Anti-AAV capsid antibodies are then used as primary antibodies that bind to denatured capsid proteins (see, e.g., Wobus et al., J. Viral. 74 (2000) 9281-9293). A secondary antibody that binds to the primary antibody contains a means for detecting the primary antibody. Binding between the primary and secondary antibodies is detected semi-quantitatively to determine the amount of capsids. Another method would be analytical HPLC with a SEC column or analytical ultracentrifuge.

***

In addition to the various embodiments depicted and claimed, the disclosed subject matter is also directed to other embodiments having other combinations of the features disclosed and claimed herein. As such, the particular features presented herein can be combined with each other in other manners within the scope of the disclosed subject matter such that the disclosed subject matter includes any suitable combination of the features disclosed herein. The foregoing description of specific embodiments of the disclosed subject matter has been presented for purposes of illustration and description. It is not intended to be exhaustive or to limit the disclosed subject matter to those embodiments disclosed.

All references mentioned herein are incorporated herewith by reference.

***

The following examples are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

## Examples

### General techniques

#### 1) Recombinant DNA techniques

Standard methods are used to manipulate DNA as described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y, (1989). The molecular biological reagents are used according to the manufacturer's instructions.

#### 2) DNA and protein sequence analysis and sequence data management

The EMBOSS (European Molecular Biology Open Software Suite) software package, Invitrogen's Vector NTI and Geneious Prime and are used for sequence creation, mapping, analysis, annotation and illustration.

**3) Gene and oligonucleotide synthesis**

Desired gene segments are prepared by chemical synthesis at Geneart GmbH (Regensburg, Germany). The synthesized gene fragments are cloned into an E. coli plasmid for propagation/amplification. The DNA sequences of subcloned gene fragments are verified by DNA sequencing. Alternatively, short synthetic DNA fragments are assembled by annealing chemically synthesized oligonucleotides or via PCR. The respective oligonucleotides are prepared by metabion GmbH (Planegg-Martinsried, Germany).

**4) Reagents**

All commercial chemicals, antibodies and kits are used as provided according to the manufacturer's protocol if not stated otherwise.

**5) Cloning**

**General**

For the plasmids, a cloning strategy via restriction enzymes was used. By selection of suitable restriction enzymes, the wanted gene of interest can be cut out and afterwards inserted into a different plasmid by ligation. Therefore, enzymes cutting in a multiple cloning site (MCS) are preferably used and chosen in a smart manner, so that a ligation of the fragments in the correct array can be conducted. If plasmid and fragment are previously cut with the same restriction enzyme, the sticky ends of fragment and plasmid fit perfectly together and can be ligated by a DNA ligase, subsequently. After ligation, competent E. coli cells are transformed with the newly generated plasmid.

**Cloning via Restriction digestion**

For the digest of plasmids with restriction enzymes the following components are pipetted together on ice:

**Table 11**: Restriction Digestion Reaction Mix

| component | ng (set point) | µL |
|---|---|---|
| purified DNA | tbd | tbd |
| CutSmart Buffer (10x) | | 5 |
| Restriction Enzyme | | 1 |
| PCR-grade Water | | ad 50 |
| Total | | 50 |

If more enzymes are used in one digestion, 1 µL of each enzyme is used and the volume is adjusted by addition of more or less PCR-grade water. All enzymes are selected on the preconditions that they are qualified for the use with CutSmart buffer from new England Biolabs (100 % activity) and have the same incubation temperature (all 37 °C).

Incubation is performed using thermomixers or thermal cyclers, allowing incubating the samples at a constant temperature (37 °C). During incubation the samples are not agitated. Incubation time is set at 60 min. Afterwards the samples are directly mixed with loading dye and loaded onto an agarose electrophoresis gel or stored at 4 °C/on ice for further use.

A 1% agarose gel is prepared for gel electrophoresis. Therefore, 1.5 g of multi-purpose agarose are weighed into a 125 Erlenmeyer shake flask and filled up with 150 mL TAE-buffer. The mixture is heated up in a microwave oven until the agarose is completely dissolved. 0.5 µg/mL ethidium bromide are added into the agarose solution. Thereafter the gel is cast in a mold. After the agarose is set, the mold is placed into the electrophoresis chamber and the chamber is filled with TAE-buffer. Afterwards the samples are loaded. In the first pocket (from the left), an appropriate DNA molecular weight marker is loaded, followed by the samples. The gel is run for around 60 minutes at <130 V. After electrophoresis, the gel is removed from the chamber and analyzed in an UV-Imager.

The target bands are cut and transferred to 1.5 mL Eppendorf tubes. For purification of the gel, the QIAquick Gel Extraction Kit from Qiagen is used according to the manufacturer's instructions. The DNA fragments are stored at -20 °C for further use.

The fragments for the ligation are pipetted together in a molar ratio of 1:2, 1:3 or 1:5 plasmid to insert, depending on the length of the inserts and the plasmid-fragments and their correlation to each other. If the fragment, that should be inserted into the plasmid is short, a 1:5-ratio is used. If the insert is longer, a smaller amount of it is used in correlation to the plasmid. An amount of 50 ng of plasmid is used in each ligation and the particular amount of insert calculated with NEBioCalculator. For ligation, the T4 DNA ligation kit from NEB is used. An example for the ligation mixture is depicted in the following Table 12.

**Table 12**: Ligation Reaction Mix

| component | ng (set point) | conc. [ng/µL] | µL |
|---|---|---|---|
| T4 DNA Ligase Buffer (10x) | | | 2 |
| Plasmid DNA (4000 bp) | 50 | 50 | 1 |
| Insert DNA (2000 bp) | 125 | 20 | 6.25 |
| Nuclease-free Water | | | 9.75 |
| T4 Ligase | | | 1 |
| Total | | | 20 |

All components are pipetted together on ice, starting with the mixing of DNA and water, addition of buffer and finally addition of the enzyme. The reaction is gently mixed by pipetting up and down, briefly microfuged and then incubated at room temperature for 10 minutes. After incubation, the T4 ligase is heat inactivated at 65 °C for 10 minutes. The sample is chilled on ice. In a final step, 10-beta competent E. coli cells are transformed with 2 µL of the ligated plasmid (see below).

**Transformation 10-beta competent E. coli cells**

For transformation, the 10-beta competent E. coli cells are thawed on ice. After that, 2 µL of plasmid DNA is pipetted directly into the cell suspension. The tube is flicked and put on ice for 30 minutes. Thereafter, the cells are placed into a 42 °C thermal block and heat-shocked for exactly 30 seconds. Directly afterwards, the cells are chilled on ice for 2 minutes. 950 µL of NEB 10-beta outgrowth medium are added to the cell suspension. The cells are incubated under shaking at 37 °C for one hour. Then, 50-100 µL are pipetted onto a pre-warmed (37 °C) LB-Amp agar plate and spread with a disposable spatula. The plate is incubated overnight at 37 °C. Only bacteria, which have successfully incorporated the plasmid, carrying the resistance

gene against ampicillin, can grow on these plates. Single colonies are picked the next day and cultured in LB-Amp medium for subsequent plasmid preparation.

**Bacterial culture**

Cultivation of E. coli is done in LB-medium, short for Luria Bertani, which is spiked with 1 mL/L 100 mg/mL ampicillin resulting in an ampicillin concentration of 0.1 mg/mL. For the different plasmid preparation quantities, the following amounts are inoculated with a single bacterial colony.

**Table 13**: E. coli cultivation volumes

| Quantity plasmid preparation | Volume LB-Amp medium [mL] | Incubation time [h] |
|---|---|---|
| Mini-Prep 96-well (EpMotion) | 1.5 | 23 |
| Mini-Prep 15 mL-tube | 3.6 | 23 |
| Maxi-Prep | 200 | 16 |

For Mini-Prep, a 96-well 2 mL deep-well plate is filled with 1.5 mL LB-Amp medium per well. The colonies are picked and the toothpick is tuck in the medium. When all colonies are picked, the plate is closed with a sticky air porous membrane. The plate is incubated in a 37 °C incubator at a shaking rate of 200 rpm for 23 hours.

For Mini-Preps a 15 mL-tube (with a ventilated lid) is filled with 3.6 mL LB-Amp medium and equally inoculated with a bacterial colony. The toothpick is not removed but left in the tube during incubation. Like the 96-well plate, the tubes are incubated at 37 °C, 200 rpm for 23 hours.

For Maxi-Prep 200 mL of LB-Amp medium are filled into an autoclaved glass 1 L Erlenmeyer flask and are inoculated with 1 mL of bacterial day-culture, that is roundabout 5 hours old. The Erlenmeyer flask is closed with a paper plug and incubated at 37 °C, 200 rpm for 16 hours.

**Plasmid preparation**

For Mini-Prep, 50 µL of bacterial suspension are transferred into a 1 mL deep-well plate. After that, the bacterial cells are centrifuged down in the plate at 3000 rpm, 4 °C for 5 min. The supernatant is removed and the plate with the bacteria pellets is

placed into an EpMotion. After approx. 90 minutes, the run is done and the eluted plasmid-DNA can be removed from the EpMotion for further use.

For Mini-Prep, the 15 mL tubes are taken out of the incubator and the 3.6 mL bacterial culture is splitted into two 2 mL Eppendorf tubes. The tubes are centrifuged at 6,800xg in a tabletop microcentrifuge for 3 minutes at room temperature. After that, Mini-Prep is performed with the Qiagen QIAprep Spin Miniprep Kit according to the manufacturer's instructions. The plasmid DNA concentration is measured with Nanodrop.

Maxi-Prep is performed using the Macherey-Nagel NucleoBond® Xtra Maxi EF Kit according to the manufacturer's instructions. The DNA concentration is measured with Nanodrop.

**Ethanol precipitation**

The volume of the DNA solution is mixed with the 2.5-fold volume ethanol 100 %. The mixture is incubated at -20 °C for 10 min. Then the DNA is centrifuged for 30 min. at 14,000 rpm, 4 °C. The supernatant is carefully removed and the pellet is washed with 70 % ethanol. Again, the tube is centrifuged for 5 min. at 14,000 rpm, 4 °C. The supernatant is carefully removed by pipetting and the pellet is dried. When the ethanol is evaporated, an appropriate amount of endotoxin-free water is added. The DNA is given time to re-dissolve in the water overnight at 4 °C. A small aliquot is taken and the DNA concentration is measured with a Nanodrop device.

**Expression cassette composition**

For the expression of an open reading frame, a transcription unit comprising at least the following functional elements is used:

- a promoter,
- a nucleic acid comprising the respective open reading frame including signal sequences, if required,
- a polyadenylation signal sequence.

Beside the expression unit/cassette including the desired gene to be expressed, the basic/standard mammalian expression plasmid contains

- an origin of replication from the plasmid pUC18 which allows replication of this plasmid in E. coli, and
- a beta-lactamase gene which confers ampicillin resistance in E. coli.

**6) Cell culture techniques**

Standard cell culture techniques are used as described in Current Protocols in Cell Biology (2000), Bonifacino, J.S., Dasso, M., Harford, J.B., Lippincott-Schwartz, J. and Yamada, K.M. (eds.), John Wiley & Sons, Inc.

**Transient transfections in HEK293 system**

Cells producing a recombinant AAV particle have been generated by transient transfection with the respective plasmids using the HEK293 system (Invitrogen, now Thermo Scientific) according to the manufacturer's instruction. Briefly, HEK293 cells (Invitrogen) growing in suspension either in a shake flask or in a stirred fermenter in serum-free FreeStyle™ 293 expression medium (Invitrogen) are transfected with a mix of the respective plasmids and 293fectin™ or fectin (Invitrogen). For 2 L shake flask (Corning) HEK293 cells are seeded at a density of $1*10^6$ cells/mL in 600 mL and are incubated at 120 rpm, 8 % $CO_2$. The day after the cells are transfected at a cell density of ca. $1.5*10^6$ cells/mL with ca. 42 mL mix of A) 20 mL Opti-MEM (Invitrogen) with 600 µg total plasmid DNA (1 µg/mL) and B) 20 mL Opti-MEM + 1.2 mL 293 fectin or fectin (2 µL/mL). According to the glucose consumption, glucose solution is added during the course of the fermentation.

**Example 1**

**no pre-processing**

procedure:

- mix 90 µL H2O and 10 µL sample
- incubate at 95 °C for 15 minutes

example:

1. process sample
2. prepare PCR mastermix
3. add mastermix to plate
4. prepare 1:10 dilutions with water
5. add template to plate
6. seal plate and vortex (1 min at 2.200 rpm) and centrifuge
7. droplet formation (20 µL final mix + 70 µL oil) and transfer to plate (42 µL) with automated droplet generator (Auto-DG)
8. seal plate and start PCR run

**Example 2**

**heat denaturation**

procedure:

- incubate the sample at 98 °C for 10 minutes

example:

1. heat denaturation
2. prepare PCR mastermix
3. add mastermix to plate
4. prepare 1:10 dilutions with water
5. add template to plate
6. seal plate and vortex (1 min at 2.200 rpm) and centrifuge
7. droplet formation (20 µL final mix + 70 µL oil) and transfer to plate (42 µL) with Auto-DG
8. seal plate and start PCR run

**Example 3**

**DNase I digestion**

reagents:

1) DNase I buffer (Promega): 400 mM Tris-HCl, pH 8, 100 mM $MgSO_4$, 10 mM $CaCl_2$

2) DNase I (Promega): 50 U/mL diluted to 1 U/µL

procedure (50 µL reaction volume):

- mix 30 µL H2O, 5 µL DNase I buffer, 5 µL DNase I, 10 µL sample
- incubate at 37 °C for 30 min.
- heat to 95 °C for 15 min.

procedure (100 µL reaction volume):

- mix 75 µL H2O, 10 µL DNase I buffer, 5 µL DNase I, 10 µL sample
- incubate at 37 °C for 30 min.
- heat to 95 °C for 15 min.

example:

1. DNase I digest;
2. add 50 µL water to the reaction mixture
3. prepare PCR mastermix
4. add PCR mastermix to plate (16.5 µL per well)
5. prepare 1:10 dilutions: 10 µL sample/plasmid/standard + 90 µL H2O
6. add template to plate (5.5 µL per well)
7. seal plate and vortex (1 min at 2.200 rpm) and centrifuge (1 min. at 1000 rcf)
8. droplet formation (20 µL final mix + 70 µL oil) and transfer to plate (42 µL) with Auto-DG
9. seal plate and start PCR run

**Example 4**

**Proteinase K digestion**

reagents:

1) Proteinase K (Roche; 17.8 mg/mL =≥ 50 U/mL): diluted to 1 U / mL
2) proteinase K buffer (BioRad): 400 mM Tris-HCl, 20 mM EDTA, 2000 mM NaCl, pH 8
3) Sodium dodecyl sulfate solution (SDS-solution): 10 %

procedure (water):

- mix 68 µL water with 10 µL sample and add 20 µL proteinase K
- incubate at 50 °C for 60 minutes
- heat to 95 °C for 15 minutes

procedure (buffer without SDS):

- mix 63 µL water with 5 µL proteinase K buffer and add 10 µL sample as well as 20 µL proteinase K
- incubate at 50 °C for 60 minutes
- heat to 95 °C for 15 minutes

procedure (buffer with SDS):

- mix 53 µL water with 5 µL proteinase K buffer and add 10 µL SDS solution, 10 µL sample as well as 20 µL proteinase K
- incubate at 50 °C for 60 minutes
- heat to 95 °C for 15 minutes

example:

1. Proteinase K digest
2. prepare PCR mastermix
3. add mastermix to plate (16.5 µL per well)
4. prepare 1:10 dilutions: 10 µL sample + 90 µL H2O

5. add template to plate (5.5 µL per well)
6. seal plate and vortex (1 min. at 2.200 rpm) and centrifuge (1 min at 1000 rcf)
7. droplet formation (20 µL final mix + 70 µL oil) and transfer to plate (42 µL) with Auto-DG
8. seal plate and start PCR run

**Example 5**

**heat denaturation followed by proteinase K digestion**

reagents:

1) Proteinase K (Roche; 17.8 mg/mL =≥ 50 U/mL): 1 U / mL
2) proteinase K buffer (BioRad): 400 mM Tris-HCl, 20 mM EDTA, 2000 mM NaCl, pH 8

procedure:

- heat denaturation: incubate the sample at 98 °C for 10 minutes
- proteinase K digest: 1 µL proteinase K per 50 µL sample; incubate at 50 °C for 30 min.; inactivate at 95 °C for 10 min.

example:

1. heat denaturation
2. proteinase K digest
3. prepare 1:10 dilutions: 10 µL sample + 90 µL H2O
4. prepare PCR mastermix
5. add mastermix to plate
6. add sample to plate
7. seal plate and vortex (1 min. at 2.200 rpm) and centrifuge
8. droplet formation (20 µL final mix + 70 µL oil) and transfer to plate (42 µL) with Auto-DG
9. seal plate and start PCR run

**Example 6**

**DNase I digestion followed by proteinase K digestion**

**METHOD 1:**

reagents:

1) DNase I buffer (Promega): 400 mM Tris-HCl, pH 8, 100 mM $MgSO_4$, 10 mM $CaCl_2$
2) DNase I (Promega): 1 U/µL
3) Proteinase K (Roche; 17.8 mg/mL =≥ 50 U/mL): 1 U / mL
4) proteinase K buffer (BioRad): 400 mM Tris-HCl, 20 mM EDTA, 2000 mM NaCl, pH 8
5) Sodium dodecyl sulfate solution (SDS-solution): 10 %

procedure:

- mix 30 µL H2O, 5 µL DNase I buffer, 5 µL DNase I, 10 µL sample
- incubate at 37 °C for 30 min.
- heat to 95 °C for 15 min.
- mix 50 µL PK-Mix (42 µL H2O + 2 µL proteinase K + 5 µL 20x proteinase K buffer + 1 µL 10 % SDS solution) with 50 µL incubated DNase I-Mix
- incubate for 60 min. at 50 °C
- heat to 95 °C for 15 min.

example:

1. DNase I digest; optionally dilute 1:10
2. Proteinase K digest; dilute 1:10
3. prepare PCR mastermix
4. add PCR mastermix to plate (16.5 µL per well)
5. prepare 1:10 dilutions: 10 µL sample + 90 µL H2O
6. add template to plate (5.5 µL per well)
7. seal plate and vortex (1 min, at 2.200 rpm) and centrifuge (1 min. at 1000 rcf)

8. droplet formation (20 µL final mix + 70 µL oil) and transfer to plate (42 µL) with Auto-DG
9. seal plate and start PCR run

**METHOD 2:**

reagents:

1) Proteinase K (NEB; approx. 20 mg/mL =≥ 800 U/mL): diluted to 16 U / mL
2) proteinase K buffer (BioRad): 400 mM Tris-HCl, 20 mM EDTA, 2000 mM NaCl
3) Sodium dodecyl sulfate solution (SDS-solution): 10 %

procedure (buffer with SDS):

- mix 42 µL water with 50 µL sample (DNase I digestion solution) and add 2 µL proteinase K, 5 µL proteinase K buffer and 1 µL SDS solution
- incubate at 50 °C for 60 minutes
- heat to 95 °C for 15 minutes

example:

1. DNase I digestion
2. proteinase K digestion
3. dilute 1:10
4. prepare PCR mastermix
5. add mastermix to plate (16.5 µL per well)
6. prepare 1:10 dilutions: 10 µL sample + 90 µL H2O
7. add template to plate (5.5 µL per well)
8. seal plate and vortex (1 min. at 2.200 rpm) and centrifuge (1 min at 1000 rcf)
9. droplet formation (20 µL final mix + 70 µL oil) and transfer to plate (42 µL) with Auto-DG
10. seal plate and start PCR run

**Example 7**

**Comparison of conditions**

The reagents and procedures were as in the previous examples outlined.

The conditions were according to the following Tables 14 and 15.

**Table 14:**

| **condition** | **A** | **B** |
|---|---|---|
| enzyme | no DNase I | DNase I: 1 U/ µL |
| buffer | - | DNase I buffer (Promega) |
| procedure | 90 µL H2O + 10 µL sample | 75 µL H2O + 10 µL buffer + 5 µL DNase I + 10 µL sample |
| incubation temperature | - | 37 °C |
| incubation time | - | 30 min |
| inactivation | 95 °C 15 min | 95 °C 15 min |

**Table 15:**

| **condition** | **W** | **X** | **Y** | **Z** |
|---|---|---|---|---|
| enzyme | no proteinase K (PK) | 1 U/mL proteinase K | 1 U/mL proteinase K | 1 U/mL proteinase K |
| buffer | - | H2O | proteinase K buffer | proteinase K buffer 10% SDS |

| condition | W | X | Y | Z |
|---|---|---|---|---|
| procedure | 90 µL H2O + 10 µL sample | 68 µL H2O + 10 µL sample + 20 µL PK | 63 µL H2O + 5 µL PK-buffer + 10 µL sample + 20 µL PK | 53 µL H2O + 5 µL PK-buffer + 10 µL 10% SDS + 10 µL sample + 20 µL PK |
| incubation temperature | - | 50 °C | 50 °C | 50 °C |
| incubation time | - | 60 min | 60 min | 60 min |
| inactivation | 95 °C 15 min, 4 °C | 95 °C 15 min, 4 °C | 95 °C 15 min, 4 °C | 95 °C 15 min, 4 °C |

**Example 8**

**ddPCR**

For viral genome titration, a duplexing ddPCR assay was performed. Primer and probes were designed against ITR sites and against the Amp resistance sequence, which is present on the backbone of all three plasmids used in the rAAV production. The PCR mastermix was prepared according to Table 16 (droplet digital PCR guide - Bio-Rad).

**Table 16:** ddPCR mastermix composition.

| components | volume per well [µL] | final concentration |
|---|---|---|
| Supermix (2x) | 11 | 1x |
| 20 µM ITR primer fwd | 0.99 | 900 nM |
| 20 µM ITR primer rev | 0.99 | 900 nM |
| 20 µM FAM-labeled ITR probe | 0.275 | 250 nM |
| 20 µM Amp primer fwd | 0.99 | 900 nM |
| 20 µM Amp primer rev | 0.99 | 900 nM |
| 20 µM HEX-labeled Amp probe | 0.275 | 250 nM |
| template | 5.5 | 1*10E4-1*10E5 copies/mL |
| water | 0.99 | - |

| components | volume per well [µL] | final concentration |
|---|---|---|
| total | 22 | |

The prepared mastermix was pipetted into a 96 well plate with 16.5 µL per well. Then, dilution series of the pretreated samples were conducted: 10 µL of samples were transferred with LoRentention Tips into 90 µL water in LoBind Tubes and thoroughly mixed. Thereafter, 5.5 µL of the samples were added to the mastermix solution in the 96 well plate in several dilution steps. The plate was sealed at 180 °C, vortexed at 2,200 rpm for 1 min. and centrifuged at 1,000 rpm for another 1 min. With an automatic droplet generator device, which takes 20 µL PCR mix out of each well, up 20,000 droplets per well were produced and transferred into another 96 well plate. After sealing the droplet plate at 180 °C, a PCR run was carried out. The respective conditions are shown in Table 17.

**Table 17:** ddPCR thermal cycling program.

| number of cycles | denaturation | annealing | final elongation | end |
|---|---|---|---|---|
| 1 | 95 °C, 10 min | | | |
| 40 | 94 °C, 30 sec | 60 °C, 1 min | | |
| 1 | | | 98 °C, 10 min | 12 °C, maintained |

In a droplet reader, the fluorescence signal was measured for each droplet in the FAM and HEX channel. The QuantaSoft software processed the reader data and calculated copy numbers per 20 µL well for both target sequences, ITR sites and Amp. Initial sample titers can be determined with following equation 1:

$$copy\ number\ \left[\frac{copies}{mL}\right] = \frac{output\left[\frac{copies}{20\mu L\ well}\right]}{5\left[\frac{\mu L\ sample}{20\mu L\ well}\right]} \cdot dilution\ factor \cdot\ 1000\left[\frac{\mu L}{mL}\right] \qquad (2)$$

## Example 9

### rAAV production

HEK293-F suspension cells were transfected with three plasmids, i.e. pAAV-transgene (EGFP or EBFP), pAAV-rep/cap and pAAV-helper. Plasmid DNA (1 µg/1 mL cell culture) and lipofection reagent PEI pro (2 µL/1 mL cell culture)

were separately mixed with OptiMEM (50 µL/1 mL cell culture) (see, e.g., Grieger, J., et al. 2016). Afterwards, both solutions were combined, incubated at RT for 15 min. and added to HEK293-F cell suspension with 1*10E6 cell/mL in F17 medium. The cells were incubated at 37 °C, 8 % CO2, 120 rpm for 48 to 72 hours (Grieger, J., et al. (2016)).

