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(54) Title: METHODS AND COMPOSITIONS FOR PURIFYING ADENO ASSOCIATED VIRUS PARTICLES

(57) Abstract: The present invention relates to compositions and methods for lysing cells and isolating and/or purifying adeno-associated virus particles using a detergent selected from the group of secondary fatty alcohol ethoxylates.



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Methods and compositions for purifying adeno associated virus particles

5 The present invention relates to compositions and methods for lysing cells and thus purifying adeno-associated virus particles using a detergent selected from the group of non-ionic surfactants, namely secondary fatty alcohol ethoxylates.

10 Adeno-associated virus (AAV) have been characterized and developed as a potent viral vector to deliver genes in vitro in cultured cells and also in vivo. AAV is meanwhile a leading platform for in vivo delivery of gene therapies. AAV is a small, non-enveloped virus containing a single-stranded DNA genome of approximately 4.7 kb, consisting of two inverted terminal repeats (ITRs) that are capable of forming T-shape secondary structure and
15 acting as origins of genome replication, one rep region that encodes four overlapping replication proteins, Rep78, Rep68, Rep52, and Rep40, and one cap region that encodes three structural proteins, VP1, VP2, and VP3, and an assembly activating protein (AAP). Naturally isolated serotypes 1-9 of the AAV viruses share the genomic structure although these serotypes
20 may display different tissue tropism. As the AAVs seem to be nonpathogenic, show an efficient transduction and a stable expression, they are regarded as being one of the most promising gene delivery vehicles.

25 AAV vectors can be produced in various cell lines in adherent or suspension cell culture formats using transient transfection or co-infection methods. Depending on specific serotypes and production times, viral particles including full, partial and empty species can be secreted out of cells into culture medium or contained inside cells at various ratios.

30 Initially, stable AAV producer cells were generated by transfection and selection of human-derived cells, like HeLa or HEK293 cells, with an rAAV

transfer vector containing the ITR cassette and a packaging construct containing Rep and Cap. Production of recombinant AAV vectors (rAAV) was then achieved by infection with auxiliary viruses such as adenoviruses (AdV) that provide the helper function. After identification of AdV genes
5 required for AAV vector packaging, a helper virus-free method was established using a duo or triple transfection protocol consisting of two or three plasmids including a constructed helper plasmid instead of an auxiliary virus. This system is widely used in research and drug development. In addition, development of baculovirus expression vectors
10 provides another method to produce rAAV viruses in insect Sf9 cells. These different technologies are shown to be able to produce sufficient quantities of rAAV viruses for use in laboratories and clinical trials.

A cell lysis step is generally required at harvest to release viral particles into
15 the supernatant. For this application, typical cell lysis reagents such as Triton™ X-100, Tween™ 20, and NaCl are broadly utilized. However, for certain serotypes (e.g., AAV2), viral particles tend to be tightly associated with insoluble cellular components thus limit the efficiency of certain cell lysis reagents in terms of virus release.

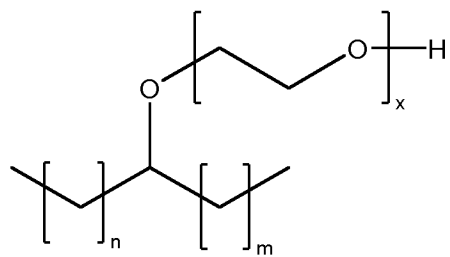
20 Current methods of releasing viruses, especially cell-associated viruses, are often time consuming like e.g. physical lysis methods, are difficult to scale, or can introduce unwanted and difficult to remove impurities, e.g., Triton™ X-100. A degradation product of Triton™ X-100 is 4-tert
25 octylphenol, which mimics the hormone estradiol and leads to harmful effects to the endocrine system of aquatic organisms. In 2017, octylphenol ethoxylates (OPE) were added to the European Authorization list (Annex XIV) of REACH (Registration, Evaluation, Authorization and Restrictions of Chemicals). Annex XIV is a list of banned substances in the EU. After
30 January 2021, OPE products, including Triton™ X-100 detergent, are prohibited in the EU by the European Chemicals Agency (ECHA), unless authorization was granted by the authorities, or the intended use is exempt

from authorization. Therefore, it would be beneficial to have an effective and sustainable reagent and method to lyse the cells and isolate and/or purify viruses like AAV from the cells in which they are produced.