Recombinant AAV particles were harvested by addition of a lysis buffer (100 µL/1 mL cell culture) containing 1 % Triton X-100, 500 mM TRIS and 20 mM $MgCl_2$ at pH 7.5. Freshly diluted Benzonase was added (10 µL/1 mL cell culture) at a final concentration of 50 U/mL. After 60 min. lysis at 37 °C with agitation, $MgSO_4$ (final concentration 37.5 mM) was added and the cell lysis broth was incubated for another 30 min. (Chahal, P., et al. (2014)). Afterwards, the lysis suspension was centrifuged at 4,000 g for 20 min. and the supernatant was filtered through a 0.22 µm filter. The obtained product was considered as crude lysate.

**Example 10**

**rAAV purification**

A YMC glass column body was packed with POROS CaptureSelect AAVx affinity resin in a column bed volume of 9.1 mL. These resin beads are coated with an antibody fragment that binds a broad range of AAV serotypes with a high specificity (POROS CaptureSelect AAV Resins – User Guide 2017).

First, the column was equilibrated with phosphate buffered saline (PBS) to produce the right binding conditions. Then, the crude, filtered lysate was loaded with 150 cm/hour. After capturing the rAAV capsids, the column was washed with 4 column volumes (CV's) PBS, following 4 CV's 0.5 M NaCl to remove impurities like cell debris and DNA residues. Another wash step with 4 CV's PBS was performed to prepare for elution conditions (POROS CaptureSelect AAV Resins – User Guide 2017).

Afterwards, the rAAV capsids were eluted in 100 mM citric acid buffer (pH 2.4) (POROS CaptureSelect AAV Resins – User Guide 2017). Fractions within the elution peak (UV detection at $\lambda = 280$ nm) were pooled. The pH-value was raised up

to pH 7.5 using 2 M TRIS (pH 9). Finally, the eluate was sterile filtered using a syringe filter with a pore size of 0.2 µm.

## Patent Claims

1. A method for the determination of viral genome DNA copy number in a sample, wherein the method comprises the steps of

   - incubating the sample with proteinase K,
   - determining the viral genome DNA copy number by digital droplet polymerase chain reaction,

   wherein the sample is free of DNA, which is not encapsidated within a viral particle,

   wherein the incubation with proteinase K is in the presence of 0.05 (w/v) % to 1.5 (w/v) % sodium dodecyl sulfate.

2. The method according to claim 1, wherein the method comprises the following steps

   - incubating the sample with a nuclease to obtain a digested sample,
   - incubating the digested sample with proteinase K to obtain a proteinase K incubated sample,
   - determining the viral genome DNA copy number in the proteinase K incubated sample by digital droplet polymerase chain reaction.

3. The method according to claim 2, wherein the digested sample is diluted most 2.5-times for the incubation with proteinase K.

4. The method according to any one of claims 2 to 3, wherein the complete digested sample is incubated with proteinase K.

5. The method according to any one of claims 1 to 4, wherein the method is performed at a temperature of at most 95 °C except for the final elongation step of the digital droplet polymerase chain reaction.

6. The method according to any one of claims 1 to 5, wherein the total amount of proteinase K employed in the incubation is 15 mU to 35 mU.

7. The method according to any one of claims 1 to 6, wherein the volume of the sample is about 10 µL.

8. The method according to any one of claims 1 to 7, wherein the incubating with proteinase K is in a total volume of 100 µL.

9. The method according to any one of claims 2 to 8, wherein the nuclease is DNase I.

10. The method according to any one of claims 2 to 9, wherein the total amount of nuclease employed in the incubation is about 5 U.

11. The method according to any one of claims 2 to 10, wherein the incubating with the nuclease is in a total volume of 50 µL.

12. The method according to any one of claims 2 to 11, wherein the incubating with the nuclease is at final concentrations of 40 mM Tris*HCl, 10 mM $MgSO_4$, 1 mM $CaCl_2$ at a pH value of about 8.

13. The method according to any one of claims 2 to 12, wherein the incubating with the nuclease is at 37 °C for 30 minutes followed by an inactivating of the nuclease at 95 °C for 15 minutes.

14. The method according to any one of claims 1 to 13, wherein the incubating with proteinase K is at final concentrations of 20 mM Tris*HCl, 1 mM EDTA, 100 mM NaCl, 1 (w/v) % sodium dodecyl sulfate at a pH value of about 8.

15. The method according to any one of claims 1 to 13, wherein the incubating with proteinase K is at final concentrations of 30 mM Tris*HCl, 5 mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.5 mM EDTA, 50 mM NaCl, 0.1 (w/v) % sodium dodecyl sulfate at a pH value of about 8.

16. The method according to any one of claims 1 to 15, wherein the incubating with proteinase K is at 50 °C for 60 minutes followed by an inactivating of the protease at 95 °C for 15 minutes.

17. The method according to any one of claims 1 to 16, wherein all steps of the method are performed at a temperature of at most 95 °C except for the final elongation step of the digital droplet polymerase chain reaction.

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# INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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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, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| Y | BINNY CHRISTOPHER J. ET AL: "Vector systems for prenatal gene therapy: principles of adeno-associated virus vector design and production", PRENATAL GENE THERAPY, vol. 891, 1 January 2012 (2012-01-01), pages 109-131, XP055850570, the whole document | 1-17 |
| Y | WO 2007/084773 A2 (ASKLEPIO BIOPHARMACEUTICAL INC [US]; SAMULSKI RICHARD J [US] ET AL.) 26 July 2007 (2007-07-26) the whole document | 1-17 |

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

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"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

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| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Botz, Jürgen |

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | WO 03/104413 A2 (UNIV FLORIDA [US])<br>18 December 2003 (2003-12-18)<br>the whole document | 1-17 |
| Y | US 11 028 372 B2 (UNIV PENNSYLVANIA [US])<br>8 June 2021 (2021-06-08)<br>the whole document | 1-17 |
| Y | US 8 652 821 B2 (OKINO STEVEN T [US]; WANG YAN [US]; BIO RAD LABORATORIES [US])<br>18 February 2014 (2014-02-18)<br>the whole document | 1-17 |
| Y | CN 109 957 561 A (WUXI APPTEC BIOTECHNOLOGY CO LTD)<br>2 July 2019 (2019-07-02)<br>the whole document | 1-17 |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

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| | | WO 2012054613 A2 | 26-04-2012 |
| CN 109957561 A | 02-07-2019 | NONE | |

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(54)发明名称

用于确定AAV基因组的方法

(57)摘要

本文报道了一种用于确定样品中病毒基因组DNA拷贝数的方法,其中所述方法包括以下步骤:将所述样品与蛋白酶K一起孵育,以及通过数字微滴式聚合酶链式反应确定所述病毒基因组DNA拷贝数,其中所述样品不含未在病毒颗粒内衣壳化的DNA,其中与蛋白酶K一起的所述孵育在存在0.05(w/v)%至1.5(w/v)%十二烷基硫酸钠的情况下进行。

CN 118974274 A

1.一种用于确定样品中病毒基因组DNA拷贝数的方法,其中所述方法包括以下步骤:

-将所述样品与蛋白酶K一起孵育,

-通过数字微滴式聚合酶链式反应确定所述病毒基因组DNA拷贝数,

其中所述样品不含未在病毒颗粒内衣壳化的DNA,

其中与蛋白酶K一起的所述孵育在存在0.05(w/v)%至1.5(w/v)%十二烷基硫酸钠的情况下进行。

2.根据权利要求1所述的方法,其中所述方法包括以下步骤:

-将所述样品与核酸酶一起孵育以获得经消化的样品,

-将所述经消化的样品与蛋白酶K一起孵育以获得经蛋白酶K孵育的样品,

-通过数字微滴式聚合酶链式反应确定所述经蛋白酶K孵育的样品中的所述病毒基因组DNA拷贝数。

3.根据权利要求2所述的方法,其中将所述经消化的样品稀释最多2.5倍以用于与蛋白酶K一起的所述孵育。

4.根据权利要求2至3中任一项所述的方法,其中将全部经消化的样品与蛋白酶K一起孵育。

5.根据权利要求1至4中任一项所述的方法,其中所述方法在至高95℃的温度进行,除了所述数字微滴式聚合酶链式反应的最终延伸步骤之外。

6.根据权利要求1至5中任一项所述的方法,其中在所述孵育中采用的蛋白酶K的总量为15mU至35mU。

7.根据权利要求1至6中任一项所述的方法,其中所述样品的体积为约10μL。

8.根据权利要求1至7中任一项所述的方法,其中与蛋白酶K一起的所述孵育在100μL的总体积中进行。

9.根据权利要求2至8中任一项所述的方法,其中所述核酸酶为DNA酶I。

10.根据权利要求2至9中任一项所述的方法,其中在所述孵育中采用的核酸酶的总量为约5U。

11.根据权利要求2至10中任一项所述的方法,其中与所述核酸酶一起的所述孵育在50μL的总体积中进行。

12.根据权利要求2至11中任一项所述的方法,其中与所述核酸酶一起的所述孵育以40mM Tris*HCl、10mM $MgSO_4$、1mM $CaCl_2$的终浓度以约8的pH值进行。

13.根据权利要求2至12中任一项所述的方法,其中与所述核酸酶一起的所述孵育在37℃持续30分钟,然后使所述核酸酶在95℃失活15分钟。

14.根据权利要求1至13中任一项所述的方法,其中与蛋白酶K一起的所述孵育以20mM Tris*HCl、1mM EDTA、100mM NaCl、1(w/v)%十二烷基硫酸钠的终浓度以约8的pH值进行。

15.根据权利要求1至13中任一项所述的方法,其中与蛋白酶K一起的所述孵育以30mM Tris*HCl、5mM $MgSO_4$、0.5mM $CaCl_2$、0.5mM EDTA、50mM NaCl、0.1(w/v)%十二烷基硫酸钠的终浓度以约8的pH值进行。

16.根据权利要求1至15中任一项所述的方法,其中与蛋白酶K一起的所述孵育在50℃持续60分钟,然后使所述蛋白酶在95℃失活15分钟。

17.根据权利要求1至16中任一项所述的方法,其中所述方法的所有步骤在至高95℃的

温度进行,除了所述数字微滴式聚合酶链式反应的最终延伸步骤之外。

# 用于确定AAV基因组的方法

[0001] 本发明属于基因疗法领域。更详细地,本文报道了用于利用ddPCR来确定过程和纯化样品中的病毒基因组拷贝数的方法,其中在存在洗涤剂的情况下在PCR之前将样品与蛋白酶K一起孵育。

## 背景技术

[0002] 腺相关病毒(AAV)颗粒因其良好的安全性概况、高治疗功效和靶标特异性工程化的可能性,通常用作用于研究和临床方法中的基因转移媒介物。对于重组生产,需要准确且稳健的用于病毒载体表征的分析方法。通常,载体基因组滴定是通过微滴式数字PCR(ddPCR)进行的,这是一种对核酸进行绝对定量的方法。

[0003] 在文献中,针对实时和微滴式数字PCR二者,报道了几种用于AAV载体基因组滴定的预处理方法。在对衣壳内的靶序列进行绝对定量之前,需要用核酸酶如DNA酶I降解游离瞬时质粒和其他未包装的核酸(如宿主细胞DNA)(Zolotukhin,S.等人1999;Furuta-Hanawa,B.等人2019;Fripont,S.等人2019;Dobnik,D.等人2019;Sanmiguel J.等人2019)。此外,衣壳需要被降解以获得更好的载体基因组可及性。AAV颗粒的热灭活以及随之产生的其衣壳蛋白的变性足以达到此目的(Wang,Y.,等人2019)。然而,在大多数公开中,描述了用蛋白酶K进行额外的蛋白质消化(Zolotukhin,S.等人1999;Fripont,S.等人2019;Dobnik,D.等人2019;Sanmiguel J.等人2019)。此外,对于用于经处理样品的后续稀释系列的稀释缓冲液存在各种建议:除了无核酸酶水(Zolotukhin,S.等人1999)、TE缓冲液(Furuta-Hanawa,B.等人2019;Wang,Y.等人2019)或PCR缓冲液(Sanmiguel J.等人2019)以外,在文献中还描述了添加剂如抗表面活性剂Pluronic F-68(Furuta-Hanawa,B.等人2019;Sanmiguel J.等人2019)和剪切鲑鱼精子(sss)DNA(Lock,M.等人2014;Sanmiguel J.等人2019)。

[0004] Suoranta,T.等人(Hum.Gen.Ther.32(2020)1270-1279)通过热变性、蛋白酶K处理以及使用qPCR和ddPCR的试剂盒提取比较了在磷酸盐缓冲盐水溶液中进行碘克沙醇梯度超速离心后AAV1、AAV2、AAV5、AAV6、AAV8和AAV9基因组的提取。试剂盒提取(其在旋转柱纯化之前在变性缓冲液中在存在额外载剂RNA的情况下含有蛋白酶K处理)显著增加了qPCR和ddPCR二者中针对所有血清型获得的滴度。重要的是,Suoranta等人发现尚无研究提出有关ddPCR中基因组可用性的结论性数据。

[0005] CN 109957561公开了一种用于从样品中提取核酸的方法,其中该方法包括以下步骤:向待提取样品中添加裂解溶液以释放核酸分子;进一步添加月桂酰肌氨酸钠盐溶液;进一步添加含有碘化钠、糖原和异丙醇的混合溶液,从而形成含有核酸的沉淀,通过离心样品回收该沉淀。

[0006] US 8,652,821公开了一种用于纯化不含RNA的DNA的试剂混合物,其包含蛋白酶、RNA酶和洗涤剂。

[0007] US11,028,372公开了一种用于血清型rh.10的AAV颗粒的可扩展纯化方法。

[0008] WO 03/104413公开了一种假型重组AAV病毒体的未进一步具体说明的斑点印迹分

析,其包括蛋白酶K孵育,之后是苯酚提取和乙醇沉淀。

[0009] WO 2007/084773公开了一种在昆虫细胞中生产的感染性细小病毒载体的未进一步具体说明的斑点印迹分析。

[0010] Binny,C.J.和Nathwani,A.C.公开了一种琼脂糖凝胶分析方法,其用于确定包装到scAAV载体中的基因组如预期的那样是短双链发夹结构还是短ssDNA还是长非折叠ssDNA(Meth.Mol.Biol.891(2012)109-131)。

[0011] US2021/0284699公开了一种用于分析已使用包括以下步骤的方法纯化的rAAV颗粒的qPCR方法:(a)生成包含多个本文提供的rAAV的病毒颗粒提取物,其中该病毒颗粒提取物包含经裂解的生产细胞的上清液或其衍生物;(b)使该病毒颗粒提取物与离子洗涤剂接触以生成第一混合物;(c)使第一混合物与酸接触以生成第二混合物;(d)离心第二混合物以生成上清液;(e)用一个或多个过滤器过滤上清液以生成滤液;(f)进行将滤液缓冲液交换为最终储存缓冲液的一次或多次循环。

## 发明内容

[0012] 本文报道了一种用于确定样品中病毒基因组DNA拷贝数的方法,其中该方法包括以下步骤:将样品与蛋白酶K一起孵育以及通过数字微滴式聚合酶链式反应确定病毒基因组DNA拷贝数,其中该样品不含未在病毒颗粒内衣壳化的DNA,其中与蛋白酶K一起的孵育是在存在0.05(w/v)%至1.5(w/v)%十二烷基硫酸钠的情况下进行的。

[0013] 非限制数量的方面(独立主题)和实施例(从属主题)为:

[0014] 1.一种用于确定样品中病毒基因组DNA拷贝数的方法,其中该方法包括以下步骤:

[0015] -通过数字微滴式聚合酶链式反应确定所述病毒基因组DNA拷贝数,

[0016] 其中所述样品不含未在病毒颗粒内衣壳化的DNA,

[0017] 其中所述样品尚未与核酸酶一起孵育,并且

[0018] 其中所述样品尚未与蛋白酶一起孵育。

[0019] 2.根据方面1所述的方法,其中所述核酸酶为限制酶。

[0020] 3.根据方面1或实施例2中任一项所述的方法,其中所述核酸酶为DNA酶I。

[0021] 4.根据方面1或实施例2至3中任一项所述的方法,其中所述蛋白酶为蛋白酶K。

[0022] 5.一种用于确定样品中病毒基因组DNA拷贝数的方法,其中所述方法

[0023] 包括以下步骤:

[0024] -将所述样品与蛋白酶K一起孵育,

[0025] -通过数字微滴式聚合酶链式反应确定所述病毒基因组DNA拷贝数。

[0026] 6.根据方面5所述的方法,其中所述样品为细胞裂解物。

[0027] 7.根据方面5或实施例6中任一项所述的方法,其中所述样品为经裂解的细胞样品。

[0028] 8.根据实施例7所述的方法,其中所述经裂解的细胞样品已通过用洗涤剂或通过化学手段裂解生产病毒的细胞获得;任选地,细胞碎片已从所述经裂解的细胞样品中去除。

[0029] 9.根据方面5或实施例6至8中任一项所述的方法,其中所述样品包含病毒颗粒且不含未在病毒颗粒内衣壳化的DNA,所述病毒DNA基因组在所述病毒颗粒中衣壳化。

[0030] 10.一种用于确定样品中病毒基因组DNA拷贝数的方法,其中所述方法包括以下步

骤:

[0031] -将所述样品与蛋白酶K一起孵育,

[0032] -通过数字微滴式聚合酶链式反应确定所述病毒基因组DNA拷贝数,

[0033] 其中所述样品不含未在病毒颗粒内衣壳化的DNA,

[0034] 其中与蛋白酶K一起的所述孵育是在存在洗涤剂的情况下进行的。

[0035] 11.根据方面10所述的方法,其中所述洗涤剂为十二烷基硫酸钠。

[0036] 12.根据方面10或实施例11中任一项所述的方法,其中在与蛋白酶K一起孵育期间所述洗涤剂的终浓度为0.05(w/v)%至1.5(w/v)%

[0037] 13.根据方面10或实施例11至12中任一项所述的方法,其中在与蛋白酶K一起孵育期间所述洗涤剂的终浓度为约0.1(w/v)%。

[0038] 14.根据方面10或实施例11至12中任一项所述的方法,其中在与蛋白酶K一起孵育期间所述洗涤剂的终浓度为约1(w/v)%。

[0039] 15.根据方面5或方面10或实施例6至9或实施例11至14中任一项所述的方法,其中所述方法包括以下步骤:

[0040] -将所述样品与核酸酶一起孵育以获得经消化的样品,

[0041] -将所述经消化的样品与蛋白酶K一起孵育以获得经蛋白酶K孵育的样品,

[0042] -通过数字微滴式聚合酶链式反应确定所述经蛋白酶K孵育的样品中的所述病毒基因组DNA拷贝数。

[0043] 16.根据实施例15所述的方法,其中将所述经消化的样品稀释最多2.5倍以用于与蛋白酶K一起的所述孵育。

[0044] 17.根据实施例15至16中任一项所述的方法,其中将全部所述经消化的样品与蛋白酶K一起孵育。

[0045] 18.根据方面1或方面5或方面10或实施例2至4或实施例6至9或实施例11至17中任一项所述的方法,其中所述数字微滴式聚合酶链式反应包括以下步骤:

[0046] a)将所述样品在约95℃孵育10分钟,

[0047] b)进行以下热循环:将所述样品在约94℃孵育30秒,之后将所述样品在约60℃孵育1分钟,

[0048] c)重复步骤b)15至60次,

[0049] d)在98℃进行最终延伸步骤10分钟。

[0050] 19.根据实施例18所述的方法,其中在步骤a)和d)中以2℃的温度斜升进行。

[0051] 20.根据方面1或方面5或方面10或实施例2至4或实施例6至9或实施例11至19中任一项所述的方法,其中将所述样品维持在95℃或更低的温度。

[0052] 21.根据方面1或方面5或方面10或实施例2至4或实施例6至9或实施例11至19中任一项所述的方法,其中不将所述样品暴露于高于95℃的温度。

[0053] 22.根据方面1或方面5或方面10或实施例2至4或实施例6至9或实施例11至19中任一项所述的方法,其中所述方法在至高95℃的温度进行,除了所述数字微滴式聚合酶链式反应的所述最终延伸步骤之外。

[0054] 23.根据方面5或方面10或实施例2至4或实施例6至9或实施例11至22中任一项所述的方法,其中在所述孵育中采用的蛋白酶K的总量为1mU至50mU。

[0055] 24.根据方面5或方面10或实施例2至4或实施例6至9或实施例11至23中任一项所述的方法,其中在所述孵育中采用的蛋白酶K的总量为10mU至40mU。

[0056] 25.根据方面5或方面10或实施例2至4或实施例6至9或实施例11至24中任一项所述的方法,其中在所述孵育中采用的蛋白酶K的总量为15mU至35mU。

[0057] 26.根据方面5或方面10或实施例2至4或实施例6至9或实施例11至25中任一项所述的方法,其中在所述孵育中采用的蛋白酶K的总量为20mU至32mU。

[0058] 27.根据方面5或方面10或实施例2至4或实施例6至9或实施例11至26中任一项所述的方法,其中在所述孵育中采用的蛋白酶K的总量为约20mU。

[0059] 28.根据方面5或方面10或实施例2至4或实施例6至9或实施例11至26中任一项所述的方法,其中在所述孵育中采用的蛋白酶K的总量为约32mU。

[0060] 29.根据方面1或方面5或方面10或实施例2至4或实施例6至9或实施例11至28中任一项所述的方法,其中所述病毒基因组DNA拷贝数的所述确定是所述病毒基因组DNA拷贝数的定量。

[0061] 30.根据方面1或方面5或方面10或实施例2至4或实施例6至9或实施例11至29中任一项所述的方法,其中所述样品的体积为约10μL。

[0062] 31.根据方面5或方面10或实施例2至4或实施例6至9或实施例11至30中任一项所述的方法,其中与蛋白酶K一起的所述孵育在100μL的总体积中进行。

[0063] 32.根据实施例15至31中任一项所述的方法,其中所述核酸酶为DNA酶I。

[0064] 33.根据实施例15至32中任一项所述的方法,其中在所述孵育中采用的核酸酶的总量为约5U。

[0065] 34.根据实施例15至33中任一项所述的方法,其中与所述核酸酶一起的所述孵育在50μL的总体积中进行。

[0066] 35.根据实施例15至34中任一项所述的方法,其中与所述核酸酶一起的所述孵育以40mM Tris*HCl、10mM $MgSO_4$、1mM $CaCl_2$的终浓度以约8的pH值进行。

[0067] 36.根据实施例15至35中任一项所述的方法,其中与所述核酸酶一起的所述孵育在37℃持续30分钟。

[0068] 37.根据实施例36所述的方法,其中在所述孵育之后是使所述核酸酶在95℃失活15分钟。

[0069] 38.根据实施例15至37中任一项所述的方法,其中与蛋白酶K一起的所述孵育以20mM Tris*HCl、1mM EDTA、100mM NaCl的终浓度以约8的pH值进行。

[0070] 39.根据实施例15至37中任一项所述的方法,其中与蛋白酶K一起的所述孵育以20mM Tris*HCl、1mM EDTA、100mM NaCl的终浓度以约8的pH值进行。

[0071] 40.根据实施例15至37中任一项所述的方法,其中与蛋白酶K一起的所述孵育以20mM Tris*HCl、1mM EDTA、100mM NaCl、1(w/v)%十二烷基硫酸钠的终浓度以约8的pH值进行。

[0072] 41.根据实施例15至37中任一项所述的方法,其中与蛋白酶K一起的所述孵育以30mM Tris*HCl、5mM $MgSO_4$、0.5mM $CaCl_2$、0.5mM EDTA、50mM NaCl、0.1(w/v)%十二烷基硫酸钠的终浓度以约8的pH值进行。

[0073] 42.根据实施例15至41中任一项所述的方法,其中与蛋白酶K一起的所述孵育在50

℃持续60分钟。

[0074] 43.根据实施例42所述的方法,其中在所述孵育之后是使所述蛋白酶在95℃失活15分钟。

[0075] 44.根据方面1或方面5或方面10或实施例2至4或实施例7至9或实施例11至43中任一项所述的方法,其中所述体积的样品为经亲和色谱法纯化的细胞裂解物。

[0076] 45.根据方面1或方面5或方面10或实施例2至4或实施例6至9或实施例11至43中任一项所述的方法,其中所述方法在不存在沉淀步骤或/和糖原的情况下进行。

## 具体实施方式

[0077] 本发明至少部分基于以下发现:对于AAV基因组拷贝数确定,在粗细胞裂解物样品包含AAV颗粒并且还包含非病毒衣壳化DNA的情况下,样品必须在PCR之前与蛋白酶K一起孵育。洗涤剂的存在可以进一步改善该确定。

[0078] 本发明至少部分基于以下发现:对于AAV基因组拷贝数确定,在经纯化的样品包含AAV颗粒并且基本上不含非病毒衣壳化的DNA的情况下,样品必须在PCR之前与蛋白酶K一起孵育。不受该理论的束缚,假设洗涤剂阻止了蛋白质片段和病毒基因组DNA的聚集体的形成,这阻止了随后通过聚合酶链式反应进行的扩增,并且从而导致对样品中病毒基因组拷贝数的低估。

[0079] 本发明至少部分基于以下发现:对于用核酸酶和蛋白酶连续处理后的AAV基因组拷贝数确定,蛋白酶处理必须在洗涤剂存在下进行。不受该理论的束缚,假设洗涤剂阻止了蛋白质片段和病毒基因组DNA的聚集体的形成,这阻止了随后通过聚合酶链式反应进行的扩增,并且从而导致样品中病毒基因组拷贝数的低估。

[0080] 本发明进一步至少部分基于以下发现:对于包含AAV颗粒并且基本上不含非病毒衣壳化的DNA的经纯化的样品,AAV基因组拷贝数的确定必须在没有核酸酶和蛋白酶预处理的情况下进行,或者在聚合酶链式反应之前,在洗涤剂的存在下进行蛋白酶K孵育。不受该理论的束缚,假设高度纯化的样品(即,不包含基本量的非病毒衣壳化的DNA)与单独的核酸酶或核酸酶与蛋白酶的组合在不存在洗涤剂的情况下一起孵育导致聚集,并且从而人为降低了AAV基因组拷贝数。

[0081] 本发明进一步至少部分基于以下发现:对于包含AAV颗粒并且基本上不含非病毒衣壳化的DNA的经纯化的样品,AAV基因组拷贝数的确定必须在没有热变性步骤的情况下在高于95℃的温度进行。不受该理论的束缚,假设高度纯化的样品(即,不包含基本量的非病毒衣壳化的DNA)在高于95℃的温度的热处理导致聚集,并且从而人为降低了AAV基因组拷贝数。

[0082] 本发明进一步至少部分基于在存在洗涤剂的情况下低量的蛋白酶K足以使样品中基本上所有AAV基因组可用于聚合酶链式反应并由此进行确定/定量。不受该理论的束缚,假设高浓度的蛋白酶K干扰聚合酶链式反应,并且通过使用显著减少的量(即低量)的蛋白酶K通过减少PCR抑制引起聚合酶链式反应的改进。尤其是在存在洗涤剂的情况下通过蛋白酶K孵育可以减少甚至消除AAV生产细胞的细胞裂解物中的蛋白质物质对聚合酶链式反应的抑制。

[0083] 本发明进一步至少部分基于以下发现:对于粗细胞裂解物样品与核酸酶和蛋白酶

的连续孵育,样品在核酸酶孵育之后不应被稀释,并且孵育混合物的总体积应用于蛋白酶孵育步骤。

[0084] 一般来说,在ddPCR方法中确定AAV基因组拷贝数之前需要的预处理步骤越多,污染的可能性就越高。

[0085] 定义

[0086] 可用于实施本发明的方法和技术描述于:例如Ausubel,F.M.(编辑),Current Protocols in Molecular Biology,第I卷至第III卷(1997);Glover,N.D.和Hames,B.D.编辑,DNA Cloning:A Practical Approach,第I卷和第II卷(1985),Oxford University Press;Freshney,R.I.(编辑),Animal Cell Culture-a practical approach,IRL Press Limited(1986);Watson,J.D.等人,Recombinant DNA,第二版,CHSL Press(1992);Winnacker,E.L.,From Genes to Clones;N.Y.,VCH Publishers(1987);Celis,J.编辑,Cell Biology,第二版,Academic Press(1998);Freshney,R.I.,Culture of Animal Cells:A Manual of Basic Technique,第二版,Alan R.Liss,Inc.,N.Y.(1987)。

[0087] 必须注意的是,如本文和所附权利要求书中所用,单数形式“一个”、“一种”和“该/所述”包括复数指代,除非上下文另外明确规定。因此,例如,提及“一个细胞”包括多个此类细胞和本领域技术人员已知的其等同物,诸如此类。同样,术语“一个/一种”、“一个或多个/一种或多种”和“至少一个/至少一种”在本文中可以互换使用。还应当注意的是,术语“包含”、“包括”和“具有”可以互换使用。

[0088] 术语“AAV辅助功能”表示AAV衍生的编码序列(蛋白质),其可以被表达以提供AAV基因产物和AAV颗粒,该AAV基因产物和AAV颗粒又反式发挥作用以进行生产性AAV复制和包装。因此,AAV辅助功能包括AAV开放阅读框(ORF),包括rep和cap以及其他,诸如用于某些AAV血清型的AAP。已证明rep基因表达产物具有许多功能,其中包括:识别、结合和切割DNA复制的AAV起点;DNA解旋酶活性;以及调节自AAV(或其他异源)启动子的转录。cap基因表达产物(衣壳)提供必要的包装功能。AAV辅助功能用于补充AAV载体基因组中缺失的反式AAV功能。

[0089] 术语“约”表示其后所跟随的数值的+/-20%范围。在某些实施例中,术语“约”表示其后所跟随的数值的+/-10%的范围。在某些实施例中,术语“约”表示其后所跟随的数值的+/-5%的范围。

[0090] 术语“包括”还涵盖术语“包含……”。

[0091] 术语“空的衣壳”和“空的颗粒”是指AAV颗粒,其具有AAV蛋白壳但其全部或部分缺乏编码蛋白质的核酸或被转录为侧翼为AAV ITR(即载体)的目标转录物的核酸。因此,空的衣壳无法起到将编码蛋白质或被转录为目标转录物的核酸转移到宿主细胞中的作用。

[0092] 术语“内源性”表示某物在细胞内天然存在;由细胞天然生产;类似地,“内源性基因座/细胞内源性基因座”是细胞中天然存在的基因座。

[0093] 如本文所用,术语“外源”是指核苷酸序列并非来源于特异性细胞,而是通过DNA递送方法(例如,通过转染、电穿孔或由病毒载体进行的转导)引入所述细胞中。因此,外源核苷酸序列是人工序列,其中人工性可以源自例如不同来源的子序列的组合(例如,具有SV40启动子的重组酶识别序列与绿色荧光蛋白质的编码序列的组合是人工核酸)或源自序列(例如仅编码膜结合受体的细胞外结构域或cDNA的序列)的部分的缺失,或者核碱基突变。

术语“内源性”是指来源于细胞的核苷酸序列。“外源性”核苷酸序列可以具有碱基组成相同的“内源性”对应物,但其中该序列例如经由重组DNA技术通过引入到细胞中变为“外源性”序列。

[0094] “经分离的”组合物是已与其天然环境的一种或多种组分分离的组合物。在一些实施例中,将组合物纯化至大于95%或99%的纯度,如通过例如电泳(例如,SDS-PAGE、等电聚焦(IEF)、毛细管电泳、CE-SDS)或色谱法(例如,尺寸排阻色谱法或离子交换或反相HPLC)确定的。关于用于评估例如抗体纯度的方法的综述,参见Flatman,S.等人,J.Chrom.B 848(2007)79-87。

[0095] “经分离的”核酸是指已与其自然环境的一种或多种组分分离的核酸分子。经分离的核酸包括这样的核酸分子,其包含在通常含有核酸分子的细胞中,但该核酸分子存在于染色体外或与其天然染色体位置不同的染色体位置处。

[0096] “经分离的”多肽或抗体是指已与其天然环境的一种或多种组分分离的多肽分子或抗体分子。

[0097] 术语“包含外源性核苷酸序列的哺乳动物细胞”涵盖已引入一种或多种外源性核酸的细胞,包括此类细胞的后代。这些可以作为进一步基因修饰的起点。因此,术语“包含外源性核苷酸序列的哺乳动物细胞”涵盖包含整合在所述哺乳动物细胞基因组的基因座内的单个位点处的外源性核苷酸序列的细胞,其中该外源性核苷酸序列至少包含侧接至少一个第一选择标志物的第一重组识别位点和第二重组识别位点(这些重组识别位点是不同的)。在某些实施例中,包含外源性核苷酸序列的哺乳动物细胞是包含整合在所述细胞基因组的基因座内的单个位点处的外源性核苷酸序列的细胞,其中该外源性核苷酸序列包含侧接至少一个第一选择标志物的第一重组识别序列和第二重组识别序列,以及位于第一重组识别序列与第二重组识别序列之间的第三重组识别序列,并且所有重组识别序列都不同。

[0098] “包含外源性核苷酸序列的哺乳动物细胞”和“重组细胞”都是“转化细胞”。该术语包括原代转化细胞以及由其衍生的后代,而不考虑传代次数。子代可能例如不与亲本细胞的核酸内容物完全一致,而是可能含有突变。涵盖了具有与在最初转化的细胞中的功能或生物活性相同的功能或生物活性的突变体后代。

[0099] “编码AAV包装蛋白的核酸”通常是指一种或多种核酸分子,其包括从AAV载体中删除的提供AAV功能的核苷酸序列,该核酸分子用于生产转导活性重组AAV颗粒。编码AAV包装蛋白的核酸通常用于提供AAV rep和/或cap基因的表达,以补充AAV复制所需的缺失的AAV功能;然而,核酸构建体缺乏AAV ITR,并且既不能自我复制也不能自我包装。编码AAV包装蛋白的核酸可以呈质粒、噬菌体、转座子、粘粒、病毒或颗粒的形式。已经描述了许多核酸构建体,诸如常用的质粒pAAV/Ad和pIM29+45,它们编码rep基因表达产物和cap基因表达产物二者。参见例如Samulski等人,J.Virol.63(1989)3822-3828;和McCarty等人,J.Virol.65(1991)2936-2945.已经描述了许多编码rep和/或cap基因表达产物的质粒(例如,US 5,139,941和US 6,376,237)。这些编码AAV包装蛋白的核酸中的任一种可以包含根据本发明的DNA元件或核酸。

[0100] 术语“编码辅助蛋白的核酸”通常指一种或多种核酸分子,其包括编码提供腺病毒辅助功能的蛋白质和/或RNA分子的核苷酸序列。具有编码辅助蛋白的核酸的质粒可以转染到合适的细胞中,其中该质粒然后能够支持在所述细胞中生产AAV颗粒。这些编码辅助蛋白

的核酸中的任一种可以包含根据本发明的DNA元件或核酸。该术语明确排除感染性病毒颗粒,因为它们存在于大自然中,诸如腺病毒、疱疹病毒或牛痘病毒颗粒。

[0101] 如本文所用,术语“可操作地连接”是指两种或更多种组分的并置,其中这些组分的关系允许它们以预期的方式发挥作用。例如,如果启动子和/或增强子起到调节编码序列/开放阅读框/基因转录的作用,则该启动子和/或增强子与编码序列/开放阅读框/基因可操作地连接。在某些实施例中,“可操作地连接”的DNA序列相邻。在某些实施例中,例如,当必须接合两个蛋白质编码区(诸如分泌前导区和多肽)时,这些序列是相邻的,并且在同一阅读框中。在某些实施例中,可操作地连接的启动子位于编码序列/开放阅读框/基因上游并且可以与该编码序列/开放阅读框/基因相邻。在某些实施例中,例如,关于调节编码序列/开放阅读框/基因表达的增强子序列,这两种组分可以可操作地连接,但并不相邻。如果增强子增加编码序列/开放阅读框/基因的转录,则该增强子与编码序列/开放阅读框/基因可操作地连接。可操作地连接的增强子可以位于编码序列/开放阅读框/基因的上游、内部或下游,并且可以位于与编码序列/开放阅读框/基因的启动子距离相当远的位置。

[0102] 术语“包装蛋白”是指非AAV衍生的病毒和/或细胞功能,AAV的复制依赖于该功能。因此,该术语体现了AAV复制所需的蛋白质和RNA,包括参与AAV基因转录激活、阶段特异性AAV mRNA剪接、AAV DNA复制、Cap表达产物合成和AAV衣壳组装的那些部分。基于病毒的辅助功能可以源自已知的辅助病毒中的任一者,诸如腺病毒、疱疹病毒(I型单纯疱疹病毒除外)和牛痘病毒。

[0103] 如本文所用,“AAV包装蛋白”是指AAV衍生的序列,其反式发挥作用以进行生产性AAV复制。因此,AAV包装蛋白由主要的AAV开放阅读框(ORF)、rep和cap编码。已证明rep蛋白具有许多功能,其中包括:识别、结合和切割DNA复制的AAV起点;DNA解旋酶活性;以及调节自AAV(或其他异源)启动子的转录。cap(衣壳)蛋白提供必要的包装功能。AAV包装蛋白在本文中用于补充AAV载体中缺失的反式AAV功能。

[0104] “质粒”是核酸或多核苷酸的一种形式,其通常具有用于质粒表达(例如,转录、复制等)或增殖(复制)的附加元件。如本文所用,质粒也可以用于指代此类核酸或多核苷酸序列。因此,在所有方面,本发明的组合物和方法适用于核酸、多核苷酸以及质粒,例如以用于产生生产病毒(例如,AAV)载体的细胞、产生病毒(例如,AAV)颗粒、产生包含病毒(例如,AAV)颗粒的细胞培养基等。

[0105] 如本文所用,术语“重组细胞”表示最终遗传修饰后的细胞,诸如例如表达目标多肽或生产目标rAAV颗粒并且可以用于以任何规模生产所述目标多肽或目标rAAV颗粒的细胞。例如,已进行过重组酶介导的盒式交换(RMCE),由此目标多肽的编码序列已被引入宿主细胞基因组中的“包含外源性核苷酸序列的哺乳动物细胞”为“重组细胞”。尽管该细胞仍能够进行进一步的RMCE反应,但并不希望这样做。

[0106] “重组AAV载体”通过使用分子方法以从病毒(例如AAV)中去除野生型基因组并用非天然核酸(诸如转录为转录物或编码蛋白质的核酸)替换该野生型基因组而源自病毒(诸如AAV)的野生型基因组。对于AAV,通常会在重组AAV载体中保留野生型AAV基因组的一个或两个反向末端重复(ITR)序列。“重组”AAV载体区别于野生型病毒AAV基因组,因为病毒基因组的全部或部分已经替换为相对于病毒基因组核酸的非天然(即,异源)序列。因此,非天然序列的掺入将病毒载体(例如,AAV)定义为“重组”载体,在AAV的情况下该载体可以称为

“rAAV载体”。

[0107] 重组载体(例如,AAV)序列可以被包装-在本文中称为“颗粒”-以用于随后离体、体外或体内的细胞感染(转导)。当重组载体序列被封装或包装到AAV颗粒中时,该颗粒也可以称为“rAAV”。此类颗粒包括封装或包装载体基因组的蛋白质。具体示例包括病毒包膜蛋白,并且在AAV的情况下,包括衣壳蛋白,诸如AAV VP1、VP2和VP3。

[0108] 如本文所用,术语“选择标志物”表示这样的基因:其允许在相应的选择性试剂的存在下特异性选择或排除携带该基因的细胞。例如,但不作为限制,选择标志物可以允许在相应选择性试剂(选择性培养条件)的存在下正选择用该选择标志物基因转化的宿主细胞;未转化的宿主细胞将不能在该选择性培养条件下生长或存活。选择标志物可以是正的、负的或双功能的。正选择标志物可以允许选择携带该标记的细胞,而负选择标志物可以允许选择性地消除携带该标记的细胞。选择标志物可以赋予对药物的抗性,或者补偿宿主细胞中的代谢或分解代谢缺陷。在原核细胞中,可以使用赋予对氨苄青霉素、四环素、卡那霉素或氯霉素的抗性的基因,以及其他基因。可用作真核细胞中的选择标志物的抗性基因包括但不限于针对氨基糖苷磷酸转移酶(APH)(例如,潮霉素磷酸转移酶(HYG)、新霉素和G418 APH)、二氢叶酸还原酶(DHFR)、胸苷激酶(TK)、谷氨酰胺合成酶(GS)、天冬酰胺合成酶、色氨酸合成酶(吲哚)、组氨醇脱氢酶(组氨醇D)的基因,以及编码对嘌呤霉素、杀稻瘟菌素、博来霉素、腐草霉素、氯霉素、Zeocin和霉酚酸的抗性的基因。另外的标记基因描述于WO 92/08796和WO 94/28143中。

[0109] 除有助于在存在相应选择性试剂的情况下进行选择之外,选择标志物还可以替代性地为通常不存在于细胞中的分子,例如绿色荧光蛋白质(GFP)、增强的GFP(eGFP)、合成的GFP、黄色荧光蛋白质(YFP)、增强的YFP(eYFP)、青色荧光蛋白质(CFP)、mPlum、mCherry、tdTomato、mStrawberry、J-red、DsRed单体、mOrange、mKO、mCitrine、Venus、YPet、Emerald、CyPet、mCFPm、Cerulean和T-Sapphire。可以例如分别通过检测到编码的多肽所发出的荧光或不存在这种荧光,来将表达这种分子的细胞与不含该基因的细胞区分开来。

[0110] 如本文所用,术语“血清型”是基于AAV衣壳在血清学上不同的区别。血清学的区别性基于一种AAV的抗体相比于另一种AAV的抗体之间缺乏交叉反应性来确定。此类交叉反应性差异通常是由于衣壳蛋白序列/抗原决定簇的差异(例如,由于AAV血清型的VP1、VP2和/或VP3序列差异)。尽管包括衣壳变体在内的AAV变体可能在血清学上与参考AAV或其他AAV血清型没有区别,但与参考AAV血清型或其他AAV血清型相比,它们至少有一个核苷酸或氨基酸残基不同。

[0111] 在传统定义下,血清型是指已经针对血清测试了目标病毒,该血清对所有现有的和经表征的血清型具有特异性以用于中和活性,并且没有发现中和目标病毒的抗体。随着发现更多天然存在的病毒分离物和/或生成衣壳突变体,与当前存在的血清型中的任一者可能存在或可能不存在血清学差异。因此,在新病毒(例如AAV)不具有血清学差异的情况下,该新病毒(例如AAV)将为对应的血清型的亚组或变体。在许多情况下,尚未对具有衣壳序列修饰的突变病毒执行中和活性的血清学测试,以确定它们是否具有根据血清型的传统定义的另一种血清型。因此,为了方便和避免重复,术语“血清型”广泛地指血清学上不同的病毒(例如AAV)以及血清学上并无不同的病毒(例如AAV)两者,它们可能在给定血清型的亚组或变体内。

[0112] 术语“转导”和“转染”是指将分子诸如核酸(病毒载体、质粒)引入细胞。当外源核酸已经被引入细胞膜内时,细胞已经被“转导”或“转染”。因此,“转导细胞”为其中已经被引入“核酸”或“多核苷酸”的细胞,或其中已经被引入外源核酸的其子代。在特定的实施例中,“转导”细胞(例如,在哺乳动物中,诸如细胞或组织或器官细胞)在掺入外源性分子例如核酸(例如,转基因)之后具有遗传变化。“转导”细胞可以进行繁殖并且转录导入的核酸和/或表达蛋白质。

[0113] 在“转导”或“转染”细胞中,核酸(病毒载体、质粒)可以或可以不整合至基因组核酸中。如果引入的核酸整合至受体细胞或生物体的核酸(基因组DNA)中,则它可以稳定地维持在该细胞或生物体中,并且进一步传递至受体细胞或生物体的子代细胞或生物体上或由其遗传.最后,引入的核酸可以在受体细胞或宿主生物体中存在于染色体外,或仅瞬时存在。许多技术为已知的,例如参见,Graham等人,Virology 52(1973)456;Sambrook等人(1989)Molecular Cloning,a laboratory manual,Cold Spring Harbor Laboratories,New York;Davis等人(1986)Basic Methods in Molecular Biology,Elsevier;以及Chu等人,Gene 13(1981)197。此类技术可以用于将一个或多个外源DNA部分引入合适的宿主细胞。

[0114] 术语“转基因”在本文中用于方便地指旨在或已经被引入细胞或生物体中的核酸。转基因包括任何核酸,诸如转录为转录物或者编码多肽或蛋白质的基因。

[0115] “载体”是指重组质粒序列的一部分,其最终直接或者以单链或RNA的形式被包装或封装以形成病毒(例如,AAV)颗粒。在重组质粒用于构建或制造重组病毒颗粒的情况下,病毒颗粒不包括与重组质粒的载体序列不对应的“质粒”部分。重组质粒的该非载体部分称为“质粒骨架”,该质粒骨架对于质粒的克隆和扩增很重要,这是繁殖和重组病毒生产所需的过程,但它本身并没有被包装或封装至病毒(例如AAV)颗粒中。因此,“载体”是指由病毒颗粒(例如,AAV)包装或封装的核酸。

[0116] 重组细胞

[0117] 一般而言,为了高效且大规模地生产目标蛋白质化合物,诸如例如rAAV颗粒或治疗性多肽,表达并且如果可能还分泌所述蛋白质化合物的细胞是必需的。此类细胞被称为“重组细胞”或“重组生产细胞”。

[0118] 为了生成“重组生产细胞”,用编码所述目标蛋白质化合物的所需核酸序列转染合适的哺乳动物细胞。可能需要转染额外的辅助多肽。

[0119] 为了生成稳定的重组生产细胞,接下来进行第二步,其中选择稳定表达目标蛋白质化合物的单细胞。这可以例如基于选择标志物的共表达来完成,该选择标志物已经与编码目标蛋白质化合物的核酸序列共转染,或者为蛋白质化合物本身的表达。

[0120] 对于表达编码序列,即开放阅读框,需要额外的调控元件,诸如启动子和多聚腺苷酸化信号(序列)。因此,开放阅读框可操作地连接至所述额外的调控元件以进行转录。这可以通过将其整合到所谓的表达盒中来实现。表达盒在哺乳动物细胞中起作用所需的最少调控元件是在所述哺乳动物细胞中起作用的启动子,该启动子位于开放阅读框的上游,即5',以及在所述哺乳动物细胞中起作用的多聚腺苷酸化信号(序列),该多聚腺苷酸化信号位于开放阅读框的下游,即3'。此外,终止子序列可能存在于多聚腺苷酸化信号(序列)的3'。对于表达,启动子、开放阅读框/编码区和多聚腺苷酸化信号序列必须以可操作连接的形式排

列。

[0121] 同样,转录成编码非蛋白质的RNA的核酸称为“RNA基因”。同样对于RNA基因的表达,需要额外的调控元件,诸如启动子和转录终止信号或多聚腺苷酸化信号(序列)。此类元件的性质和定位取决于旨在驱动RNA基因表达的RNA聚合酶。因此,RNA基因通常也整合到表达盒中。