- 5 The inventors have surprisingly found that a certain group of sustainable detergents is especially suitable for releasing cell associated viruses.

The present invention is thus directed to a method for lysing cells enclosing adeno-associated virus (AAV) particles by contacting a suspension of said
 10 cells with an effective amount of a composition comprising one or more compounds of Formula I to promote cell lysis and release of the AAV particles from the cells whereby the viral particles remain unaffected.

15



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Formula I

with $x = 7$ to 15

and $n + m = 8$ to 12

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Preferably $x = 8$ to 10 , in a very preferred embodiment $x = 9$.

An effective amount is preferably an amount that after contacting with the suspension of cells results at a final overall concentration of compounds according to Formula I above their CMC.

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Typically, this lysis method is part of a method for purifying viruses whereby further isolation or purification steps are performed in addition to the lysis step.

- 5 The present invention is further directed to a method for purifying viral particles of an adeno-associated virus (AAV) from a sample comprising cells enclosing the viral particles by
- a) contacting a suspension of the cells and the viral particles with an effective amount of a composition comprising one or more compounds of
- 10 Formula I to promote cell lysis and release of the AAV particles from the cells
- b) isolating and/or purifying the AAV particles.

15 In a preferred embodiment, the suspension is contacted with an effective amount of a composition comprising one or more compounds of Formula I and a salt like sodium chloride either as a combination or subsequently. In case that a salt like sodium chloride is added subsequently, the suspension is typically incubated with the composition for a certain time, preferably 1 to 2 hours, prior to the addition of the one or more salts like sodium chloride.

20 In a very preferred embodiment, the composition comprises a C11-C15 secondary alcohol ethoxylate according to CAS 68131-40-8 and/or a C12-C14 secondary alcohol ethoxylate according to CAS 84133-50-60.

25 In another preferred embodiment, in step a) the cells are contacted with the composition for 30 to 180 minutes, preferably for 60 to 120 minutes.

In another embodiment the viral particles are AAVs, especially an AAV2, AAV5 or AAV9 serotype.

30 In one embodiment the composition is an aqueous solution comprising one or more compounds of Formula I and a salt like sodium chloride.

Preferably, the composition contains only water, one or more compounds of Formula I, sodium chloride and optionally buffer components.

5 In a preferred embodiment, the concentration of the one or more compounds of Formula I in the composition is such that the concentration in the mixture with the cells is between 0.1% and 4%, preferably between 0.25% to 1% (w/w).

10 In another preferred embodiment the concentration of the salt like sodium chloride in the composition is such that the concentration in the mixture with the cells is between 0.05 and 1 mol/l, preferably between 0.1 and 1 mol/l.

In one embodiment step b) is performed by filtration or centrifugation.

15

In one embodiment, the method of the present invention comprises one or more of the following steps:

- Clarification

- filtration

20

- dialysis/ diafiltration

- tangential flow filtration

- treatment with nuclease, e.g. RNase and/or DNase

- treatment with chloroform

- ion exchange chromatography

25

- affinity chromatography

- hydrophobic interaction chromatography

- centrifugation

- PEG precipitation

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The present invention is further directed to a composition comprising one or more compounds of Formula I and a salt like sodium chloride.

In a preferred embodiment the composition is an aqueous solution comprising 5 to 30 % (w/w) of a C11-C15 secondary alcohol ethoxylate according to CAS 68131-40-8 and/or a C12-C14 secondary alcohol ethoxylate according to CAS 84133-50-60 and 1 to 6 mol/l of a salt like NaCl, preferably NaCl.

In a preferred embodiment the composition further comprises a nuclease, preferably a salt tolerant nuclease, most preferred WT *Vibrio salmonicida* EndA nuclease.

In a preferred embodiment the composition has a pH between 6 and 9.

The present invention is further directed to a kit comprising the composition of the present invention as well as a nuclease.

Figure 1 shows AAV2 viral genome titers after cell lysis with the different detergents. Tween™ 20 and Triton™ X-100 are benchmark comparisons. The lysis procedure included a nuclease and a subsequent step with 0.5 M NaCl. Further details can be found in Example 1.

Figure 2 shows the plot static surface tension as a function of secondary alcohol ethoxylate concentration. This plot is employed to determine the critical micelle concentration of the detergent (CMC). Further details can be found in Example 2.

Figure 3 shows the percentage of viable HEK 293 adherent cells after the lysis. Tween™ 20 and Triton™ X-100 are benchmark comparison, the condition without detergent is the control. The lysis procedure included Benzonase® endonuclease and no NaCl was added to the amount present in the cell culture media. The values were calculated based on the total and viable cells counted by the Vi-CELL (Beckmann Coulter). Further details can be found in Example 3.

Figure 4 shows the percentage of viable HEK 293 adherent cells after the lysis. Tween™ 20 and Triton™ X-100 are benchmark comparison, the condition without detergent is the control. The lysis procedure included Benzonase® salt tolerant endonuclease and NaCl was added to reach a
5 total concentration of 0.5 M. The values were calculated based on the total and viable cells counted by the Vi-CELL (Beckmann Coulter). Further details can be found in Example 3.

Figure 5 shows the percentage of viable HEK 293 suspension cells after the lysis. Tween™ 20 and Triton™ X-100 are benchmark comparison, the
10 condition without detergent is the control. The lysis procedure included Benzonase® endonuclease and no NaCl was added to the amount present in the cell culture media. The values were calculated based on the total and viable cells counted by the Vi-CELL (Beckmann Coulter). Further details
15 can be found in Example 3.

Figure 6 shows the percentage of viable HEK 293 suspension cells after the lysis. Tween™ 20 and Triton™ X-100 are benchmark comparison, the condition without detergent is the control. The lysis procedure included Benzonase® salt tolerant endonuclease and NaCl was added to reach a
20 total concentration of 0.5 M. The values were calculated based on the total and viable cells counted by the Vi-CELL (Beckmann Coulter). Further details can be found in Example 3.

Figure 7 shows AAV2 viral genome titers after HEK 293 adherent cell lysis with the different detergents. Tween™ 20 and Triton™ X-100 are
25 benchmark comparisons. The lysis procedure included a Benzonase® nuclease and no NaCl was added to the amount present in the cell culture media. Further details can be found in Example 3.

Figure 8 shows AAV2 viral genome titers after HEK 293 adherent cell lysis with the different detergents. Tween™ 20 and Triton™ X-100 are
30 benchmark comparisons. The lysis procedure included a Benzonase® salt

tolerant endonuclease and NaCl was added to reach a total concentration of 0.5 M. Further details can be found in Example 3.

Figure 9 shows AAV5 viral genome titers after HEK 293 suspension cell lysis with the different detergents. Tween™ 20 and Triton™ X-100 are benchmark comparisons. The lysis procedure included a Benzonase®
5 nuclease and no NaCl was added to the amount present in the cell culture media. Further details can be found in Example 3.

Figure 10 shows AAV5 viral genome titers after HEK 293 suspension cell lysis with the different detergents. Tween™ 20 and Triton™ X-100 are
10 benchmark comparisons. The lysis procedure included a Benzonase® salt tolerant endonuclease and NaCl was added to reach a total concentration of 0.5 M. Further details can be found in Example 3.

Figure 11 shows AAV2 viral genome titers after Sf-RVN® cell lysis with the different detergents. Tween™ 20 and Triton™ X-100 are benchmark
15 comparisons. The lysis procedure included a Benzonase® nuclease and no NaCl was added to the amount present in the cell culture media. Further details can be found in Example 3.

Figure 12 shows AAV2 viral genome titers after Sf-RVN® cell lysis with the different detergents. Tween™ 20 and Triton™ X-100 are benchmark
20 comparisons. The lysis procedure included a Benzonase® salt tolerant endonuclease and NaCl was added to reach a total concentration of 0.5 M. Further details can be found in Example 3.

Figure 13 Infectivity of AAV2 after the lysis of HEK293 adherent cells with detergents. The lysis procedure included a Benzonase® nuclease and no
25 NaCl was added to the amount present in the cell culture media. The infectivity was determined with the transduction unit assay.