[0122] 如果目标蛋白质化合物是AAV颗粒,该AAV颗粒由不同的(单体)衣壳多肽和单链DNA分子构成,并且另外需要其他腺病毒辅助功能来进行生产和封装,则需要多个表达盒,这些表达盒的不同之处在于所含的开放阅读框/编码序列。在这种情况下,至少需要用于转基因、形成AAV载体的衣壳的不同多肽、所需的辅助功能以及VA RNA中的每一者的表达盒。因此,需要用于辅助E1A、E1B、E2A、E4orf6、VA RNA、rep和cap基因中的每一者的单独表达盒。

[0123] 如前面段落中概述的,分别地,目标蛋白质化合物越复杂或额外所需的辅助多肽和/或RNA的数量越高,所需的不同表达盒的数量越高。核酸的总大小也与表达盒的数量固有地相关。然而,可转移的核酸大小存在实际上限,其范围为约15kbps(千碱基对)。超过此限制,处理和加工效率就会大幅下降。该问题可以通过使用两个或更多个单独的质粒来解决。由此,不同的表达盒被分配给不同的质粒,由此每个质粒仅包含表达盒中的一些。

[0124] 在所有方面和实施例的某些实施例中,表达盒中的每一者沿5’至3’方向包含启动子、开放阅读框/编码序列或RNA基因和多聚腺苷酸化信号序列、和/或终止子序列。在某些实施例中,开放阅读框编码多肽并且表达盒包含具有或不具有附加终止子序列的多聚腺苷酸化信号序列。在某些实施例中,表达盒包含RNA基因,启动子是2型Pol III启动子并且存在多聚腺苷酸化信号序列或polyU终止子。参见例如Song等人Biochemical and Biophysical Research Communications 323(2004)573-578。在某些实施例中,表达盒包含RNA基因,启动子是2型Pol III启动子和polyU终止子序列。

[0125] 在所有方面和实施例的某些实施例中,开放阅读框编码多肽,启动子为具有或没有内含子A的人CMV启动子,多聚腺苷酸化信号序列为bGH(牛生长激素)polyA信号序列,并且终止子为hGT(人胃泌素终止子)。

[0126] 在所有方面和实施例的某些实施例中,启动子是具有内含子A的人CMV启动子,多聚腺苷酸化信号序列是bGH多聚腺苷酸化信号序列并且终止子是hGT,RNA基因的表达盒和选择标志物的表达盒除外,其中对于选择标志物,启动子是SV40启动子并且多聚腺苷酸化信号序列是SV40多聚腺苷酸化信号序列并且不存在终止子,并且其中对于RNA基因,启动子是野生型2型聚合酶III启动子并且终止子是聚合酶II或III终止子。

[0127] 腺相关病毒(AAV)

[0128] AAV和腺病毒或疱疹辅助功能的一般综述参见Berns和Bohensky,Advances in Virus Research,Academic Press.,32(1987)243-306。Srivastava等人,J.Virol.,45(1983)555-564中描述了AAV的基因组。US 4,797,368中描述了用于构建重组AAV载体的设计注意事项(也参见WO 93/24641)。描述AAV载体的其他参考文献为West等人,Virol.160(1987)38-47;Kotin,Hum.Gene Ther.5(1994)793-801;和Muzyczka J.Clin.Invest.94(1994)1351。US 5,173,414;Lebkowski等人,Mol.Cell.Biol.8(1988)3988-3996;Tratschin等人,Mol.Cell.Biol.5(1985)3251-3260;Tratschin等人,Mol.Cell.Biol.,4

(1994)2072-2081;Hermonat和Muzyczka Proc.Natl.Acad.Sci.USA 81(1984)6466-6470;Samulski等人J.Virol.63(1989)3822-3828中描述了重组AAV载体的构建

[0129] 腺相关病毒(AAV)是复制缺陷型细小病毒。它只能在细胞中复制,其中某些病毒功能由共感染的辅助病毒提供,诸如腺病毒、疱疹病毒以及在一些情况下的痘病毒,诸如牛痘。尽管如此,只要存在适当的辅助病毒功能,AAV几乎可以在人类、猿猴或啮齿动物来源的任何细胞系中复制。

[0130] 如果不存在辅助病毒基因,则AAV在其宿主细胞中建立潜伏期。其基因组整合到19号染色体中的特定位点[(Chr)19(q13.4)],称为腺相关病毒整合位点1(AAVS1)。对于特定血清型,诸如AAV-2,已发现其他整合位点,诸如例如在5号染色体上[(Chr)5(p13.3)],称为AAVS2,以及在3号染色体上[(Chr)3(p24.3)],称为AAVS3。

[0131] AAV分为不同的血清型。这些是根据参数诸如血凝、致瘤性和DNA序列同源性进行分配的。迄今为止,已鉴定出超过10种不同的血清型和超过一百种对应AAV不同分支的序列。

[0132] 衣壳蛋白类型和对称性决定了相应AAV的组织向性。例如,AAV-2、AAV-4和AAV-5对视网膜具有特异性,AAV-2、AAV-5、AAV-8、AAV-9和AAVrh-10对脑具有特异性,AAV-1、AAV-2、AAV-6、AAV-8和AAV-9对心脏组织具有特异性,AAV-1、AAV-2、AAV-5、AAV-6、AAV-7、AAV-8、AAV-9和AAV-10对肝脏具有特异性,AAV-1、AAV-2、AAV-5和AAV-9对肺具有特异性。

[0133] 假型包装表示包括AAV基因组在不同血清型之间交叉包装的过程,即基因组用不同来源的衣壳蛋白包装。

[0134] 野生型AAV基因组大小为约4.7kb。AAV基因组进一步包含两个重叠基因,称为rep和cap,其包含多个开放阅读框(参见例如,Srivastava等人,J.Viral.,45(1983)555-564;Hermonat等人,J.Viral.51(1984)329-339;Tratschin等人,J.Virol.,51(1984)611-619)。编码Rep蛋白的开放阅读框提供了四种不同大小的蛋白质,它们被称为Rep78、Rep68、Rep52和Rep40。这些涉及AAV的复制、拯救和整合。编码Cap蛋白的开放阅读框提供四种蛋白质,称为VP1、VP2、VP3和AAP。VP1、VP2和VP3是AAV颗粒的蛋白质衣壳的一部分。组合的rep和cap开放阅读框在其5'端和3'端侧接所谓的反向末端重复序列(ITR)。对于复制,AAV除了Rep和Cap蛋白之外还需要腺病毒的基因E1A、E1B、E4orf6、E2A和VA的产物或另一辅助病毒的相应因子。

[0135] 例如,在血清型2(AAV-2)的AAV的情况下,ITR各自具有145个核苷酸的长度并且侧接约4470个核苷酸的编码序列区。ITR的145个核苷酸中,125个核苷酸具有回文序列,并且可以形成T形发夹结构。该结构在病毒复制期间具有引物的功能。其余20个非配对核苷酸表示为D序列。

[0136] AAV基因组含有三个转录启动子P5、P19和P40(Laughlin等人,Proc.Natl.Acad.Sci.USA 76(1979)5567-5571)以用于表达rep和cap基因。

[0137] ITR序列必须以顺式存在于编码区。ITR提供功能性复制起点(ori),整合到靶细胞基因组中所需的信号,以及自宿主细胞染色体或重组质粒的有效切除和拯救。ITR进一步包含复制起点样元件,诸如Rep蛋白结合位点(RBS)和末端解析位点(TRS)。已经发现ITR本身可以具有AAV载体中转录启动子的功能(Flotte等人,J.Biol.Chem.268(1993)3781-3790;Flotte等人,Proc.Natl.Acad.Sci.USA 93(1993)10163-10167)。

[0138] 对于病毒单链DNA基因组的分别复制和衣壳化,需要rep和cap基因产物的反式组织。

[0139] rep基因座包含两个内部启动子,称为P5和P19。它包含针对四种蛋白质的开放阅读框。启动子P5可操作地连接至提供编码Rep蛋白Rep78(阻滞细胞周期的染色质切口酶)的非剪接的4.2kb mRNA和编码Rep蛋白Rep68(位点特异性核酸内切酶)的剪接的3.9kb mRNA的核酸序列。启动子P19可操作地连接至提供编码Rep蛋白Rep52的非剪接的mRNA和编码Rep蛋白Rep40(用于积累和包装的DNA解旋酶)的剪接的3.3kb mRNA的核酸序列。

[0140] 两个较大的Rep蛋白Rep78和Rep68对于AAV双链体DNA复制至关重要,而较小的Rep蛋白Rep52和Rep40似乎对于后代单链DNA积累至关重要(Chejanovsky&Carter,Virology 173(1989)120-128)。

[0141] 较大的Rep蛋白Rep68和Rep78可以特异性结合AAV ITR的发夹构象。它们表现出确定的酶活性,这是解决AAV末端处复制所需的。Rep78或Rep68的表达可能足以形成感染性颗粒(Holscher,C.等人J.Virol.68(1994)7169-7177和69(1995)6880-6885)。

[0142] 认为所有Rep蛋白,主要是Rep78和Rep68,都表现出调控活性,诸如AAV基因的诱导和抑制以及对细胞生长的抑制作用(Tratschin等人,Mol.Cell.Biol.6(1986)2884-2894;Labow等人,Mol.Cell.Biol.,7(1987)1320-1325;Khleif等人,Virology,181(1991)738-741)。

[0143] Rep78的重组过表达产生由诱导DNA损伤引起的细胞生长减少的表型。由此,宿主细胞被阻滞在S期,从而促进病毒的潜伏感染(Berthet,C.等人,Proc.Natl.Acad.Sci.USA 102(2005)13634-13639)。

[0144] Tratschin等人报道了P5启动子由Rep78或Rep68负向自动调节(Tratschin等人,Mol.Cell.Biol.6(1986)2884-2894)。由于Rep蛋白表达的毒性作用,据报道,在AAV稳定整合后,针对某些细胞系的表达仅非常低(参见例如Mendelson等人,Virol.166(1988)154-165)。

[0145] cap基因座包含一个启动子,称为P40。启动子P40可操作地连接至提供2.6kb mRNA的核酸序列,该mRNA通过选择性剪接和使用可替代的起始密码子来编码Cap蛋白VP1(87kDa,非剪接的mRNA转录物)、VP2(72kDa,来自剪接的mRNA转录物)和VP3(61kDa,来自可替代的起始密码子)。VP1至VP3构成病毒衣壳的组成部分。衣壳具有结合至细胞表面受体并允许病毒的细胞内运输的功能。VP3占病毒颗粒总蛋白的约90%。然而,所有三种蛋白质对于有效衣壳生产都是必需的。

[0146] 据报道,所有三种衣壳蛋白VP1至VP3的失活阻止单链后代AAV DNA的积累。VP1氨基末端的突变(“Lip阴性”或“Inf阴性”)仍然允许单链DNA组装成病毒颗粒,从而大大降低感染性滴度。

[0147] AAP开放阅读框编码组装激活蛋白(AAP)。它的大小为约22kDa,并将天然VP蛋白转运到核仁区域以进行衣壳组装。该开放阅读框位于VP3蛋白编码序列的上游。

[0148] 单个AAV颗粒中仅含有一个单链DNA分子。这可以是“正”链或“负”链。含有DNA分子的AAV病毒颗粒具有感染性。在受感染的细胞内,亲本感染单链转化为双链,随后被扩增。扩增产生大量双链DNA分子,由其置换单链并包装到衣壳中。

[0149] 腺相关病毒(AAV)载体可以转导分裂细胞和静息细胞。可以假设使用AAV载体导入

靶细胞的转基因将长期表达。使用AAV载体的一个缺点是可以引入细胞的转基因大小受到限制。

[0150] 病毒载体诸如细小病毒颗粒,包括AAV血清型及其变体,提供了将核酸离体、体外和体内递送至编码蛋白质的细胞中的方式,使得细胞表达所编码的蛋白质。AAV是可用作基因疗法载体的病毒,因为它们可以穿透细胞并引入核酸/遗传物质,使得核酸/遗传物质可以稳定地维持在细胞中。此外,例如,这些病毒可以将核酸/遗传物质引入特定位点。由于AAV与人类的致病性疾病无关,因此AAV载体能够向人类患者递送异源多核苷酸序列(例如,治疗性蛋白质和药剂),而不会引起实质性的AAV发病机制或疾病。

[0151] 可以使用的病毒载体包括但不限于多种血清型(例如,AAV-1至AAV-12等)的腺相关病毒(AAV)颗粒和杂合/嵌合AAV颗粒。

[0152] 有利地,AAV颗粒可以用作有效基因递送的媒介物。此类颗粒具有许多此类应用所需的特征,包括针对分裂和非分裂细胞的向性。关于这些载体的早期临床经验也表明没有持续的毒性,并且免疫应答小或无法检测到。已知AAV通过受体介导的内吞作用或通过转胞吞作用在体内和体外感染多种细胞类型。这些载体系统已在人类中靶向视网膜上皮、肝脏、骨骼肌、气道、脑、关节和造血干细胞进行了测试。

[0153] 重组AAV颗粒通常不包括与发病机制相关的病毒基因。此类载体通常具有全部或部分缺失的野生型AAV基因中的一者或多者,例如rep和/或cap基因,但保留至少一个功能性侧翼ITR序列,这是将重组载体拯救、复制和包装到AAV颗粒中所必须的。例如,仅包括载体的基本部分,例如分别为ITR和LTR元件。因此,AAV载体基因组将包括复制和包装所需的顺式序列(例如,功能性ITR序列)。

[0154] 重组AAV载体及其方法和用途包括任何病毒株系或血清型。作为非限制性实例,重组AAV载体可以基于任何AAV基因组,诸如例如AAV-1、-2、-3、-4、-5、-6、-7、-8、-9、-10、-11、-12、2i8、AAV rh74或AAV 7m8。此类载体可以基于相同的株系或血清型(或亚组或变体),或彼此不同。作为非限制性实例,基于一种血清型基因组的重组AAV载体可以与包装该载体的衣壳蛋白中的一者或多者相同。另外,重组AAV载体基因组可以基于与包装载体的AAV衣壳蛋白中的一者或多者不同的AAV(例如,AAV2)血清型基因组。例如,AAV载体基因组可以基于AAV2,而三种衣壳蛋白中的至少一者可以为AAV1、AAV3、AAV4、AAV5、AAV6、AAV7、AAV8、AAV9、AAV10、AAV11、AAV12、AAV-2i8、AAV rh74、AAV 7m8或其变体。AAV变体包括AAV1、AAV2、AAV3、AAV4、AAV5、AAV6、AAV7、AAV8、AAV9、AAV10、AAV11、AAV12、AAV-2i8、AAV rh74和AAV 7m8衣壳的变体和嵌合体。

[0155] 在所有方面和实施例的某些实施例中,腺相关病毒(AAV)载体包括AAV1、AAV2、AAV3、AAV4、AAV5、AAV6、AAV7、AAV8、AAV9、AAV10、AAV11、AAV12、AAV-2i8、AAV rh74和AAV 7m8及其变体(例如,衣壳变体,诸如氨基酸插入、添加、取代和缺失),例如,如WO 2013/158879,WO 2015/013313和US2013/0059732(公开了LK01、LK02、LK03等)所示。

[0156] AAV和AAV变体(例如,衣壳变体)血清型(例如,VP1、VP2和/或VP3序列)可以不同于或可以不不同于其他AAV血清型,包括例如AAV1-AAV12(例如,不同于AAV1至AAV12血清型中任一者的VP1、VP2和/或VP3序列)。

[0157] 在所有方面和实施例的某些实施例中,与参考血清型相关的AAV颗粒具有多核苷酸、多肽或其子序列,其包括以下项或由以下项组成:与一种或多种AAV1、AAV2、AAV3、AAV4、

AAV5、AAV6、AAV7、AAV8、AAV9、AAV10、AAV11、AAV12、AAV-2i8、AAV rh74或AAV 7m8(例如,诸如ITR,或VP1、VP2和/或VP3序列)至少80%或更多(例如,85%、90%、95%、96%、97%、98%、99%、99.1%、99.2%、99.3%、99.4%、99.5%等)相同的序列。

[0158] 本发明的组合物、方法和用途包括表现出与参考AAV血清型(诸如AAV1、AAV2、AAV3、AAV4、AAV5、AAV6、AAV7、AAV8、AAV9、AAV10、AAV11、AAV12、AAV-2i8、AAV rh74或AAV 7m8)的序列同一性小于100%,但与已知的AAV基因或蛋白质(诸如AAV1、AAV2、AAV3、AAV4、AAV5、AAV6、AAV7、AAV8、AAV9、AAV10、AAV11、AAV12、AAV-2i8、AAV rh74或AAV 7m8基因或蛋白质等)不同且不相同的AAV序列(多肽和核苷酸)及其子序列。在所有方面和实施例的某些实施例中,AAV多肽或其子序列包括以下项或由以下项组成:与任何参考AAV序列或其子序列(诸如AAV1、AAV2、AAV3、AAV4、AAV5、AAV6、AAV7、AAV8、AAV9、AAV10、AAV11、AAV12、AAV-2i8、AAV rh74或AAV 7m8(例如,VP1、VP2和/或VP3衣壳或ITR))至少75%或更多相同(例如,80%、85%、85%、87%、88%、89%、90%、91%、92%、93%、94%、95%、96%、97%、98%、99%、99.1%、99.2%、99.3%、99.4%、99.5%等、直至100%相同)的序列。在某些实施例中,AAV变体具有1、2、3、4、5、5至10、10至15、15至20或更多个氨基酸取代。

[0159] 重组AAV颗粒,包括AAV1、AAV2、AAV3、AAV4、AAV5、AAV6、AAV7、AAV8、AAV9、AAV10、AAV11、AAV12、AAV-2i8、AAV rh74或AAV 7m8,以及变体、相关、杂合和嵌合序列,可以使用本领域技术人员已知的重组技术来构建,以包括侧接一个或多个功能性AAV ITR序列的一个或多个核酸序列(转基因)。

[0160] 重组颗粒(例如,rAAV颗粒)可以掺入药物组合物中。此类药物组合物尤其可用于体内或离体向受试者施用和递送。在某些实施例中,药物组合物含有药用载剂或赋形剂。此类赋形剂包括本身不诱导对接受组合物的个体有害的免疫应答并且可以在没有过度毒性的情况下施用的任何药剂。

[0161] 用于生成腺病毒载体的方案已在US 5,998,205;US 6,228,646;US 6,093,699;US 6,100,242;WO 94/17810和WO 94/23744中描述,这些参考文献的全部内容通过引用并入本文。

[0162] 根据本发明的方法的具体实施例

[0163] 为了允许进行病毒基因组拷贝确定,必须使病毒基因组可访问,即必须打开屏蔽衣壳。为此,热变性是方便且常用的。然而,已经发现热变性会导致人为降低病毒基因组拷贝数。

[0164] 本发明人已经证明,在高于95℃(诸如例如98℃)的温度进行热变性导致确定的病毒拷贝基因组数的减少。这已使用ATCC AAV2标准VR-1616进行了例证。实验中使用的批次的标称病毒基因组拷贝数(vgcn)为3.28*10E10 vg/mL。结果在以下表1中示出。

[0165] 表1:

[0166]

| 条件 | 绝对vgcn | 相对vgcn |
|---|---|---|
| 无预处理 | 2.735-3.306*10E10/mL | 83-101% |
| 热变性,98℃,10分钟 | 1.012*10E10/mL | 31% |

[0167] 同样,本发明人已经证明,在不存在洗涤剂的情况下,在水溶液或缓冲溶液中与蛋白酶K(PK)一起孵育也会导致确定的病毒拷贝基因组数的减少。这已使用ATCC AAV2标准VR-1616进行了例证。实验中使用的批次的标称病毒基因组拷贝数(vgcn)为3.28*10E10

vg/mL。结果在以下表2中示出。

[0168] 表2：

[0169]

| 条件 | 绝对 vgcn | 相对 vgcn |
|---|---|---|
| 无预处理 | 2.735-3.306*10E10/mL | 83-101% |
| 2 mU PK，在水中，50℃，60 分钟；95℃，15 分钟；4℃ | 1.202*10E9/mL | 4% |
| 2 mU PK，在 20 mM Tris-HCl、1 mM EDTA、100 mM NaCl 中，50℃，60 分钟；95℃，15 分钟；4℃ | 1.327*10E10/mL | 40% |

[0170] 结合起来，本发明人已经证明，在高于95℃(诸如例如98℃)的温度进行热变性，即使与在没有洗涤剂的情况下在缓冲液中与蛋白酶K(PK)一起孵育的情况组合，也会导致确定的病毒拷贝基因组数的减少。这已使用ATCC AAV2标准VR-1616进行了例证。实验中使用的批次的标称病毒基因组拷贝数(vgcn)为3.28*10E10 vg/mL。结果在以下表3中示出。

[0171] 表3：

[0172]

| 条件 | 绝对 vgcn | 相对 vgcn |
|---|---|---|
| 无预处理 | 2.735-3.306*10E10/mL | 83-101% |
| 热变性，98℃，10 分钟；1 mU PK，在 20 mM Tris-HCl、1 mM EDTA、100 mM NaCl 中，50℃，60 分钟；95℃，15 分钟；4℃ | 1.814*10E10/mL | 55% |

[0173] 本发明人现已发现，确定的病毒基因组拷贝基因组数的减少可以通过进行在存在十二烷基硫酸钠(SDS)的情况下与蛋白酶K一起孵育来克服。本实例中使用ATCC AAV2标准VR-1616对此进行了证明。实验中使用的批次的标称病毒基因组拷贝数(vgcn)为3.28*10E10 vg/mL。结果在以下表4中示出。

[0174] 表4：

[0175]

| 条件 | 绝对 vgcn | 相对 vgcn |
|---|---|---|
| 无预处理 | 2.735-3.306*10E10/mL | 83-101% |
| 2 mU PK，在 20 mM Tris-HCl、1 mM EDTA、100 mM NaCl、1% SDS 中，50℃，60 分钟；95℃，15 分钟；4℃ | 3.520*10E10/mL | 107% |

[0176] 在表4的条件下获得的回收率满足根据EMA和FDA指南进行测定程序验证的验收标准,即在标称值的+/-15%内。

[0177] 本发明人已进一步表明,在病毒基因组数确定之前用DNA酶I处理导致确定的病毒拷贝基因组数的减少。这已使用ATCC AAV2标准VR-1616进行了例证。实验中使用的批次的标称病毒基因组拷贝数(vgcn)为3.28*10E10 vg/mL。结果在以下表5中示出。

[0178] 表5：

[0179]

| 条件 | 绝对 vgcn | 相对 vgcn |
|---|---|---|
| 无预处理 | 2.735-3.306*10E10/mL | 83-101% |
| 5 U DNA 酶 I，40 mM Tris-HCl，pH 8.0，10 mM MgSO4，1 mM CaCl2，37℃，30 分钟；95℃，15 分钟。 | 1.795*10E10/mL | 55% |

[0180] DNA酶I处理引起的减少与样品的进一步处理无关,即与热变性或蛋白酶K孵育无关。表6中总结了不同的测试条件,这些条件都显示出确定的病毒拷贝数的减少。已使用ATCC AAV2标准VR-1616。实验中使用的批次的标称病毒基因组拷贝数(vgcn)为3.28*10E10 vg/mL。

[0181] 表6：

[0182]

| 条件 | 绝对 vgcn | 相对 vgcn |
|---|---|---|
| 无预处理 | 2.735-3.306*10E10/mL | 83-101% |