Figure 14 Infectivity of AAV2 after the lysis of HEK293 adherent cells with detergents. The lysis procedure included a Benzonase® salt tolerant
30 endonuclease and NaCl was added to reach a total concentration of 0.5 M. The infectivity was determined with the transduction unit assay.

Before describing the present invention in detail, it is to be understood that this invention is not limited to specific compositions or process steps, as such may vary. It must be noted that, as used in this specification and the appended claims, the singular form "a", "an" and "the" include plural
5 referents unless the context clearly dictates otherwise. Thus, for example, reference to "a virus" includes a plurality of viruses and reference to "an antibody" includes a plurality of antibodies and the like.

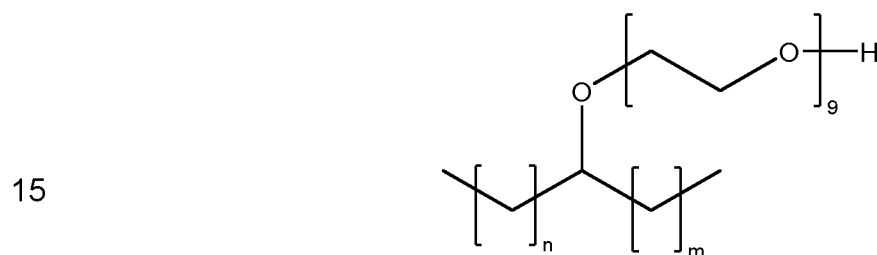
Unless defined otherwise, all technical and scientific terms used herein
10 have the same meaning as commonly understood by one of ordinary skill in the art to which this invention is related. The following terms are defined for purposes of the invention as described herein.

Surfactants are defined as "surface active molecules". The term "surfactant"
15 as used herein refers to compounds that lower the surface tension between two liquids or between a liquid and a solid. Surfactants may act as detergents, wetting agents, emulsifiers, foaming agents, and dispersants. A detergent is defined as a surfactant or a mixture containing one or more surfactants having cleaning properties in dilute solutions. Detergents
20 especially have the ability to permeabilize lipid membranes.

Micelles are defined as "surfactant aggregates formed in solution, which exist in equilibrium with the molecules or ions from which they are formed". The critical micelle concentration (CMC) is defined as "relatively small
25 range of concentrations separating the limit below which virtually no micelles are detected and the limit above which virtually all additional surfactant molecules form micelles". (IUPAC. 2008a. "Critical Micelle Concentration." *The IUPAC Compendium of Chemical Terminology* 1077:2014. / IUPAC. 2008b. "Micelle." *The IUPAC Compendium of*
30 *Chemical Terminology* 1077:3889.). Above the CMC virtually all additional detergents added to the system go to micelles. The value of the CMC for a given detergent in a given medium depends on temperature, pressure, and

(sometimes strongly) on the presence and concentration of other surface-active substances and electrolytes.

5 Secondary alcohol alkoxylates contain an ethylene oxide chain attached to a secondary alcohol. The secondary alcohol alkoxylates to be used in the method of the present invention are secondary alcohol ethoxylates made from secondary alcohols with 11 to 15 carbons and carrying 7 to 15 ethylene oxide units. An especially preferred group of such secondary alcohol ethoxylates is shown in Formula II comprising 9 ethylene oxide
10 units.



Formula II

Such compounds are commercially available as Tergitol™ 15-S-9, The Dow
20 Chemical Company or Deviron® 13-S9, Merck KGaA, Germany.

These compounds are preferably used at concentrations above their critical micelle concentration.

25 Examples of salts like sodium chloride are salts comprising a metal cation like K^+ , Na^+ , Li^+ , Mg^{2+} , Ca^{2+} and an anionic component like F^- , SO_4^{2-} , HPO_4^{2-} , acetate, Cl^- . Preferred are salts comprising monoatomic ions. Especially preferred are chloride salts like sodium chloride and potassium chloride, whereby sodium chloride is most preferred.

30

Adeno-associated virus (AAV) is a member of the Parvoviridae family. The AAV genome is composed of a linear single-stranded DNA molecule which

contains approximately 4.7 kilobases (kb) and consists of two major open reading frames encoding the non-structural Rep (replication) and structural Cap (capsid) proteins. Flanking the AAV coding regions are two cis-acting inverted terminal repeat (ITR) sequences, approximately 145 nucleotides in length, with interrupted palindromic sequences that can fold into hairpin structures that function as primers during initiation of DNA replication. In addition to their role in DNA replication, the ITR sequences have been shown to be necessary for viral integration, rescue from the host genome, and encapsidation of viral nucleic acid into mature virions (Muzyczka, (1992) Curr. Top. Micro. Immunol. 158:97-129).

Multiple serotypes of AAV exist and offer varied tissue tropism. Known serotypes include, for example, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10 and AAV11.

Vectors derived from AAV are particularly attractive for delivering genetic material because they are able to infect (transduce) a wide variety of non-dividing and dividing cell types including muscle fibers and neurons and they are devoid of the virus structural genes, thereby eliminating the natural host cell responses to virus infection, e.g., interferon-mediated responses. In addition, wild-type viruses have never been associated with any pathology in humans.

According to the present invention scAAV are also within the group of AAVs. Self-complementary adeno-associated vectors (scAAV) are viral vectors engineered from the naturally occurring adeno-associated virus (AAV) for use in gene therapy. ScAAV is termed "self-complementary" because the coding region has been designed to form an intramolecular double-stranded DNA template.

Thus, in some embodiments, by an "AAV " is meant a vector or virus derived from an adeno-associated virus serotype, including without limitation, AAV-1, AAV-2, AAV-3, AAV-4, AAV -5, AAV- 6, AAV-7, AAV -8,

AAV-9, AAV-10 and AAV-11. AAV vectors can have one or more of the AAV wild-type genes deleted in whole or part, e.g., the rep and/or cap genes, but retain functional flanking ITR sequences. Functional ITR sequences are necessary for the rescue, replication and packaging of the AAV virion. Thus, an AAV vector is defined herein to include at least those sequences that provide for replication and packaging (e.g., functional ITRs) of the virus. The ITRs need not be the wild-type nucleotide sequences, and may be altered, e.g., by the insertion, deletion or substitution of nucleotides, so long as the sequences provide for functional rescue, replication and packaging. In one embodiment, the vector is an AAV-9 vector, with AAV-2 derived ITRs. Also by an "AAV" is meant the protein shell or capsid, which provides an efficient vehicle for delivery of vector nucleic acid to the nucleus of target cells.

The term "AAV" as used herein is intended to also encompass recombinant AAV.

As used herein, the term "cell" or "cell line" refers to a single cell or to a population of cells capable of continuous or prolonged growth and division in vitro. In some embodiments, e.g. the terms "HEK293 cells", "293 cells" or their grammatical equivalents are used interchangeably here and refer to the host/packaging cell line used in the methods disclosed herein.

Suitable cells and cell lines have been described for use in production of AAVs. The cells themselves may be selected from any biological organism, including prokaryotic (e.g., bacterial) cells and eukaryotic cells including insect cells, yeast cells and mammalian cells. Particularly desirable host cells are selected from among any mammalian species, including, without limitation, A549, WEHI, 3T3, 10T1/2, BHK, MDCK, COS 1, COS 7, BSC 1, BSC 40, BMT 10, VERO, WI38, HeLa, a HEK 293 cell, Saos, C2C12, L cells, HT1080, HepG2 and primary fibroblast, hepatocyte and myoblast cells derived from mammals including human, monkey, mouse, rat, rabbit, and hamster.

Generally, the expression cassette is composed of, at a minimum, a 5' AAV inverted terminal repeat (ITR), a nucleic acid sequence encoding a desirable therapeutic, immunogen, or antigen operably linked to regulatory sequences which direct expression thereof, and a 3' AAV ITR. In one
5 embodiment, the 5' and/or 3' ITRs of AAV serotype 2 are used. However, 5' and 3' ITRs from other suitable sources may be selected. It is this expression cassette that is packaged into capsid proteins to form an AAV virus or particle.

In addition to the expression cassette, the cells contain the sequences
10 which drive expression of AAVs in the cells (cap sequences) and rep sequences of the same source as the source of the AAV ITRs found in the expression cassette, or a cross-complementing source. The AAV cap and rep sequences may be independently selected from different AAV parental sequences and be introduced into the host cell in a suitable manner known
15 to one in the art. While the full-length rep gene may be utilized, it has been found that smaller fragments thereof, i.e., the rep78/68 and the rep52/40 are sufficient to permit replication and packaging of the AAV.