[0183]

| 条件 | 绝对 vgcn | 相对 vgcn |
|---|---|---|
| 5 U DNA 酶 I，40 mM Tris-HCl，pH 8.0，10 mM MgSO4，1 mM CaCl2，37°C，30 分钟；95°C，15 分钟。 | 1.795*10E10/mL | 55% |
| 5 U DNA 酶 I，40 mM Tris-HCl，pH 8.0，10 mM MgSO4，1 mM CaCl2，37°C，30 分钟；95°C，15 分钟；2 mU PK，在水中，50°C，60 分钟；95°C，15 分钟；4°C | 4.947*10E9/mL | 15% |
| 5 U DNA 酶 I，40 mM Tris-HCl，pH 8.0，10 mM MgSO4，1 mM CaCl2，37°C，30 分钟；95°C，15 分钟；2 mU PK，在 20 mM Tris-HCl、1 mM EDTA、100 mM NaCl 中，50°C，60 分钟；95°C，15 分钟；4°C | 9.590*10E9/mL | 29% |
| 5 U DNA 酶 I，40 mM Tris-HCl，pH 8.0，10 mM MgSO4，1 mM CaCl2，37°C，30 分钟；95°C，15 分钟；2 mU PK，在 20 mM Tris-HCl、1 mM EDTA、100 mM NaCl、1% SDS 中，50°C，60 分钟；95°C，15 分钟；4°C | 2.347*10E10/mL | 72% |

[0184] 本发明人的发现使用生产AAV2的HEK293细胞培养的裂解物得到证实。裂解物中确定的病毒基因组拷贝数(vgcn)为1.746*10E10 vg/mL(裂解物18)。结果在以下表7中示出。

[0185] 可以看出,与蛋白酶K一起孵育允许病毒基因组回收率为96％。通过在SDS存在下将样品与蛋白酶K一起孵育可以将回收率进一步增加至100％。

[0186] 表7：

[0187]

| 条件 | 绝对 vgcn | 相对 vgcn |
|---|---|---|
| 无预处理 | 3.594*10E9/mL | 21% |
| 热变性，98℃，10 分钟 | 3.514*10E9/mL | 20% |
| 2 mU PK，在水中，50℃，60 分钟；95℃，15 分钟；4℃ | 7.636*10E9/mL | 44% |
| 热变性，98℃，10 分钟；2 mU PK，在 20 mM Tris-HCl、1 mM EDTA、100 mM NaCl 中，50℃，60 分钟；95℃，15 分钟；4℃ | 7.020*10E9/mL | 40% |
| 2 mU PK，在 20 mM Tris-HCl、1 mM EDTA、100 mM NaCl 中，50℃，60 分钟；95℃，15 分钟；4℃ | 1.671*10E10/mL | 96% |
| 2 mU PK，在 20 mM Tris-HCl、1 mM EDTA、100 mM NaCl、1% SDS 中，50℃，60 分钟；95℃，15 分钟；4℃ | 1.746*10E10/mL | 100% |

[0188] 必须指出的是,没有对蛋白酶K孵育的裂解物进行进一步纯化,即,例如,没有基于柱的纯化或提取纯化。

[0189] 经额外DNA酶I处理的确定的病毒基因组拷贝数仅在4％至16％的范围内,如下表8所示。

[0190] 表8：

[0191]

| 条件 | 绝对 vgcn | 相对 vgcn |
|---|---|---|
| 无预处理 | 3.594*10E9/mL | 21% |
| 5 U DNA 酶 I，40 mM Tris-HCl，pH 8.0，10 mM MgSO4，1 mM CaCl2，37°C，30 分钟；95°C，15 分钟。 | 6.960*10E8/mL | 4% |
| 5 U DNA 酶 I，40 mM Tris-HCl，pH 8.0，10 mM MgSO4，1 mM CaCl2，37°C，30 分钟；95°C，15 分钟；2 mU PK，在水中，50°C，60 分钟；95°C，15 分钟；4°C | 1.179*10E9/mL | 7% |
| 5 U DNA 酶 I，40 mM Tris-HCl，pH 8.0，10 mM MgSO4，1 mM CaCl2，37°C，30 分钟；95°C，15 分钟；2 mU PK，在 20 mM Tris-HCl、1 mM EDTA、100 mM NaCl 中，50°C，60 分钟；95°C，15 分钟；4°C | 1.746*10E9/mL | 10% |
| 5 U DNA 酶 I，40 mM Tris-HCl，pH 8.0，10 mM MgSO4，1 mM CaCl2，37°C，30 分钟；95°C，15 分钟；2 mU PK，在 20 mM Tris-HCl、1 mM EDTA、100 mM NaCl、1% SDS 中，50°C，60 分钟；95°C，15 分钟；4°C | 2.716*10E9/mL | 16% |

[0192] 本发明人已发现,对于与DNA酶I和蛋白酶K的连续孵育,有利的是使用DNA酶I孵育的整个孵育混合物进行蛋白酶K孵育。从而确定的病毒基因组拷贝数可以增加到既定数量的80％。裂解物中既定的病毒基因组拷贝数(vgcn)为1.746*10E10 vg/mL(裂解物18)和

2.340*10E9 vg/mL(裂解物31)。此类效果在纯化样品中可能看不到,诸如ATCC标准品(标称病毒基因组拷贝数(vgcn)为3.28*10E10 vg/mL)。结果如表9所示。

[0193] 表9:

| 条件 | 绝对 vgcn | 相对 vgcn |
|---|---|---|
| 10 μL 裂解物 18，用于 DNA 酶 I 孵育；1:10 稀释，用于 PK 孵育 | 2.716*10E9/mL | 16% |
| 10 μL 裂解物 31，用于 DNA 酶 I 孵育；PK 孵育无需稀释 | 1.872*10E9/mL | 80% |
| 10 μL ATCC 标准品，用于 DNA 酶 I 孵育；1:10 稀释，用于 PK 孵育 | 2.347*10E10/mL | 72% |
| 10 μL ATCC 标准品，用于 DNA 酶 I 孵育；PK 孵育无需稀释 | 2.468*10E10/mL | 75% |

[0194]

[0195] 本发明人已经发现,使用DNA酶I和蛋白酶K孵育的组合可以实现超过80%的病毒基因组回收率,而无需使用亲和色谱法纯化的细胞裂解物进行稀释。亲和力纯化的裂解物中既定的病毒基因组拷贝数(vgcn)为5.110*10E10 vg/mL(亲和力纯化的裂解物31)和7.320*10E10 vg/mL(亲和力纯化的裂解物33)。结果如表10所示。

[0196] 表10:

| 条件 | 绝对 vgcn | 相对 vgcn |
|---|---|---|
| 10 μL 亲和力纯化的裂解物 31，用于 DNA 酶 I 孵育；PK 孵育无需稀释 | 4.430*10E10/mL | 87% |

[0197]

| | | |
|---|---|---|
| 10 μL 亲和力纯化的裂解物 33，用于 DNA 酶 I 孵育；PK 孵育无需稀释 | 7.320*10E10/mL | 100% |

[0198]

[0199] 病毒基因组定量

[0200] 为了允许进行正确的病毒基因组定量,样品必须不含质粒DNA以及未包装的载体基因组,两者都至少含有部分病毒基因组。此外,包装的AAV基因组必须可由第一个PCR循环获得。

[0201] 为了去除可能干扰ddPCR过程的未包装的DNA,通常使用DNA酶I消化。

[0202] 为了使封装的DNA可进行ddPCR,需要打开衣壳。这可以通过高温孵育或蛋白酶K消化来完成。本领域对蛋白酶K消化的需求进行了大量讨论。

[0203] 微滴式数字聚合酶链式反应

[0204] 微滴式数字PCR(ddPCR)允许对病毒基因组进行绝对定量,而无需生成标准曲线。

[0205] 更详细地,微滴式数字聚合酶链式反应(ddPCR)通过将PCR反应混合物随机分配到离散的分区中来实现核酸的绝对定量,其中一些分区没有核酸靶序列,并且其他分区存在一个或多个模板拷贝(Hindson,B.等人,Anal.Chem.83(2011)8604-8610)。由于分区,热循环期间会进行数千个独立的PCR反应。在终点,读出靶阳性分区的分数并用于计算初始模板DNA浓度(Pinheiro,L.等人,Anal.Chem.84(2011)1003-1011)。

[0206] 首先,在水-油乳液中形成多达20,000个体积为约1nL的液滴。因此,将PCR反应混合物分区,该PCR反应混合物由核酸模板、正向(fwd)和反向(rev)引物、TaqMan探针和ddPCR超混合液构成,该ddPCR超混合液含有水生栖热菌(Thermus aquaticus)(Taq)DNA聚合酶、dNTP和PCR缓冲液(例如参见Hindson,B.等人2011;Taylor,S等人,Sci.Rep.7(2017)2409)。在热循环期间,根据DNA靶标的存在与否,在每个液滴中进行单独的PCR反应。

[0207] 在含有模板DNA的液滴中,扩增靶序列。在扩增期间,Taq聚合酶的5'至3'核酸外切酶活性水解结合至模板链的TaqMan探针。由于探针降解成更小的片段,位于5'的荧光团不再靠近其位于3'的猝灭剂。从而消除信号猝灭并生成荧光信号。缺少模板序列的分区未显示扩增,并且因此分别没有TaqMan探针的水解和荧光生成,因为位于5'的荧光团的荧光保持猝灭(参见例如Holland,P.等人,Proc.Natl.Acad.Sci.USA 88(1991)7276-7580)。由于可以使用具有不同荧光团的探针,这些探针具有不同的激发和发射波长,因此ddPCR反应可以在一个液滴内作为多重反应进行。二维ddPCR的常用荧光团是6-羧基荧光素(FAM)和六氯-6-羧基荧光素(HEX),两者均由黑洞猝灭剂1(BHQ1)猝灭(参见例如Furuta-Hanawa,B.等人Hum.Gen.Therap.Meth.30(2019)127-136)。

[0208] 作为终点分析,读出热循环后每个液滴的荧光信号。可以使用泊松统计根据等式1由阳性读数与总读数的比率(p)计算靶序列的拷贝数(λ)(参见例如Hindson,B.等人(2011))。

[0209] $\lambda = -\ln(1-p)$ (1)

[0210] 由于ddPCR依赖于终点测量,因此靶序列定量在一定程度上独立于PCR反应效率。这与实时PCR(qPCR)形成对比,该qPCR通常用于病毒基因组滴定(参见例如Taylor,S等人(2017))。此外,不需要使用标准品或校准样品(参见例如Dorange,F.,Bec,C.,Cell Gen.Therap.Ins.4(2018)119-129)。

[0211] 重组AAV颗粒

[0212] 本领域已知用于生成rAAV颗粒的不同方法。例如,使用AAV质粒和AAV辅助序列的转染以及与一种AAV辅助病毒(例如,腺病毒、疱疹病毒或牛痘病毒)的共感染,或使用重组AAV质粒、AAV辅助质粒和辅助功能质粒的转染。用于生成rAAV颗粒的非限制性方法描述于例如US 6,001,650、US 6,004,797、WO 2017/096039和WO 2018/226887中。在重组rAAV颗粒生产(即在细胞培养系统中的颗粒生成)后,可以从宿主细胞和细胞培养上清液中获得rAAV颗粒并进行纯化。

[0213] 对于重组AAV颗粒的生成,需要在单个哺乳动物细胞中表达Rep和Cap蛋白、辅助蛋白E1A、E1B、E2A和E4orf6以及腺病毒VA RNA。辅助蛋白E1A、E1B、E2A和E4orf6可以使用任何启动子表达,如Matsushita等人(Gene Ther.5(1998)938-945)所示,尤其是CMV IE启动子。

因此,可以使用任何启动子。

[0214] 一般来说,为了生产重组AAV颗粒,将不同的互补质粒共转染到宿主细胞中。这些质粒中的一者包含夹在两个顺式作用AAV ITR之间的转基因。后代重组基因组复制和后续包装所需的缺失AAV元件(即针对Rep和Cap蛋白的开放阅读框)以反式含在第二质粒上。Rep蛋白的过度表达引起对细胞生长的抑制作用(Li,J.等人,J.Virol.71(1997)5236-5243)。此外,AAV复制需要包含辅助病毒(即来自腺病毒的E1、E4orf6、E2A和VA)的基因的第三质粒。

[0215] 为了减少所需质粒的数量,Rep、Cap和腺病毒辅助基因可以组合在单个质粒上。

[0216] 可替代地,宿主细胞可能已经稳定表达E1基因产物。此类细胞是HEK293细胞。表示为293的人胚肾克隆早在1977年就通过将腺病毒DNA整合到人胚肾细胞(HEK细胞)中而生成(Graham,F.L.等人,J.Gen.Virol.36(1977)59-74)。HEK293细胞系包含腺病毒血清型5基因组的碱基对1至4344。这涵盖E1A和E1B基因以及腺病毒包装信号(Louis,N.等人,Virology 233(1997)423-429)。

[0217] 当使用HEK293细胞时,缺失的E2A、E4orf6和VA基因可以通过与腺病毒共感染或通过与表达E2A、E4orf6和VA的质粒共转染来引入(参见例如Samulski,R.J.等人,J.Virol.63(1989)3822-3828;Allen,J.M.等人,J.Virol.71(1997)6816-6822;Tamayose,K.等人,Hum.Gene Ther.7(1996)507-513;Flotte,T.R.等人,Gene Ther.2(1995)29-37;Conway,J.E.等人,J.Virol.71(1997)8780-8789;Chiorini,J.A.等人,Hum.Gene Ther.6(1995)1531-1541;Ferrari,F.K.等人,J.Virol.70(1996)3227-3234;Salvetti,A.等人,Hum.Gene Ther.9(1998)695-706;Xiao,X.等人,J.Virol.72(1998)2224-2232;Grimm,D.等人,Hum.Gene Ther.9(1998)2745-2760;Zhang,X.等人,Hum.Gene Ther.10(1999)2527-2537)。可替代地,可以使用腺病毒/AAV或单纯疱疹病毒/AAV杂合载体(参见例如Conway,J.E.等人,J.Virol.71(1997)8780-8789;Johnston,K.M.等人,Hum.Gene Ther.8(1997)359-370;Thrasher,A.J.等人,Gene Ther.2(1995)481-485;Fisher,J.K.等人,Hum.Gene Ther.7(1996)2079-2087;Johnston,K.M.等人,Hum.Gene Ther.8(1997)359-370)。

[0218] 因此,其中整合并表达rep基因的细胞系往往生长缓慢或以非常低的水平表达Rep蛋白。

[0219] 为了将转基因活性限制在特定组织中,即限制整合位点,可以将转基因可操作地连接至诱导型或组织特异性启动子(参见例如Yang,Y.等人Hum.Gene.Ther.6(1995)1203-1213)。

[0220] rAAV颗粒生产的一个困难是rAAV载体包装效率低下,导致滴度低。由于多种原因,包装一直很困难,该原因包括:

[0221] -野生型AAV基因组在存在时的优选衣壳化;

[0222] -由于与rep基因产物相关联的抑制作用,难以生成足够的补充功能,诸如由野生型rep和cap基因提供的功能;

[0223] -质粒构建体共转染的效率有限。

[0224] 所有这一切都是基于Rep蛋白的生物学特性。尤其是Rep蛋白的抑制(细胞抑制和细胞毒性)特性以及逆转培养细胞永生化表型的能力是有问题的。另外,当采用广泛使用的AAV P5启动子时,Rep蛋白下调它们自身的表达(参见例如Tratschin等人,

Mol.Cell.Biol.6(1986)2884-2894)。

[0225] 在所有方面和实施例的某些实施例中,rAAV颗粒源自选自由以下项组成的组的AAV:AAV1、AAV2、AAV3、AAV4、AAV5、AAV6、AAV7、AAV8、AAV9、AAV10、Rh.10、Rh74和7m8。

[0226] 在所有方面和实施例的某些实施例中,rAAV颗粒包含与AAV1、AAV2、AAV3、AAV4、AAV5、AAV6、AAV7、AAV8、AAV9、AAV10、Rh.10、Rh74或7m8衣壳序列具有70%或更高序列同一性的衣壳序列。

[0227] 在所有方面和实施例的某些实施例中,rAAV颗粒包含与AAV1、AAV2、AAV3、AAV4、AAV5、AAV6、AAV7、AAV8、AAV9或AAV10 ITR序列具有70%或更高序列同一性的ITR序列。

[0228] E1A、E1B、E2和E4

[0229] E1A和E1B的编码序列(开放阅读框)可以源自人腺病毒,诸如例如特别是人腺病毒血清型2或血清型5。人Ad5(腺病毒血清型5)的示例性序列见于GenBank条目X02996、AC_000008,并且示例性人Ad2的序列见于GenBank条目AC_000007。核苷酸505至3522包含编码人腺病毒血清型5的E1A和E1B的核酸序列。EP 1 230 354中报道的质粒pSTK146以及WO 2007/056994中报道的质粒pGS119和pGS122也可以用作E1A和E1B开放阅读框的来源。

[0230] E1A是腺病毒DNA进入细胞核后表达的第一个病毒辅助基因。E1A基因编码12S和13S蛋白,它们通过选择性剪接基于相同的E1A mRNA。12S和13S蛋白的表达引起其他病毒功能E1B、E2、E3和E4的激活。此外,12S和13S蛋白的表达迫使细胞进入细胞周期的S期。如果仅表达E1A衍生的蛋白质,细胞就会死亡(凋亡)。

[0231] E1B是表达的第二个病毒辅助基因。它由E1A衍生的蛋白12S和13S激活。E1B基因衍生的mRNA可以通过两种不同的方式进行剪接,从而产生第一个55kDa转录物和第二个19kDa转录物。E1B 55kDa蛋白参与调节细胞周期、阻止感染后期细胞mRNA运输以及阻止E1A诱导的细胞凋亡。E1B 19kDa蛋白参与阻止E1A诱导的细胞凋亡。

[0232] E2基因编码不同的蛋白质。E2A转录物编码单链结合蛋白(SSBP),这对于AAV复制至关重要

[0233] E4基因还编码多种蛋白质。E4基因衍生的34kDa蛋白(E4orf6)与E1B 55kDa蛋白一起阻止细胞mRNA在细胞质中积累,但也促进病毒RNA从细胞核转运到细胞质。

[0234] 腺病毒VA RNA基因

[0235] 病毒相关RNA(VA RNA)是腺病毒(Ad)的非编码RNA,调节翻译。腺病毒基因组包含两个独立的拷贝:VAI(VA RNAI)和VAII(VA RNAII)。两者均由RNA聚合酶III(参见例如Machitani,M.等人,J.Contr.相对154(2011)285-289)从2型聚合酶III启动子转录。对于重组生产,腺病毒VA RNA基因可以由任何启动子驱动。

[0236] Ma,Y.和Mathews,M.B.(J.Virol.70(1996)5083-5099)使用系统发育方法研究了腺病毒相关RNA的结构、功能和进化。他们提供了基于47种已知人腺病毒血清型的比对以及共有VA RNA序列。所述公开内容通过引用以其整体随此并入本申请中。

[0237] VA RNA、VAI和VAII由157至160个核苷酸(nt)组成。

[0238] 根据血清型,腺病毒含有一个或两个VA RNA基因。VA RNAI被认为发挥着主要的促病毒作用,而VA RNAII可以部分补偿VA RNAI的缺失(Vachon,V.K.和Conn,G.L.,Virus Res.212(2016)39-52)。

[0239] VA RNA不是必需的,但通过克服细胞抗病毒机制,在有效病毒生长中发挥重要作

用。也就是说,虽然VA RNA对于病毒生长不是必需的,但VA RNA缺失的腺病毒在载体生成的初始步骤期间无法生长,其中每个细胞仅存在病毒基因组的几个拷贝,可能是因为除VA RNA之外的阻断细胞抗病毒机制的病毒基因可能无法充分表达(参见Maekawa,A.等人Nature Sci.Rep.3(2013)1136)。

[0240] Maekawa,A.等人(Nature Sci.Rep.3(2013)1136)报道了缺乏扰乱细胞RNAi机制的病毒相关RNA的基因的腺病毒载体的高效生产,其中组成和高度表达翻转酶重组酶的HEK293细胞被感染,以通过FLP重组酶介导的VA RNA基因座切除获得VA RNA缺失的腺病毒。

[0241] 人腺病毒2VA RNAI对应于GenBank条目AC_000007序列的核苷酸10586-10810。人腺病毒5VA RNAI对应于GenBank条目AC_000008序列的核苷酸10579-10820。

[0242] 用于生产rAAV颗粒的方法

[0243] Carter等人已经表明,野生型AAV基因组中的整个rep和cap开放阅读框可以缺失并用转基因替换(Carter,B.J.,"Handbook of Parvoviruses",P.Tijssen编辑,CRC Press,pp.155-168(1990))。此外,据报道,必须维持ITR以保留将转基因复制、拯救、包装和整合到靶细胞基因组中的功能。

[0244] 当包含相应病毒辅助基因的细胞被AAV载体转导时,或者反之亦然,当包含整合的AAV原病毒的细胞被合适的辅助病毒转导时,则AAV原病毒被激活并再次进入裂解感染周期(Clark,K.R.等人,Hum.Gene Ther.6(1995)1329-1341;Samulski,R.J.,Curr.Opin.Genet.Dev.3(1993)74-80)。

[0245] 本发明的方面是用包含用于生产重组AAV颗粒的所有所需元件的核酸(例如,质粒)转导细胞的方法,其中病毒基因组拷贝数用根据本发明的方法确定。因此,当质粒编码病毒包装蛋白和/或辅助蛋白时,细胞可以生产重组病毒颗粒,其包括编码目标蛋白质的核酸或包含转录为目标转录物的序列的核酸。

[0246] 本发明提供了一种病毒(例如,AAV)颗粒生产平台,其包括通过使用根据本发明的方法将其与当前'行业标准'病毒(例如,AAV)颗粒生产过程区分开的特征。

[0247] 更一般地,用DNA转染或转导以用于重组生产AAV颗粒的细胞可以称为"重组细胞"。此类细胞可以是例如酵母细胞、昆虫细胞或哺乳动物细胞,并且已被用作编码包装蛋白(诸如AAV包装蛋白)的核酸(质粒)、编码辅助蛋白的核酸(质粒)以及编码蛋白质或转录成目标转录物的核酸(质粒)(即放置在两个AAV ITR之间的转基因)的受体。该术语包括已被转导或转染的原始细胞的后代。应当理解,由于自然、偶然或故意的突变,单个亲本细胞的后代在形态学或基因组或总核酸互补方面可能不一定与原始亲本完全相同。

[0248] 许多适合维持细胞活力或提供细胞生长和/或增殖的细胞生长培养基是可商业获得的。此类培养基的实例包括无血清真核生长培养基,诸如用于维持哺乳动物(例如人)细胞活力或提供哺乳动物(例如人)细胞生长的培养基。非限制性实例包括Ham's F12或F12K培养基(Sigma-Aldrich)、FreeStyle(FS)F17培养基(Thermo-Fisher Scientific)、MEM、DMEM、RPMI-1640(Thermo-Fisher Scientific)及其混合物。此类培养基可以补充有维生素和/或微量矿物质和/或盐和/或氨基酸,诸如用于哺乳动物(例如,人)细胞的必需氨基酸。

[0249] 辅助蛋白质粒可以呈质粒、噬菌体、转座子或粘粒的形式。特别地,已经证明辅助功能不需要腺病毒基因的完全互补。例如,无法进行DNA复制和晚期基因合成的腺病毒突变体已被证明允许AAV复制。Ito等人,J.Gen.Virol.9(1970)243;Ishibashi等人,Virology

45(1971)317。

[0250] E2B和E3区域内的突变体已被证明支持AAV复制,指示E2B和E3区域可能不参与提供辅助功能。Carter等人,Virology 126(1983)505。然而,E1区域有缺陷或E4区域缺失的腺病毒无法支持AAV复制。因此,对于腺病毒辅助蛋白,E1A和E4区域可能是AAV复制直接或间接所必需的(参见例如Laughlin等人,J.Virol.41(1982)868;Janik等人,Proc.Natl.Acad.Sci.USA 78(1981)1925;Carter等人,Virology 126(1983)505)。其他特征性腺病毒突变体包括:E1B(Laughlin等人(1982),同上;Janik等人(1981),同上;Ostrove等人,Virology 104(1980)502);E2A(Handa等人,J.Gen.Virol.29(1975)239;Strauss等人,J.Virol.17(1976)140;Myers等人,J.Virol.35(1980)665;Jay等人,Proc.Natl.Acad.Sci.USA 78(1981)2927;Myers等人,J.Biol.Chem.256(1981)567);E2B(Carter,Adeno-Associated Virus Helper Functions,I CRC Handbook of Parvoviruses(P.Tijssen编辑,1990));E3(Carter等人(1983),同上);和E4(Carter等人(1983),同上;Carter(1995))。

[0251] 对E1B突变的腺病毒提供的辅助蛋白的研究已报道,E1B 55kDa蛋白是AAV颗粒生产所必需的,而E1B 19kDa则不是。此外,WO 97/17458和Matshushita等人(Gene Therapy 5(1998)938-945)描述了编码各种腺病毒基因的辅助功能质粒。辅助质粒的实例包含腺病毒VA RNA编码区、腺病毒E4orf6编码区、腺病毒E2A 72kDa编码区、腺病毒E1A编码区和缺乏完整E1B 55kDa编码区的腺病毒E1B区域(参见例如WO 01/83797)。

[0252] 因此,本文提供了一种用于使用根据本发明的用于病毒基因组数确定的方法来生产重组AAV载体或包含所述重组AAV载体的AAV颗粒的方法,该重组AAV载体包含编码蛋白质或转录成目标转录物的核酸。

[0253] 本发明的一个方面是一种用于生产重组AAV载体或包含所述重组AAV载体的AAV颗粒的方法,该重组AAV载体包含编码蛋白质或转录成目标转录物的核酸,该方法包括以下步骤:

[0254] (i)提供一种或多种包含编码AAV包装蛋白的核酸和/或编码辅助蛋白的核酸的质粒;

[0255] (ii)提供包含编码目标蛋白质或转录成目标转录物的核酸的质粒;

[0256] (iii)使一种或多种哺乳动物或昆虫细胞与经提供的质粒接触;

[0257] (iv)进一步添加转染试剂并任选地孵育质粒/转染试剂/细胞混合物;

[0258] 或提供物理手段,诸如电流,以将核酸引入细胞中;

[0259] (v)培养经转染的细胞;

[0260] (vi)收获经培养的细胞和/或来自经培养的细胞的培养基以生产细胞和/或培养基收获物;以及

[0261] (vii)裂解细胞并任选地从细胞和/或培养基收获物裂解物中分离重组AAV载体或AAV颗粒;

[0262] (viii)用根据本发明的方法在步骤(vi)或/和步骤(vii)中或之后确定病毒基因组拷贝数;

[0263] 从而生产包含编码目标蛋白质或转录成目标转录物的核酸的重组AAV载体或AAV颗粒。

[0264] 本发明的一个方面是一种用于生产重组AAV载体或包含所述重组AAV载体的AAV颗粒的方法,该重组AAV载体包含编码蛋白质或转录成目标转录物的核酸,该方法包括以下步骤:

[0265] (i)提供一种或多种包含编码AAV包装蛋白的核酸和/或编码辅助蛋白的核酸的质粒;

[0266] (ii)提供包含编码目标蛋白质或转录成目标转录物的核酸的质粒;

[0267] (iii)

[0268] (a)通过以下方式生成稳定转染细胞

[0269] -使一种或多种哺乳动物或昆虫细胞与(i)的经提供的质粒接触;

[0270] -进一步添加转染试剂并任选地孵育质粒/转染试剂/细胞混合物;或提供物理手段,诸如电流,以将核酸引入细胞中;

[0271] -选择第一稳定转染细胞;

[0272] -使经选择的第一稳定转染细胞与(ii)的经提供的质粒接触;以及

[0273] -进一步添加转染试剂并任选地孵育质粒/转染试剂/细胞混合物;或提供物理手段,诸如电流,以将核酸引入细胞中;

[0274] (b)或通过以下方式生成瞬时转染细胞

[0275] -使一种或多种哺乳动物或昆虫细胞与(i)和(ii)的经提供的质粒接触;以及

[0276] -进一步添加转染试剂并任选地孵育质粒/转染试剂/细胞混合物;或提供物理手段,诸如电流,以将核酸引入细胞中;

[0277] (iv)培养(iii)的经转染的细胞;

[0278] (v)收获经培养的细胞和/或来自经培养的细胞的培养基以生产细胞和/或培养基收获物;

[0279] (vi)裂解细胞并任选地从细胞和/或培养基收获物裂解物中分离重组AAV载体或AAV颗粒;

[0280] (vii)用根据本发明的方法在步骤(v)或/和步骤(vi)中或之后确定病毒基因组拷贝数;

[0281] 从而生产包含编码目标蛋白质或转录成目标转录物的核酸的重组AAV载体或AAV颗粒。

[0282] 本发明的一个方面是一种用于生产重组AAV载体或包含所述重组AAV载体的AAV颗粒的方法,该重组AAV载体包含编码蛋白质或转录成目标转录物的核酸,该方法包括以下步骤:

[0283] (i)提供包含编码AAV包装蛋白的核酸和/或编码辅助蛋白的核酸的哺乳动物或昆虫细胞;

[0284] (ii)提供包含编码目标蛋白质或转录成目标转录物的核酸的质粒;

[0285] (iii)

[0286] (a)通过以下方式生成稳定转染细胞

[0287] -使一种或多种哺乳动物或昆虫细胞与(i)的经提供的质粒接触;

[0288] -进一步添加转染试剂并任选地孵育质粒/转染试剂/细胞混合物;或提供物理手段,诸如电流,以将核酸引入细胞中;

[0289] -选择第一稳定转染细胞;

[0290] -使经选择的第一稳定转染细胞与(ii)的经提供的质粒接触;以及

[0291] -进一步添加转染试剂并任选地孵育质粒/转染试剂/细胞混合物;或提供物理手段,诸如电流,以将核酸引入细胞中;

[0292] (b)或通过以下方式生成瞬时转染细胞

[0293] -使一种或多种哺乳动物或昆虫细胞与(i)和(ii)的经提供的质粒接触;以及

[0294] -进一步添加转染试剂并任选地孵育质粒/转染试剂/细胞混合物;或提供物理手段,诸如电流,以将核酸引入细胞中;

[0295] (iv)培养(iii)的经转染的细胞;

[0296] (v)收获经培养的细胞和/或来自经培养的细胞的培养基以生产细胞和/或培养基收获物;

[0297] (vi)裂解细胞并任选地从细胞和/或培养基收获物中分离和/或纯化重组AAV载体或AAV颗粒;以及

[0298] (vii)用根据本发明的方法在步骤(v)或/和步骤(vi)中或之后确定病毒基因组拷贝数;

[0299] 从而生产包含编码目标蛋白质或转录成目标转录物的核酸的重组AAV载体或AAV颗粒。

[0300] 将核酸(质粒)引入细胞可以通过多种方式进行。

[0301] 本领域已报道了将DNA转移至哺乳动物细胞的多种方法。这些都可用于根据本发明的方法。在所有方面和实施例的某些实施例中,使用用于核酸转移/转染的电穿孔、核转染或显微注射。在所有方面和实施例的某些实施例中,使用用于核酸转移/转染的无机物质(诸如例如磷酸钙/DNA共沉淀)、阳离子聚合物(诸如例如聚乙烯亚胺、DEAE-葡聚糖)或阳离子脂质(脂转染)。磷酸钙和聚乙烯亚胺是用于较大规模核酸转移的转染中最常用的试剂(参见例如Baldi等人,Biotechnol.Lett.29(2007)677-684),其中优选聚乙烯亚胺。

[0302] 在所有方面和实施例的某些实施例中,核酸(质粒)以与聚乙烯亚胺(PEI)组合、任选地与细胞组合的组合物的形式提供。在某些实施例中,组合物包括质粒/PEI混合物,其具有多种组分:(a)一种或多种包含编码AAV包装蛋白的核酸和/或编码辅助蛋白的核酸的质粒;(b)包含编码蛋白质或转录成目标转录物的核酸的质粒;以及(c)聚乙烯亚胺(PEI)溶液。在某些实施例中,质粒的摩尔比范围为约1:0.01至约1:100,或者摩尔比范围为约100:1至约1:0.01,并且组分(a)、(b)和(c)的混合物任选地已孵育约10秒至约4小时的时间段。

[0303] 在所有方面和实施例的某些实施例中,组合物进一步包含细胞。在某些实施例中,使细胞与组分(a)、(b)和/或(c)的质粒/PEI混合物接触。

[0304] 在所有方面和实施例的某些实施例中,任选地与细胞组合的组合物进一步包含游离PEI。在某些实施例中,使细胞与游离PEI接触。

[0305] 在所有方面和实施例的某些实施例中,细胞已与组分(a)、(b)和/或(c)的混合物接触至少约4小时,或约4小时至约140小时,或约4小时至约96小时。在一个优选的实施例中,细胞已与组分(a)、(b)和/或(c)以及任选的游离PEI的混合物接触至少约4小时。

[0306] 该组合物可以进一步包含质粒或/和细胞。此类质粒和细胞可以与游离PEI接触。在某些实施例中,质粒和/或细胞已与游离PEI接触至少约4小时,或约4小时至约140小时,

或约4小时至约96小时。

[0307] 本发明还提供了用于生产转染细胞的方法。该方法包括提供一种或多种质粒;提供包含聚乙烯亚胺(PEI)的溶液;以及将质粒与PEI溶液混合以生产质粒/PEI混合物的步骤。在某些实施例中,将此类混合物孵育约10秒至约4小时范围内的时段。在此类方法中,然后使细胞与质粒/PEI混合物接触以生产质粒/PEI细胞培养物;然后将游离PEI添加至所生产的质粒/PEI细胞培养物中,以生产游离PEI/质粒/PEI细胞培养物;然后将生产的游离PEI/质粒/PEI细胞培养物孵育至少约4小时,从而生产转染细胞。在某些实施例中,质粒包含rep开放阅读框、cap开放阅读框、E1A、E1B、E2和E4orf6开放阅读框以及编码蛋白质或转录成目标转录物的核酸中的一者或多者或全部。

[0308] 进一步提供了用于生产转染细胞的方法,该转染细胞生产重组AAV载体或AAV颗粒,该方法包括提供一种或多种包含编码AAV包装蛋白的核酸和/或编码辅助蛋白的核酸的质粒;提供包含编码蛋白质或转录成目标转录物的核酸的质粒;提供包含聚乙烯亚胺(PEI)的溶液;将上述质粒与PEI溶液混合,其中质粒的摩尔比范围为约1:0.01至约1:100,或者摩尔比范围为约100:1至约1:0.01,以生产质粒/PEI混合物(并且任选地将质粒/PEI混合物孵育约10秒至约4小时范围内的时段);使细胞与质粒/PEI混合物接触以生产质粒/PEI细胞培养物;将游离PEI添加至生产的质粒/PEI细胞培养物中以生产游离PEI/质粒/PEI细胞培养物;以及孵育游离PEI/质粒/PEI细胞培养物至少约4小时,从而生产转染细胞,该转染细胞生产包含编码蛋白质或转录成目标转录物的核酸的重组AAV载体或颗粒,其中用根据本发明的方法确定病毒基因组拷贝数。

[0309] 另外提供了用于生产包含编码蛋白质或转录成目标转录物的核酸的重组AAV载体或AAV颗粒的方法,该方法包括提供一种或多种包含编码AAV包装蛋白的核酸和/或编码辅助蛋白的核酸的质粒;提供包含编码目标蛋白质或转录成目标转录物的核酸的质粒;提供包含聚乙烯亚胺(PEI)的溶液;将上述质粒与PEI溶液混合,其中质粒的摩尔比范围为约1:0.01至约1:100,或者摩尔比范围为约100:1至约1:0.01,以生产质粒/PEI混合物(并且任选地将质粒/PEI混合物孵育约10秒至约4小时范围内的时间段);使细胞与如上所述生产的质粒/PEI混合物接触以生产质粒/PEI细胞培养物;将游离PEI添加至如上所述生产的质粒/PEI细胞培养物中以生产游离PEI/质粒/PEI细胞培养物;将生产的质粒/PEI细胞培养物或游离PEI/质粒/PEI细胞培养物孵育至少约4小时以生产转染细胞;收获生产的转染细胞和/或来自生产的转染细胞的培养基以生产细胞和/或培养基收获物;裂解细胞并任选地从细胞和/或培养基收获物裂解物中分离重组AAV载体或颗粒,由此用根据本发明的方法确定裂解物或分离的AAV颗粒中的病毒基因组拷贝数;从而生产包含编码蛋白质或转录成目标转录物的核酸的重组AAV载体或颗粒。

[0310] 用于使用根据本发明的方法生产重组AAV载体或AAV颗粒的方法可以包括一个或多个另外的步骤或特征。示例性步骤或特征包括但不限于收获生产的培养细胞和/或收获来自生产的培养细胞的培养基以生产细胞和/或培养基收获物的步骤。额外的示例性步骤或特征包括但不限于裂解收获的细胞并任选地从细胞和/或培养基收获物裂解物中分离重组AAV载体或AAV颗粒;其中使用根据本发明的方法确定病毒基因组拷贝数;从而生产包含编码蛋白质或转录成目标转录物的核酸的重组AAV载体或AAV颗粒。

[0311] 在所有方面和实施例的某些实施例中,在不同时间点将PEI添加到质粒和/或细胞

中。在某些实施例中,在使质粒/PEI混合物与细胞接触之前、同时或之后将游离PEI添加到细胞中。

[0312] 在所有方面和实施例的某些实施例中,当与质粒/PEI混合物接触时和/或当与游离PEI接触时,细胞处于特定密度和/或细胞生长期和/或活力。在一个优选的实施例中,当与质粒/PEI混合物接触时和/或当与游离PEI接触时,细胞的密度在约1x10E5个细胞/mL至约1x10E8个细胞/mL的范围内。在某些实施例中,当与质粒/PEI混合物或与游离PEI接触时,细胞活力为约60%或大于60%,或者其中当与质粒/PEI混合物接触时,细胞处于对数生长期,或者当与质粒/PEI混合物或与游离PEI接触时,细胞活力为约90%或大于90%,或者其中当与质粒/PEI混合物或与游离PEI接触时,细胞处于对数生长期。

[0313] 在所有方面和实施例的某些实施例中,编码的AAV包装蛋白包括AAV rep和/或AAV cap。在所有方面和实施例的某些实施例中,此类AAV包装蛋白包括任何AAV血清型的AAV rep和/或AAV cap蛋白。

[0314] 在所有方面和实施例的某些实施例中,编码的辅助蛋白包括腺病毒E1A和E1B、腺病毒E2和/或E4、VA RNA和/或非AAV辅助蛋白。

[0315] 在所有方面和实施例的某些实施例中,以特定量或比率使用核酸(质粒)。在某些实施例中,包含编码蛋白质或转录成目标转录物的核酸的质粒和包含编码AAV包装蛋白的核酸和/或编码辅助蛋白的核酸的一种或多种质粒的总量在每mL细胞约0.1μg至约15μg的范围内。在某些实施例中,包含编码蛋白质或转录成目标转录物的核酸的质粒与包含编码AAV包装蛋白的核酸和/或编码辅助蛋白的核酸的一种或多种质粒的摩尔比在约1:5至约1:1的范围内,或者在约1:1至约5:1的范围内。

[0316] 在所有方面和实施例的某些实施例中,第一质粒包含编码AAV包装蛋白的核酸并且第二质粒包含编码辅助蛋白的核酸。

[0317] 在所有方面和实施例的某些实施例中,在共转染中包含编码蛋白质或转录成目标转录物的核酸的质粒与包含编码AAV包装蛋白的核酸的第一质粒与包含编码辅助蛋白的核酸的第二质粒的摩尔比在约1-5:1:1或1:1-5:1或1:1:1-5的范围内。

[0318] 在所有方面和实施例的某些实施例中,细胞为真核细胞。在某些实施例中,真核细胞为哺乳动物细胞。在一个优选的实施例中,细胞为HEK293细胞或CHO细胞。

[0319] 可以使用约37℃、95%湿度和8体积%$CO_2$的用于培养真核细胞的常用条件进行培养。培养可以在含血清或无血清培养基、贴壁培养物或悬浮培养物中进行。悬浮培养可以在任何发酵容器中进行,诸如例如在搅拌釜反应器、波动反应器、摇摆生物反应器、摇床容器或旋转容器或所谓的滚瓶中进行。转染可以分别以高通量格式和筛选进行,例如以96或384孔格式进行。

[0320] 根据本发明的方法包括任何血清型的AAV颗粒或其变体。在所有方面和实施例的某些实施例中,重组AAV颗粒包含以下项中的任一者:AAV血清型1至12,AAV VP1、VP2和/或VP3衣壳蛋白,或者经修饰的或变体AAV VP1、VP2和/或VP3衣壳蛋白,或者野生型AAV VP1、VP2和/或VP3衣壳蛋白。在所有方面和实施例的某些实施例中,AAV颗粒包含AAV血清型或AAV假型,其中AAV假型包含不同于ITR血清型的AAV衣壳血清型。

[0321] 提供或包括AAV载体或颗粒的根据本发明的方法还可以包括其他元件。此类元件的实例包括但不限于:内含子、表达控制元件、一个或多个腺相关病毒(AAV)反向末端重复

序列(ITR)和/或填充/填充物多核苷酸序列。此类元件可以位于编码蛋白质或转录成目标转录物的核酸内或侧接该核酸,或者表达控制元件可以可操作地连接至编码蛋白质或转录成目标转录物的核酸,或者AAV ITR可以侧接编码蛋白质或转录成目的转录物的核酸的5'或3'末端侧翼,或者填充多核苷酸序列可以侧接编码蛋白质或转录成目标转录物的核酸的5'或3'末端。

[0322] 表达控制元件包括组成型或可调节控制元件,例如组织特异性表达控制元件或启动子。

[0323] ITR可以是AAV2或AAV6或AAV8或AAV9血清型中的任一者,或其组合。AAV颗粒可以包括与AAV1、AAV2、AAV3、AAV4、AAV5、AAV6、AAV10、AAV11、AAV-2i8、AAV rh74或AAV 7m8 VP1、VP2和/或VP3衣壳蛋白中的任一者具有75%或更高序列同一性的任何VP1、VP2和/或VP3衣壳蛋白,或者包含选自以下项中的任一者的经修饰的或变体VP1、VP2和/或VP3衣壳蛋白:AAV1、AAV2、AAV3、AAV4、AAV5、AAV6、AAV10、AAV11、AAV-2i8、AAV rh74和AAV 7m8 AAV血清型。

[0324] 在生产如本文所述的重组病毒(例如,AAV)颗粒后,如果需要,可以使用多种常规方法从宿主细胞中纯化和/或分离病毒(例如,rAAV)颗粒。此类方法包括柱色谱法、CsCl梯度、碘克沙醇梯度等。

[0325] 例如,可以使用多个柱纯化步骤,诸如通过阴离子交换柱、亲和柱和/或阳离子交换柱进行纯化。(参见例如WO 02/12455和US 2003/0207439)。可替代地或另外,可以使用碘克沙醇或CsCl梯度步骤(参见例如US2012/0135515;和US2013/0072548)。此外,如果使用感染性病毒来表达包装蛋白和/或辅助蛋白,则可以使用各种方法使残留病毒失活。例如,可以通过加热至约60℃的温度持续例如20分钟或更长时间来使腺病毒失活。这种治疗有效地使辅助病毒失活,因为AAV是热稳定的,而辅助腺病毒是热不稳定的。

[0326] rAAV载体生产和纯化系统的一个目标是实施策略,以最小化/控制生成生产相关杂质,诸如蛋白质、核酸和载体相关杂质,包括野生型/伪野生型AAV物种(wtAAV)和AAV封装的残留DNA杂质。

[0327] 考虑到rAAV颗粒仅占生物量的一小部分,rAAV颗粒需要纯化至可以用作临床人类基因疗法产品的纯度水平(参见例如Smith P.H.等人,Mo.Therapy 7(2003)8348;Chadeuf G.等人,Mo.Therapy 12(2005)744;来自CHMP gene therapy expert group meeting的报告,European Medicines Agency EMEA/CHMP 2005,183989/2004)。

[0328] 作为初始步骤,通常收获产生rAAV颗粒的培养细胞,任选地将其与收获细胞培养物上清液(培养基)组合,在该细胞培养物上清液(培养基)中已经培养了产生rAAV颗粒的细胞(悬浮细胞或贴壁细胞)。收获的细胞和任选的细胞培养物上清液可以按原样使用,视情况裂解或浓缩。此外,如果利用感染来表达辅助功能,则可以使残留的辅助病毒失活。例如,腺病毒可以通过加热至约60℃的温度持续例如20分钟或更长时间来失活,这仅使辅助病毒失活,因为AAV是热稳定的,而辅助腺病毒是热不稳定的。

[0329] 通过破坏细胞(例如通过化学或物理手段,诸如洗涤剂、微流化和/或均质化)来裂解细胞和/或收获物的上清液以释放rAAV颗粒。在细胞裂解期间同时或在细胞裂解之后随后,添加核酸酶(诸如例如benzonase)以降解污染的DNA。通常,对所得裂解物进行澄清以去除细胞碎片,例如通过过滤或离心去除,以得到澄清的细胞裂解物。在特定的实例中,用微

米直径孔径过滤器(诸如0.1μm至10.0μm孔径过滤器,例如,0.45μm和/或孔径0.2μm过滤器)过滤裂解物,以产生澄清的裂解物。

[0330] 裂解物(任选澄清的)含有AAV颗粒(包含rAAV载体以及空衣壳)和生产/过程相关的杂质,诸如来自宿主细胞的可溶性细胞组分,其尤其可以包括细胞蛋白、脂质和/或核酸,以及细胞培养基组分。然后对任选澄清的裂解物进行纯化步骤,以使用色谱法从杂质中纯化AAV颗粒(包含rAAV载体)。在第一色谱法步骤之前,可以用适当的缓冲液稀释或浓缩澄清的裂解物。

[0331] 在细胞裂解、任选的澄清和任选的稀释或浓缩之后,可以使用多个随后的和连续的色谱法步骤来纯化rAAV颗粒。

[0332] 第一色谱法步骤可以是阳离子交换色谱法或阴离子交换色谱法。如果第一色谱法步骤是阳离子交换色谱法,则第二色谱法步骤可以是阴离子交换色谱法或尺寸排阻色谱法(SEC)。因此,在所有方面和实施例的某些实施例中,rAAV颗粒纯化是经由阳离子交换色谱法进行的纯化,然后是经由阴离子交换色谱法进行的纯化。

[0333] 可替代地,如果第一色谱法步骤是阳离子交换色谱法,则第二色谱法步骤可以是尺寸排阻色谱法(SEC)。因此,在所有方面和实施例的某些实施例中,rAAV颗粒纯化是经由阳离子交换色谱法进行的纯化,然后是经由尺寸排阻色谱法(SEC)进行的纯化。

[0334] 仍然可替代地,第一色谱法步骤可以是亲和色谱法。如果第一色谱法步骤是亲和色谱法,则第二色谱法步骤可以是阴离子交换色谱法。因此,在所有方面和实施例的某些实施例中,rAAV颗粒纯化是经由亲和色谱法进行的纯化,然后是经由阴离子交换色谱法进行的纯化。

[0335] 任选地,可以将第三色谱法添加到前述色谱法步骤中。通常,任选的第三色谱法步骤在阳离子交换、阴离子交换、尺寸排阻或亲和色谱法之后。

[0336] 因此,在所有方面和实施例的某些实施例中,rAAV颗粒纯化是经由阳离子交换色谱法进行的纯化,然后是经由阴离子交换色谱法进行的纯化,然后是经由尺寸排阻色谱法(SEC)进行的纯化。

[0337] 此外,在所有方面和实施例的某些实施例中,进一步的rAAV颗粒纯化是经由阳离子交换色谱法进行的纯化,然后是经由尺寸排阻色谱法(SEC)进行的纯化,然后是经由阴离子交换色谱法进行的纯化。

[0338] 在所有方面和实施例的又进一步的实施例中,rAAV颗粒纯化是经由亲和色谱法进行的纯化,然后是经由阴离子交换色谱法进行的纯化,然后是经由尺寸排阻色谱法(SEC)进行的纯化。

[0339] 在所有方面和实施例的又进一步的实施例中,rAAV颗粒纯化是经由亲和色谱法进行的纯化,然后是经由尺寸排阻色谱法(SEC)进行的纯化,然后是经由阴离子交换色谱法进行的纯化。

[0340] 阳离子交换色谱法的作用是将AAV颗粒与来自亲和色谱法或尺寸排阻色谱法的澄清的裂解物和/或柱洗脱物中存在的细胞和其他组分分离。能够在宽pH范围内结合rAAV颗粒的强阳离子交换树脂的实例包括但不限于由磺酸盐官能团的存在指示的任何基于磺酸的树脂,包括芳基和烷基取代的磺酸盐,诸如磺丙基或磺乙基树脂。代表性基质包括但不限于POROS HS、POROS HS 50、POROS XS、POROS SP和POROS S(强阳离子交换剂,可从Thermo

Fisher Scientific,Inc.,Waltham,MA,USA获得)。另外的实例包括Capto S、Capto S ImpAct、Capto S ImpRes(强阳离子交换剂,可从GE Healthcare,Marlborough,MA,USA获得),以及可从Aldrich Chemical Company(Milliwaukee,WI,USA)获得的树脂的商业DOWEX®、AMBERLITE®和AMBERLYST®家族。弱阳离子交换树脂包括但不限于任何基于羧酸的树脂。示例性的阳离子交换树脂包括羧甲基(CM)树脂、磷酸盐树脂(基于磷酸盐官能团)、甲基磺酸盐(S)树脂和磺丙基(SP)树脂。

[0341] 阴离子交换色谱法的作用是将AAV颗粒与来自亲和或阳离子交换或尺寸排阻色谱法的澄清的裂解物和/或柱洗脱物中存在的蛋白质、细胞和其他组分分离。阴离子交换色谱法还可以用于减少并从而控制洗脱物中空衣壳的量。例如,可以用包含适中浓度(例如,约100mM至125mM,诸如110mM至115mM)的NaCl的溶液洗涤具有与其结合的rAAV颗粒的阴离子交换柱,并且在流过时可以洗脱一部分空衣壳而不大量洗脱rAAV颗粒。随后,可以使用包含较高浓度的NaCl(例如,约130mM至300mM NaCl)的溶液洗脱结合至阴离子交换柱的rAAV颗粒,从而产生具有减少或耗尽量的空衣壳和成比例增加量的包含rAAV载体的rAAV颗粒的柱洗脱物。

[0342] 示例性阴离子交换树脂包括但不限于基于聚胺树脂和其他树脂的那些。强阴离子交换树脂的实例包括通常基于季铵化氮原子的那些,包括但不限于季铵盐树脂,诸如三烷基苄基铵树脂。合适的交换色谱法材料包括但不限于MACRO PREP Q(强阴离子交换剂,可从BioRad,Hercules,CA,USA获得);UNOSPHERE Q(强阴离子交换剂,可从BioRad,Hercules,CA,USA获得);POROS 50HQ(强阴离子交换剂,可从Applied Biosystems,Foster City,CA,USA获得);POROS XQ(强阴离子交换剂,可从Applied Biosystems,Foster City,CA,USA获得);POROS SOD(弱阴离子交换剂,可从Applied Biosystems,Foster City,CA,USA获得);POROS 50PI(弱阴离子交换剂,可从Applied Biosystems,Foster City,CA,USA获得);Capto Q、Capto XQ、Capto Q ImpRes和SOURCE 30Q(强阴离子交换剂,可从GE healthcare,Marlborough,MA,USA获得);DEAE SEPHAROSE(弱阴离子交换剂,可从Amersham Biosciences,Piscataway,NJ,USA获得);Q SEPHAROSE(强阴离子交换剂,可从Amersham Biosciences,Piscataway,NJ,USA获得)。另外的示例性阴离子交换树脂包括氨基乙基(AE)、二乙基氨基乙基(DEAE)、二乙基氨基丙基(DEPE)和季氨基乙基(QAE)。

[0343] 纯化意图作为治疗人类疾病的产品的重组AAV颗粒的制造过程应实现以下目标:1)一致的颗粒纯度、效力和安全性;2)制造过程可扩展性;以及3)可接受的制造成本。

[0344] WO 2019/006390中报道了重组AAV颗粒纯化的示例性过程。

[0345] 下面概述的重组腺相关病毒颗粒(rAAV颗粒)纯化和生产方法可扩展到大规模。例如,到悬浮培养物的体积为5、10、10-20、20-50、50-100、100-200或更多升。重组腺相关病毒颗粒纯化和生产方法适用于多种AAV血清型/衣壳变体。

[0346] 在所有方面和实施例的某些实施例中,rAAV颗粒的纯化包括以下步骤:

[0347] (a)收获包含rAAV颗粒的细胞和/或细胞培养物上清液以生产收获物;

[0348] (b)任选地浓缩步骤(a)中生产的收获物以生产经浓缩的收获物;

[0349] (c)裂解步骤(a)中生产的收获物或步骤(b)中生产的经浓缩的收获物以生产裂解物;

[0350] (d)处理步骤(c)中生产的裂解物以减少裂解物中的污染核酸,从而生产核酸减少

的裂解物;

[0351] (e)任选地过滤步骤(d)中生产的核酸减少的裂解物以生产经澄清的裂解物,并且任选地稀释经澄清的裂解物以生产经稀释的澄清的裂解物;

[0352] (f)使步骤(d)的核酸减少的裂解物、步骤(e)的经澄清的裂解物或步骤(e)中生产的经稀释的澄清的裂解物经历阳离子交换柱色谱法以生产包含rAAV颗粒的柱洗脱物,从而将rAAV颗粒与蛋白质杂质或其他生产/过程相关杂质分离,并且任选地稀释柱洗脱物以生产经稀释的柱洗脱物;

[0353] (g)使步骤(f)中生产的柱洗脱物或经稀释的柱洗脱物经历阴离子交换色谱法,以生产包含rAAV颗粒的第二柱洗脱物,从而将rAAV颗粒与蛋白质杂质或生产/过程相关杂质分离,并且任选地浓缩第二柱洗脱物以生产经浓缩的第二柱洗脱物;

[0354] (h)使步骤(g)中生产的第二柱洗脱物或经浓缩的第二柱洗脱物经历尺寸排阻柱色谱法(SEC)以生产包含rAAV颗粒的第三柱洗脱物,从而将rAAV颗粒与蛋白质杂质或生产/过程相关的杂质分离,并且任选地浓缩第三柱洗脱物以生产经浓缩的第三柱洗脱物;以及

[0355] (i)过滤步骤(h)中生产的第三柱洗脱物或经浓缩的第三柱洗脱物,从而生产经纯化的rAAV颗粒;

[0356] 其中在步骤(a)至(i)的一个或多个步骤中或之后用根据本发明的方法确定病毒基因组拷贝数。

[0357] 在某些实施例中,步骤(a)至(f)被保留并与以下步骤组合:

[0358] (g)使步骤(f)中生产的柱洗脱物或经浓缩的柱洗脱物经历尺寸排阻柱色谱法(SEC)以生产包含rAAV颗粒的第二柱洗脱物,从而将rAAV颗粒与蛋白质杂质或其他生产/过程相关的杂质分离,并且任选地稀释第二柱洗脱物以生产经浓缩的第二柱洗脱物;

[0359] (h)使步骤(g)中生产的第二柱洗脱物或经稀释的第二柱洗脱物经历阴离子交换色谱法以生产包含rAAV颗粒的第三柱洗脱物,从而将rAAV颗粒与蛋白质杂质生产/过程相关的杂质分离,并且任选地稀释第三柱洗脱物以生产经稀释的第三柱洗脱物;以及

[0360] (i)过滤步骤(h)中生产的第三柱洗脱物或经浓缩的第三柱洗脱物,从而生产经纯化的rAAV颗粒;

[0361] 其中在步骤(a)至(i)的一个或多个步骤中或之后用根据本发明的方法确定病毒基因组拷贝数。

[0362] 在某些实施例中,步骤(a)至(g)被保留并与以下步骤组合:

[0363] (h)过滤步骤(g)中生产的第二柱洗脱物或经浓缩的第二柱洗脱物,从而生产经纯化的rAAV颗粒;

[0364] 其中在步骤(a)至(h)的一个或多个步骤中或之后用根据本发明的方法确定病毒基因组拷贝数。

[0365] 在实施例中,步骤(a)至(e)被保留并与以下步骤组合:

[0366] (f)使步骤(d)中的核酸减少的裂解物或步骤(e)中生产的经澄清的裂解物或经稀释的澄清的裂解物经历AAV亲和柱色谱法,以生产包含rAAV颗粒的柱洗脱物,从而将rAAV颗粒与蛋白质杂质或其他与生产/过程相关的杂质分离,并且任选地浓缩柱洗脱物以生产经浓缩的柱洗脱物;

[0367] (g)使步骤(f)中生产的柱洗脱物或经浓缩的柱洗脱物经历尺寸排阻柱色谱法

(SEC)以生产包含rAAV颗粒的第二柱洗脱物,从而将rAAV颗粒与蛋白质杂质或其他生产/过程相关的杂质分离,并且任选地稀释第二柱洗脱物以生产经稀释的第二柱洗脱物;

[0368] (h)任选地使步骤(g)中生产的第二柱洗脱物或经稀释的第二柱洗脱物经历阴离子交换色谱法以生产包含rAAV颗粒的第三柱洗脱物,从而将rAAV颗粒与蛋白质杂质或其他生产/过程相关的杂质分离,并且任选地稀释第三柱洗脱物以生产经稀释的第三柱洗脱物;以及

[0369] (i)过滤步骤(g)中生产的第二柱洗脱物或经稀释的第二柱洗脱物,或过滤步骤(h)中生产的第三柱洗脱物或经浓缩的第三柱洗脱物,从而生产经纯化的rAAV颗粒;

[0370] 其中在步骤(a)至(i)的一个或多个步骤中或之后用根据本发明的方法确定病毒基因组拷贝数。

[0371] 在所有方面和实施例的某些实施例中,步骤(b)和/或步骤(f)和/或步骤(g)和/或步骤(h)的浓缩是经由超滤/渗滤进行的,例如通过切向流过滤(TFF)进行。

[0372] 在所有方面和实施例的某些实施例中,步骤(b)的浓缩使收获的细胞和细胞培养物上清液的体积减少约2至20倍。

[0373] 在所有方面和实施例的某些实施例中,步骤(f)和/或步骤(g)和/或步骤(h)的浓缩使柱洗脱物的体积减少约5至20倍。

[0374] 在所有方面和实施例的某些实施例中,步骤(a)中生产的收获物或步骤(b)中生产的经浓缩的收获物的裂解是通过物理或化学手段进行的。物理手段的非限制性实例包括微流化和均质化。化学手段的非限制性实例包括洗涤剂。洗涤剂包括非离子型和离子型洗涤剂。非离子型洗涤剂的非限制性实例包括Triton X-100。洗涤剂浓度的非限制性实例介于约0.1%(v/v)或(w/v)与1.0%(v/v)或(w/v)之间,包括端值。

[0375] 在所有方面和实施例的某些实施例中,步骤(d)包括用核酸酶处理从而减少污染核酸。核酸酶的非限制性实例包括benzonase。

[0376] 在所有方面和实施例的某些实施例中,步骤(e)的经澄清的裂解物或经稀释的澄清的裂解物的过滤是经由过滤器进行的。过滤器的非限制性实例是孔径介于约0.1微米与10.0微米之间(包括端值)的那些。

[0377] 在所有方面和实施例的某些实施例中,步骤(e)的经澄清的裂解物的稀释是用水性缓冲磷酸盐、乙酸盐或Tris溶液进行的。溶液pH的非限制性实例介于约pH 4.0与pH 7.4之间,包括端值。Tris溶液pH的非限制性实例大于pH 7.5,诸如介于约pH 8.0与pH 9.0之间,包括端值。

[0378] 在所有方面和实施例的某些实施例中,步骤(f)的柱洗脱物或步骤(g)的第二柱洗脱物的稀释是用水性缓冲磷酸盐、乙酸盐或Tris溶液进行的。溶液pH的非限制性实例介于约pH 4.0与pH 7.4之间,包括端值。Tris溶液pH的非限制性实例大于pH 7.5,诸如介于约pH 8.0与pH 9.0之间,包括端值。

[0379] 在所有方面和实施例的某些实施例中,将步骤(i)产生的rAAV颗粒与表面活性剂一起配制以生产rAAV颗粒制剂。

[0380] 在所有方面和实施例的某些实施例中,步骤(f)、(g)和/或(h)的阴离子交换柱色谱法包括聚乙二醇(PEG)调节的柱色谱法。

[0381] 在所有方面和实施例的某些实施例中,在从柱中洗脱rAAV颗粒之前,用PEG溶液洗

涤步骤(g)和/或(h)的阴离子交换柱色谱法。

[0382] 在所有方面和实施例的某些实施例中,PEG的平均分子量在约1,000g/mol至80,000g/mol(包括端值)的范围内。

[0383] 在所有方面和实施例的某些实施例中,PEG的浓度为约4%(w/v)至约10%(w/v),包括端值。

[0384] 在所有方面和实施例的某些实施例中,在从柱中洗脱rAAV颗粒之前,用表面活性剂水溶液洗涤步骤(g)和/或(h)的阴离子交换柱。

[0385] 在所有方面和实施例的某些实施例中,在从柱中洗脱rAAV颗粒之前,用表面活性剂溶液洗涤步骤(f)的阳离子交换柱。

[0386] 在所有方面和实施例的某些实施例中,PEG溶液和/或表面活性剂溶液包含水性Tris-HCl/NaCl缓冲液、水性磷酸盐/NaCl缓冲液或水性乙酸盐/NaCl缓冲液。

[0387] 在所有方面和实施例的某些实施例中,缓冲液或溶液中的NaCl浓度在介于约20mM至300mM NaCl之间(包括端值)或介于约50mM至250mM NaCl之间(包括端值)的范围内。

[0388] 在所有方面和实施例的某些实施例中,表面活性剂包含阳离子或阴离子表面活性剂。

[0389] 在所有方面和实施例的某些实施例中,表面活性剂包含十二碳链表面活性剂。

[0390] 在所有方面和实施例的某些实施例中,表面活性剂包含十二烷基三甲基氯化铵(DTAC)或Sarkosyl。

[0391] 在所有方面和实施例的某些实施例中,用水性Tris-HCl/NaCl缓冲液从步骤(f)、(g)和/或(h)的阴离子交换柱洗脱rAAV颗粒。

[0392] 在所有方面和实施例的某些实施例中,Tris-HCl/NaCl缓冲液包含100mM至400mM NaCl(包括端值),任选地pH在约pH 7.5至约pH 9.0(包括端值)的范围内。

[0393] 在所有方面和实施例的某些实施例中,步骤(f)、(g)和/或(h)的阴离子交换柱用水性Tris-HCl/NaCl缓冲液洗涤。

[0394] 在所有方面和实施例的某些实施例中,水性Tris-HCl/NaCl缓冲液中的NaCl浓度在约75mM至125mM(包括端值)的范围内。

[0395] 在所有方面和实施例的某些实施例中,水性Tris-HCl/NaCl缓冲液具有约pH 7.5至约pH 9.0(包括端值)的pH。

[0396] 在所有方面和实施例的某些实施例中,将步骤(f)、(g)和/或(h)的阴离子交换柱洗涤一次或多次以减少第二或第三柱洗脱物中的空衣壳的量。

[0397] 在所有方面和实施例的某些实施例中,阴离子交换柱洗涤在rAAV颗粒洗脱之前和/或代替rAAV颗粒洗脱从柱中去除空衣壳,从而减少第二或第三柱洗脱物中空衣壳的量。

[0398] 在所有方面和实施例的某些实施例中,在rAAV颗粒洗脱之前和/或代替rAAV颗粒洗脱,阴离子交换柱洗涤从柱中除去总空衣壳的至少约50%,从而将第二或第三柱洗脱物中空衣壳的量减少约50%。

[0399] 在所有方面和实施例的某些实施例中,水性Tris-HCl/NaCl缓冲液中的NaCl浓度在约110mM至120mM(包括端值)的范围内。

[0400] 在所有方面和实施例的某些实施例中,洗脱的rAAV颗粒和空衣壳的比率和/或量通过洗涤缓冲液控制。

[0401] 在所有方面和实施例的某些实施例中,由步骤(f)的阳离子交换柱在水性磷酸盐/NaCl缓冲液或水性乙酸盐/NaCl缓冲液中洗脱rAAV颗粒。缓冲液中的非限制性NaCl浓度在约125mM至500mM NaCl(包括端值)的范围内。缓冲液pH的非限制性实例介于约pH 5.5至约pH 7.5之间,包括端值。

[0402] 在所有方面和实施例的某些实施例中,步骤(f)、(g)和/或(h)的阴离子交换柱包含季铵官能团,诸如季铵化聚乙烯亚胺。

[0403] 在所有方面和实施例的某些实施例中,步骤(g)和/或(h)的尺寸排阻柱(SEC)具有约10,000g/mol至约600,000g/mol(包括端值)的分离/分级范围(分子量)。

[0404] 在所有方面和实施例的某些实施例中,步骤(f)的阳离子交换柱包含磺酸或官能团,诸如磺丙基。

[0405] 在所有方面和实施例的某些实施例中,AAV亲和柱包含结合AAV衣壳蛋白的蛋白质或配体。蛋白质的非限制性实例包括结合AAV衣壳蛋白的抗体。更具体的非限制性实例包括结合AAV衣壳蛋白的单链美洲驼抗体(骆驼科)。

[0406] 在所有方面和实施例的某些实施例中,该方法不包括氯化铯梯度超速离心的步骤。

[0407] 在所有方面和实施例的某些实施例中,该方法从步骤(a)中生产的收获物或步骤(b)中生产的经浓缩的收获物中回收约50%至90%的总rAAV颗粒。

[0408] 在所有方面和实施例的某些实施例中,该方法生产的rAAV颗粒具有比通过单个AAV亲和柱纯化生产或纯化的rAAV颗粒更高的纯度。

[0409] 在所有方面和实施例的某些实施例中,步骤(c)和(d)基本上同时进行。

[0410] 在所有方面和实施例的某些实施例中,在步骤(c)之后但在步骤(f)之前,将NaCl浓度调节至在约100mM至400mM NaCl(包括端值)的范围内,或在约140mM至300mM NaCl(包括端值)的范围内。

[0411] 在所有方面和实施例的某些实施例中,细胞是悬浮生长或贴壁生长的细胞。

[0412] 在所有方面或实施例的某些实施例中,细胞为哺乳动物细胞。非限制性实例包括HEK细胞,诸如HEK-293细胞和CHO细胞,诸如CHO-K1细胞。

[0413] 确定含有转基因的rAAV颗粒的感染滴度的方法是本领域已知的(参见例如Zhen等人,Hum.Gene Ther.15(2004)709)。用于测定空衣壳和具有包装的转基因的rAAV颗粒的方法是已知的(参见例如Grimm等人,Gene Therapy 6(1999)1322-1330;Sommer等人,Malec.Ther.7(2003)122-128)。

[0414] 为了确定降解/变性衣壳的存在或量,可以使经纯化的rAAV颗粒经历SDS-聚丙烯酰胺凝胶电泳,该电泳由能够分离三种衣壳蛋白的任何凝胶(例如梯度凝胶)组成,然后运行凝胶直到样品分离,并将凝胶印迹到尼龙或硝酸纤维素膜上。然后将抗AAV衣壳抗体用作结合变性衣壳蛋白的一抗(参见例如Wobus等人,J.Viral.74(2000)9281-9293)。结合一抗的二抗含有用于检测一抗的工具。半定量检测介于一抗与二抗之间的结合以确定衣壳的量。另一种方法是使用SEC柱的分析型HPLC或分析型超速离心机。

[0415] ***

[0416] 除了所描绘和要求保护的各种实施例之外,所公开的主题还涉及具有本文所公开和要求保护的特征的其他组合的其他实施例。这样,本文所呈现的具体特征可以在所公开

的主题的范围内以其他方式彼此组合,使得所公开的主题包括本文所公开的特征的任何合适的组合。出于图示和描述的目的,已经呈现了所公开主题的具体实施例的前文描述。其并不旨在穷举或将所公开的主题限制为所公开的那些实施例。

[0417] 本文提及的所有参考文献均通过引用随此并入。

[0418] ***

[0419] 提供以下实例以帮助理解本发明,本发明的真正范围在所附权利要求中阐明。应当理解,在不脱离本发明的精神的情况下,可对所阐述的程序进行修改。

[0420] 实例

[0421] 一般技术

[0422] 1)重组DNA技术

[0423] 使用标准方法来操纵DNA,如在Sambrook等人,Molecular Cloning:A Laboratory Manual,第二版,Cold Spring Harbor Laboratory Press,Cold Spring Harbor,N.Y,(1989)中所述的。根据制造商的说明来使用分子生物学试剂。

[0424] 2)DNA和蛋白质序列分析及序列数据管理

[0425] EMBOSS(欧洲分子生物学开放软件套件)软件包和Invitrogen的Vector NTI以及Geneious Prime用于序列创建、映射、分析、注释和图示。

[0426] 3)基因和寡核苷酸合成

[0427] 在Geneart GmbH(Regensburg,Germany)处通过化学合成来制备所需的基因区段。将合成的基因片段克隆到大肠杆菌质粒中进行繁殖/扩增。通过DNA测序来验证亚克隆基因片段的DNA序列。可替代地,通过对化学合成的寡核苷酸进行退火或经由PCR来组装短的合成DNA片段。各个寡核苷酸由metabion GmbH(Planegg-Martinsried,Germany)制备。

[0428] 4)试剂

[0429] 如果没有另外说明,则所有商业化学品、抗体和试剂盒如根据制造商的规程所提供的使用。

[0430] 5)克隆

[0431] 常规

[0432] 对于质粒,使用经由限制酶的克隆策略。通过选择合适的限制酶,可以切除所要的目标基因,并且然后通过连接将其插入不同的质粒中。因此,优选使用并以巧妙的方式选择在多克隆位点(MCS)中切割的酶,以便可以在正确的阵列中进行片段的连接。如果质粒和片段之前用相同的限制酶切割,则片段和质粒的粘性末端完美地契合在一起,并且随后可以通过DNA连接酶连接。连接之后,用新生成的质粒转化感受态大肠杆菌细胞。

[0433] 通过限制性消化进行克隆

[0434] 对于用限制酶消化质粒,将以下组分一起移取到冰上:

[0435] 表11:限制性消化反应混合物

[0436]

| 组分 | ng（设定点） | μL |
|---|---|---|
| 纯化的 DNA | 待测定 | 待测定 |
| CutSmart 缓冲液 (10x) | | 5 |
| 限制酶 | | 1 |
| PCR 级水 | | ad 50 |
| 总计 | | 50 |

[0437] 如果在一次消化中使用更多酶,则每种酶使用1μL,并通过添加更多或更少的PCR级水来调整体积。选择所有酶的前提是它们有资格与来自新英格兰生物实验室的CutSmart缓冲液(100%活性)一起使用,并具有相同的孵育温度(全部为37℃)。

[0438] 使用热混合器或热循环仪进行孵育,允许在恒定温度(37℃)孵育样品。在孵育期间,不搅动样品。孵育时间设定为60分钟。然后将样品直接与加载染料混合并加载到琼脂糖电泳凝胶上或在4℃/冰上储存以备进一步使用。

[0439] 制备1%的琼脂糖凝胶用于凝胶电泳。因此,将1.5g多用途琼脂糖称重到125锥形摇瓶中,并用150mL TAE缓冲液填充。在微波炉中将混合物加热直至琼脂糖完全溶解。将0.5μg/mL的溴化乙锭添加到琼脂糖溶液中。随后将凝胶浇铸在模具中。琼脂糖定型后,将模具放入电泳室,并用TAE缓冲液填充该室。随后装载样品。在第一个口袋中(从左侧开始),加载适当的DNA分子量标志物,然后是样品。凝胶在<130V下运行约60分钟。电泳后,将凝胶从室中取出并在UV-Imager中进行分析。

[0440] 切割条带并将其转移到1.5mL Eppendorf管中。对于凝胶的纯化,根据制造商的说明使用来自Qiagen的QIAquick凝胶提取试剂盒。将DNA片段储存在-20℃以备进一步使用。

[0441] 用于连接的片段以1:2、1:3或1:5的质粒/插入物摩尔比移取到一起,这取决于插入物和质粒片段的长度以及它们彼此之间的相关性。如果应该插入到质粒中的片段短,则使用1:5的比率。如果插入物较长,则使用较少量的质粒。每次连接中使用50ng的质粒量,并使用NEBioCalculator计算插入物的特定量。对于连接,使用来自NEB的T4 DNA连接试剂盒。下表12描绘了连接混合物的实例。

[0442] 表12:连接反应混合物

[0443]

| 组分 | ng（设定点） | 浓度 [ng/μL] | μL |
|---|---|---|---|
| T4 DNA 连接酶缓冲液 (10x) | | | 2 |
| 质粒 DNA (4000 bp) | 50 | 50 | 1 |
| 插入物 DNA (2000 bp) | 125 | 20 | 6.25 |
| 无核酸酶水 | | | 9.75 |
| T4 连接酶 | | | 1 |
| 总计 | | | 20 |

[0444] 从混合DNA和水开始,添加缓冲液且最后添加酶,将所有组分一起移取到冰上。通过上下移取来温和地混合反应物,短暂微量离心,且然后在室温孵育10分钟。孵育后,将T4连接酶在65℃热灭活10分钟。将样品在冰上冷却。在最后一步中,用2μL的连接的质粒来转化10-β感受态大肠杆菌细胞(见下文)。

[0445] 转化10-β感受态大肠杆菌细胞

[0446] 对于转化,将10-β感受态大肠杆菌细胞在冰上解冻。之后,将2μL的质粒DNA直接移

取到细胞悬液中。轻弹试管并置于冰上30分钟。此后,将细胞放入42℃的热块中并热激恰好30秒。紧接着,将细胞在冰上冷却2分钟。将950μL的NEB 10-β生长培养基添加至细胞悬液。将细胞在37℃振荡孵育一小时。然后,将50μL至100μL移取到预热(37℃)的LB-Amp琼脂板上并用一次性抹刀铺板。将板在37℃孵育过夜。只有成功掺入携带针对氨苄青霉素的抗性基因的质粒的细菌才能在这些板上生长。次日挑取单菌落并在LB-Amp培养基中培养,以用于随后的质粒制备。

[0447] 细菌培养

[0448] 大肠杆菌的培养在LB培养基(Luria Bertani的缩写)中进行,该培养基中掺入1mL/L 100mg/mL的氨苄青霉素,使得氨苄青霉素浓度为0.1mg/mL。对于不同的质粒制备数量,用单个细菌菌落接种以下量。

[0449] 表13:大肠杆菌培养体积

[0450]

| 数量质粒制备 | 体积LB-Amp培养基[mL] | 孵育时间[h] |
|---|---|---|
| 小量制备96孔(EpMotion) | 1.5 | 23 |
| 小量制备15mL-管 | 3.6 | 23 |
| 大量制备 | 200 | 16 |

[0451] 对于小量制备,将96孔2mL深孔板每孔填充1.5mL的LB-Amp培养基。挑取菌落并将牙签塞入培养基中。当挑取所有菌落时,用粘性空气多孔膜将板封闭。将板在200rpm振荡速度下于37℃孵育器中孵育23小时。

[0452] 对于小量制备,在15mL管(带通风盖)中填充3.6mL的LB-Amp培养基并等同地接种细菌菌落。在孵育期间,牙签没有被移除而是留在管中。与96孔板一样,将管在37℃、200rpm下孵育23小时。

[0453] 对于大量制备,将200mL的LB-Amp培养基填充到高压灭菌的1L Erlenmeyer玻璃烧瓶中,并接种1mL细菌日间培养物,大约是在5小时后。将Erlenmeyer烧瓶用纸塞封闭并在37℃、200rpm下孵育16小时。

[0454] 质粒制备

[0455] 对于小量制备,将50μL的细菌悬液转移到1mL的深孔板中。之后,将细菌细胞在板中以3000rpm、4℃离心5min。去除上清液,并将带有细菌颗粒的板置于EpMotion中。在大约90分钟之后,完成运行,并且可以从EpMotion中取出经洗脱的质粒DNA以备进一步使用。

[0456] 对于小量制备,从孵育器中取出15mL管,并将3.6mL的细菌培养物分装到两个2mL的Eppendorf管中。在室温,在台式微量离心机中以6,800xg将管离心3分钟。之后,根据制造商的说明使用Qiagen QIAprep Spin小量制备试剂盒进行小量制备。用Nanodrop测量质粒DNA的浓度。

[0457] 大量制备是根据制造商的说明使用Macherey-Nagel NucleoBond®Xtra Maxi EF试剂盒进行的。用Nanodrop测量DNA浓度。

[0458] 乙醇沉淀

[0459] 将一定体积的DNA溶液与2.5倍体积的100%乙醇混合。将混合物在-20℃孵育10分钟。然后以14,000rpm、4℃将DNA离心30分钟。小心去除上清液,并用70%乙醇洗涤沉淀。再次以14,000rpm、4℃将管离心5分钟。通过移液小心去除上清液并干燥沉淀。当乙醇蒸发时,添加适量的无内毒素水。给予DNA时间以重新溶解在4℃水中过夜。取一小部分并用

Nanodrop装置测量DNA浓度。

[0460] 表达盒组成

[0461] 对于开放阅读框的表达,使用至少包含以下功能性元件的转录单位:

[0462] -启动子,

[0463] -包含相应开放阅读框的核酸,如果需要的话,该开放阅读框包括信号序列,

[0464] -多聚腺苷酸化信号序列。

[0465] 除了包括所期望的待表达基因的表达单元/盒外,基础/标准哺乳动物表达质粒还包含

[0466] -来自质粒pUC18的复制起点,其允许在大肠杆菌中进行该质粒的复制,以及

[0467] -β-内酰胺酶基因,其赋予大肠杆菌中的氨苄青霉素抗性。

[0468] 6)细胞培养技术

[0469] 使用标准细胞培养技术,如Current Protocols in Cell Biology(2000),Bonifacino,J.S.,Dasso,M.,Harford,J.B.,Lippincott-Schwartz,J.和Yamada,K.M.(编辑),John Wiley&Sons,Inc.所述。

[0470] HEK293系统中的瞬时转染

[0471] 生产重组AAV颗粒的细胞已经根据制造商的说明,使用HEK293系统(Invitrogen,现为Thermo Scientific),通过用相应的质粒瞬时转染生成。简而言之,将在摇瓶或搅拌发酵管中在无血清FreeStyle™ 293表达培养基(Invitrogen)中悬浮生长的HEK293细胞(Invitrogen)用相应质粒和293fectin™或fectin(Invitrogen)的混合物转染。对于2L摇瓶(Corning),将HEK293细胞以$1*10^6$个细胞/mL的密度接种于600mL中并在120rpm、8% $CO_2$下孵育。后一天,将细胞以约$1.5*10^6$个细胞/mL的细胞密度用约42mL的以下物质的混合物转染:A)具有600μg总质粒DNA(1μg/mL)的20mL Opti-MEM(Invitrogen)和B)20mL Opti-MEM+1.2mL293fectin或fectin(2μL/mL)。根据葡萄糖消耗量,在发酵过程期间添加葡萄糖溶液。

[0472] 实例1

[0473] 无预处理

[0474] 程序:

[0475] -混合90μL H2O和10μL样品

[0476] -在95℃孵育15分钟

[0477] 实例:

[0478] 1.过程样品

[0479] 2.制备PCR主混物

[0480] 3.将主混物添加至板中

[0481] 4.用水制备1:10稀释液

[0482] 5.将模板添加到板中

[0483] 6.密封板并涡旋(1分钟,2.200rpm)并离心

[0484] 7.利用自动液滴发生器(Auto-DG)形成液滴(20μL最终混合物+70μL油)并转移到板(42μL)

[0485] 8.密封板并开始PCR运行

[0486] 实例2

[0487] 热变性

[0488] 程序:

[0489] -将样品在98℃孵育10分钟

[0490] 实例:

[0491] 1.热变性

[0492] 2.制备PCR主混物

[0493] 3.将主混物添加至板中

[0494] 4.用水制备1:10稀释液

[0495] 5.将模板添加到板中

[0496] 6.密封板并涡旋(1分钟,2.200rpm)并离心

[0497] 7.利用Auto-DG形成液滴(20μL最终混合物+70μL油)并转移到板(42μL)

[0498] 8.密封板并开始PCR运行

[0499] 实例3

[0500] DNA酶I消化

[0501] 试剂:

[0502] 1)DNA酶I缓冲液(Promega):400mM Tris-HCl,pH 8,100mM $MgSO_4$,10mM $CaCl_2$

[0503] 2)DNA酶I(Promega):50U/mL稀释至1U/μL

[0504] 程序(50μL反应体积):

[0505] -混合30μL H2O、5μL DNA酶I缓冲液、5μL DNA酶I、10μL样品

[0506] -在37℃孵育30分钟。

[0507] -加热至95℃持续15分钟。

[0508] 程序(100μL反应体积):

[0509] -混合75μL H2O、10μL DNA酶I缓冲液、5μL DNA酶I、10μL样品

[0510] -在37℃孵育30分钟。

[0511] -加热至95℃持续15分钟。

[0512] 实例:

[0513] 1.DNA酶I消化;

[0514] 2.将50μL水添加到反应混合物中

[0515] 3.制备PCR主混物

[0516] 4.将PCR主混物添加到板中(每孔16.5μL)

[0517] 5.制备1:10稀释液:10μL样品/质粒/标准品+90μL H2O

[0518] 6.将模板添加到板中(每孔5.5μL)

[0519] 7.密封板并涡旋(1分钟,2.200rpm)并离心机(1分钟,1000rcf)

[0520] 8.利用Auto-DG形成液滴(20μL最终混合物+70μL油)并转移到板(42μL)

[0521] 9.密封板并开始PCR运行

[0522] 实例4

[0523] 蛋白酶K消化

[0524] 试剂:

[0525] 1)蛋白酶K(Roche;17.8mg/mL＝≥50U/mL):稀释至1U/mL

[0526] 2)蛋白酶K缓冲液(BioRad):400mM Tris-HCl,20mM EDTA,2000mM NaCl,pH 8

[0527] 3)十二烷基硫酸钠溶液(SDS溶液):10%

[0528] 程序(水):

[0529] -将68μL水与10μL样品混合并添加20μL蛋白酶K

[0530] -在50℃孵育60分钟

[0531] -加热至95℃持续15分钟

[0532] 程序(不含SDS的缓冲液):

[0533] -将63μL水与5μL蛋白酶K缓冲液混合,并添加10μL样品以及20μL蛋白酶K

[0534] -在50℃孵育60分钟

[0535] -加热至95℃持续15分钟

[0536] 程序(含SDS的缓冲液):

[0537] -将53μL水与5μL蛋白酶K缓冲液混合,并添加10μL SDS溶液、10μL样品以及20μL蛋白酶K

[0538] -在50℃孵育60分钟

[0539] -加热至95℃持续15分钟

[0540] 实例:

[0541] 1.蛋白酶K消化

[0542] 2.制备PCR主混物

[0543] 3.将主混物添加到板中(每孔16.5μL)

[0544] 4.制备1:10稀释液:10μL样品+90μL H2O

[0545] 5.将模板添加到板中(每孔5.5μL)

[0546] 6.密封板并涡旋(1分钟,2.200rpm)并离心(1分钟,1000rcf)

[0547] 7.利用Auto-DG形成液滴(20μL最终混合物+70μL油)并转移到板(42μL)

[0548] 8.密封板并开始PCR运行

[0549] 实例5

[0550] 热变性,然后进行蛋白酶K消化

[0551] 试剂:

[0552] 1)蛋白酶K(Roche;17.8mg/mL＝≥50U/mL):1U/mL

[0553] 2)蛋白酶K缓冲液(BioRad):400mM Tris-HCl,20mM EDTA,2000mM NaCl,pH 8

[0554] 程序:

[0555] -热变性:将样品在98℃孵育10分钟

[0556] -蛋白酶K消化:每50μL样品1μL蛋白酶K;在50℃孵育30分钟;在95℃失活10分钟。

[0557] 实例:

[0558] 1.热变性

[0559] 2.蛋白酶K消化

[0560] 3.制备1:10稀释液:10μL样品+90μL H2O

[0561] 4.制备PCR主混物

[0562] 5.将主混物添加至板中

[0563] 6.将样品添加到板中

[0564] 7.密封板并涡旋(1分钟,2.200rpm)并离心

[0565] 8.利用Auto-DG形成液滴(20μL最终混合物+70μL油)并转移到板(42μL)

[0566] 9.密封板并开始PCR运行

[0567] 实例6

[0568] DNA酶I消化,然后进行蛋白酶K消化

[0569] 方法1:

[0570] 试剂:

[0571] 1)DNA酶I缓冲液(Promega):400mM Tris-HCl,pH 8,100mM $MgSO_4$,10mM $CaCl_2$

[0572] 2)DNA酶I(Promega):1U/μL

[0573] 3)蛋白酶K(Roche;17.8mg/mL＝≥50U/mL):1U/mL

[0574] 4)蛋白酶K缓冲液(BioRad):400mM Tris-HCl,20mM EDTA,2000mM NaCl,pH 8

[0575] 5)十二烷基硫酸钠溶液(SDS溶液):10%

[0576] 程序:

[0577] -混合30μL H2O、5μL DNA酶I缓冲液、5μL DNA酶I、10μL样品

[0578] -在37℃孵育30分钟。

[0579] -加热至95℃持续15分钟。

[0580] -将50μL PK-混合物(42μL H2O+2μL蛋白酶K+5μL 20x蛋白酶K缓冲液+1μL 10%SDS溶液)与50μL经孵育的DNA酶I-混合物混合

[0581] -在50℃孵育60分钟

[0582] -加热至95℃持续15分钟。

[0583] 实例:

[0584] 1.DNA酶I消化;任选地稀释1:10

[0585] 2.蛋白酶K消化;稀释1:10

[0586] 3.制备PCR主混物

[0587] 4.将PCR主混物添加到板中(每孔16.5μL)

[0588] 5.制备1:10稀释液:10μL样品+90μL H2O

[0589] 6.将模板添加到板中(每孔5.5μL)

[0590] 7.密封板并涡旋(1分钟,2.200rpm)并离心(1分钟,1000rcf)

[0591] 8.利用Auto-DG形成液滴(20μL最终混合物+70μL油)并转移到板(42μL)

[0592] 9.密封板并开始PCR运行

[0593] 方法2:

[0594] 试剂:

[0595] 1)蛋白酶K(NEB;约20mg/mL＝≥800U/mL):稀释至16U/mL

[0596] 2)蛋白酶K缓冲液(BioRad):400mM Tris-HCl、20mM EDTA、2000mM NaCl

[0597] 3)十二烷基硫酸钠溶液(SDS溶液):10%

[0598] 程序(含SDS的缓冲液):

[0599] -将42μL水与50μL样品(DNA酶I消化溶液)混合,并添加2μL蛋白酶K、5μL蛋白酶K缓冲液和1μL SDS溶液

[0600] -在50℃孵育60分钟

[0601] -加热至95℃持续15分钟

[0602] 实例：

[0603] 1.DNA酶I消化

[0604] 2.蛋白酶K消化

[0605] 3.稀释1:10

[0606] 4.制备PCR主混物

[0607] 5.将主混物添加到板中(每孔16.5μL)

[0608] 6.制备1:10稀释液:10μL样品+90μL H2O

[0609] 7.将模板添加到板中(每孔5.5μL)

[0610] 8.密封板并涡旋(1分钟,2.200rpm)并离心(1分钟,1000rcf)

[0611] 9.利用Auto-DG形成液滴(20μL最终混合物+70μL油)并转移到板(42μL)

[0612] 10.密封板并开始PCR运行实例7

[0613] 条件比较

[0614] 试剂和程序如先前实例中所概述。

[0615] 条件依据下表14和15。

[0616] 表14：

[0617]

| **条件** | **A** | **B** |
|---|---|---|
| 酶 | 无 DNA 酶 I | DNA 酶 I: 1 U/ μL |
| 缓冲液 | - | DNA 酶 I 缓冲液 (Promega) |
| 程序 | 90 μL H2O + 10 μL 样品 | 75 μL H2O + 10 μL 缓冲液 + 5 μL DNA 酶 I + 10 μL 样品 |
| 孵育温度 | - | 37 ℃ |
| 孵育时间 | - | 30 分钟 |
| 失活 | 95℃，15 min | 95℃，15 min |

[0618] 表15：

[0619]

| **条件** | **W** | **X** | **Y** | **Z** |
|---|---|---|---|---|
| 酶 | 无蛋白酶 K (PK) | 1 U/mL 蛋白酶 K | 1 U/mL 蛋白酶 K | 1 U/mL 蛋白酶 K |
| 缓冲液 | - | H2O | 蛋白酶 K 缓冲液 | 蛋白酶 K 缓冲液 10% SDS |

[0620]

| 条件 | W | X | Y | Z |
|---|---|---|---|---|
| 程序 | 90 µL H2O + 10 µL 样品 | 68 µL H2O + 10 µL 样品 + 20 µL PK | 63 µL H2O + 5 µL PK 缓冲液 + 10 µL 样品 + 20 µL PK | 53 µL H2O + 5 µL PK 缓冲液 + 10 µL 10% SDS + 10 µL 样品 + 20 µL PK |
| 孵育温度 | - | 50 ℃ | 50 ℃ | 50 ℃ |
| 孵育时间 | - | 60 min | 60 min | 60 min |
| 失活 | 95℃ 15 分钟，4℃ | 95℃ 15 分钟，4℃ | 95℃ 15 分钟，4℃ | 95℃ 15 分钟，4℃ |

[0621] 实例8

[0622] ddPCR

[0623] 对于病毒基因组滴定,进行了双重ddPCR测定。引物和探针是针对ITR位点和Amp抗性序列设计的,该序列存在于rAAV生产中使用的所有三种质粒的主链上。根据表16(微滴式数字PCR指南-Bio-Rad)制备PCR主混物。

[0624] 表16:ddPCR主混物组成。

[0625]

| 组分 | 每孔体积[µL] | 最终浓度 |
|---|---|---|
| 超混物(2x) | 11 | 1x |
| 20µM ITR正向引物 | 0.99 | 900nM |
| 20µM ITR反向引物 | 0.99 | 900nM |
| 20µM FAM标记的ITR探针 | 0.275 | 250nM |
| 20µM Amp正向引物 | 0.99 | 900nM |
| 20µM Amp反向引物 | 0.99 | 900nM |
| 20µM HEX标记的Amp探针 | 0.275 | 250nM |
| 模板 | 5.5 | 1*10E4-1*10E5个拷贝/mL |
| 水 | 0.99 | - |
| 总计 | 22 | |

[0626] 将制备的主混物移入96孔板中,每孔16.5µL。然后,对预处理样品进行系列稀释:使用LoRentention Tips将10µL样品转移到LoBind管中的90µL水中并充分混合。此后,通过几个稀释步骤将5.5µL样品添加到96孔板中的主混物溶液中。将板在180℃密封,以2,200rpm涡旋1分钟并以1,000rpm再离心1分钟。使用自动液滴发生器装置从每个孔中取出20µL PCR混合物,每孔生产多达20,000个液滴,并转移到另一个96孔板中。在180℃密封液滴板后,进行PCR运行。相应的条件在下表17中示出。

[0627] 表17:ddPCR热循环程序。

[0628]

| 循环次数 | 变性 | 退火 | 最终延伸 | 结束 |
|---|---|---|---|---|
| 1 | 95℃,10分钟 | | | |

| | | | | |
|---|---|---|---|---|
| 40 | 94℃,30秒 | 60℃,1min | | |
| 1 | | | 98℃,10min | 12℃,维持 |

[0629] 在液滴读取器中,测量FAM和HEX通道中针对每个液滴的荧光信号。QuantaSoft软件处理读取器数据并计算每20μL孔中针对靶序列、ITR位点和Amp的拷贝数。初始样品滴度可通过以下等式1确定:

[0630] $$拷贝数\left[\frac{拷贝}{mL}\right]=\frac{输出\left[\frac{拷贝}{20\mu L\ 孔}\right]}{5\left[\frac{\mu L\ 样品}{20\mu L\ 孔}\right]}\cdot 稀释因子\cdot 1000\left[\frac{\mu L}{mL}\right] \quad (2)$$

[0631] 实例 9

[0632] rAAV 生产

[0633] HEK293-F悬浮细胞用三种质粒转染,即pAAV-转基因(EGFP或EBFP)、pAAV-rep/cap和pAAV-辅助。将质粒DNA(1μg/1mL细胞培养物)和脂转染试剂PEI pro(2μL/1mL细胞培养物)分别与OptiMEM(50μL/1mL细胞培养物)混合(参见例如Grieger,J.等人2016)。然后,将两种溶液合并,在室温孵育15分钟并添加到F17培养基中的HEK293-F细胞悬液(1*10E6个细胞/mL)中。将细胞在37℃、8% CO2、120rpm下孵育48至72小时(Grieger,J.等人(2016))。

[0634] 通过添加含有1% Triton X-100、500mM TRIS和20mM $MgCl_2$(pH 7.5)的裂解缓冲液(100μL/1mL细胞培养物)收获重组AAV颗粒。添加新鲜稀释的Benzonase(10μL/1mL细胞培养物),终浓度为50U/mL。60分钟后,在37℃搅拌下裂解,添加$MgSO_4$(终浓度37.5mM),并将细胞裂解液体培养基再孵育30分钟(Chahal,P.等人(2014))。然后,将裂解悬浮液以4,000g离心20分钟并将上清液通过0.22μm过滤器过滤。所得产物被认为是粗裂解物。

[0635] 实例 10

[0636] rAAV 纯化

[0637] YMC玻璃柱体填充有POROS CaptureSelect AAVx亲和树脂,柱床体积为9.1mL。这些树脂珠涂有抗体片段,能够以高特异性结合多种AAV血清型(POROS CaptureSelect AAV树脂-用户指南2017)。

[0638] 首先,用磷酸盐缓冲盐水(PBS)平衡柱以生产正确的结合条件。然后,以150cm/小时加载粗过滤的裂解物。捕获rAAV衣壳后,将柱用4个柱体积(CV)的PBS洗涤,然后用4CV的0.5M NaCl洗涤,以去除如细胞碎片和DNA残留物的杂质。使用4CV的PBS进行另一个洗涤步骤,以准备洗脱条件(POROS CaptureSelect AAV树脂-用户指南2017)。

[0639] 然后,在100mM柠檬酸缓冲液(pH 2.4)中洗脱rAAV衣壳(POROS CaptureSelect AAV树脂-用户指南2017)。合并洗脱峰内的级分(在λ=280nm处进行UV检测)。使用2M TRIS(pH 9)将pH值升高至pH 7.5。最后,使用孔径为0.2μm的注射器过滤器对洗脱物进行无菌过滤。

## Abstract

Herein is reported a method for the determination of viral genome DNA copy number in a sample, wherein the method comprises the steps of incubating the sample with proteinase K and determining the viral genome DNA copy number by digital droplet polymerase chain reaction, wherein the sample is free of DNA, which is not encapsidated within a viral particle, wherein the incubation with proteinase K is in the presence of 0.05(w/v)% to 1.5(w/v)% sodium dodecyl sulfate.

## 摘要

本文报道了一种用于确定样品中病毒基因组 DNA 拷贝数的方法，其中所述方法包括以下步骤：将所述样品与蛋白酶 K 一起孵育，以及通过数字微滴式聚合酶链式反应确定所述病毒基因组 DNA 拷贝数，其中所述样品不含未在病毒颗粒内衣壳化的 DNA，其中与蛋白酶 K 一起的所述孵育在存在 0.05(w/v)％至 1.5(w/v)％十二烷基硫酸钠的情况下进行。