The cells also require helper functions in order to package the AAV of the invention. Optionally, these helper functions may be supplied by a
20 herpesvirus. In another embodiment, the necessary helper functions are each provided from a human or non-human primate adenovirus source, such as are available from a variety of sources, including the American Type Culture Collection (ATCC), Manassas, Va. (US).

25 A buffer or a buffer solution is a solution of a certain pH used to prevent pH changes. Examples of buffers are CO₂/HCO₃ (carbonate), phosphate, HEPES, PIPES, ACES, BES, TES, MOPS and TRIS.

30 During the manufacturing of AAVs, a percentage of capsids might not incorporate any of the transgenes and are referred to as empty capsids or empty AAVs. Additionally, capsids that contain fragments of the transgene are called partial capsids or partial AAVs. These undesired product-related

impurities are co-produced with the full capsids or full AAVs which contain the full length of the desired transgene.

5 Purification means to increase the degree of purity of a target molecule, in this case the AAVs, e.g., by removing or reducing the amount of one or more impurities.

10 The term "impurity" or "contaminant" as used herein, refers to any foreign or objectionable molecules or species, including a biological macromolecules such as DNA, RNA, one or more host cell proteins, nucleic acids, endotoxins, lipids, impurities of synthetic origin like detergents, partial and/or empty AAVs as well as one or more additives which may be present in a sample containing the viral particles to be purified and thus to be separated from one or more of the impurities.

15 A bioreactor is any vessel or tank in which cells can be cultured. Incubation is typically done under suitable conditions like suitable temperature etc and with a suitable medium for supporting the growth/culturing of the cells. A person skilled in the art is aware of suitable incubation conditions for supporting or maintaining the growth/culturing of cells.

20

The present invention is based on the finding that a certain type of composition comprising at least one or more compounds of Formula I as a detergent is especially suitable for lysing cells and setting free viral particles. AAV vectors can be produced in various cell lines in adherent or suspension cell culture formats using transient transfection, infection, or co-infection methods. Depending on specific serotypes and production times, viral particles including full, partial, and empty species can be secreted out of cells into culture medium or contained inside cells at various ratios. A cell lysis step is generally required at harvest to release viral particles into the supernatant. Sometimes, the viral particles tend to be tightly associated

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with insoluble cellular components which limits the efficiency of certain cell lysis reagents in terms of virus release.

5 It has been found one or more compounds of Formula I alone or in combination with salts like sodium chloride are especially suitable to promote cell lysis and isolation and/or purification of the viral particles.

10 The production of cells comprising AAVs is known to a person skilled in the art. Typically, the selected cells are expanded in suitable culture media in a bioreactor under suitable conditions. The cells may be grown as adherent or suspension culture. For example, in suspension culture of HEK293 cells suitable seeding numbers before transfection are 0.5 to 2 e6 viable cells per ml. For adherent HEK 293 cells, typically a lower seeding density is required.

15 Suitable methods for the transduction are known in the art. In one embodiment, cells can be transduced in vitro by combining a rAAV with the cells, e.g., in appropriate media, and screening for those cells harboring the DNA of interest using conventional techniques such as Southern blots and/or PCR, or by using selectable markers.

20 Transfection can be performed using any of the techniques known in the art, including but not limited to electroporation, lipofection, e.g., with a lipofectamine, cationic polymers and cationic lipids. Any suitable transfection media may be used. In one embodiment of the transfection process, adherent or suspension human embryonic kidney (HEK293) cells are transfected with a double or triple DNA plasmid polyethylenimine (PEI) co-precipitation.

30 In an embodiment, the present disclosure provides a method for manufacturing an AAV based viral vector comprising the steps of (i) culturing cells in a bioreactor, (ii) transfecting the cells with plasmids to

enable production of the AAV particles, (iii) contacting a mixture of the cells and the viral particles with an effective amount of a composition comprising one or more compounds of Formula I and optionally a salt like sodium chloride to promote cell lysis and release of the viral particles from the cells
5 iv) isolating and/or purifying the viral particles.

After a suitable virus production period post transfection or infection, the cells are lysed, and the viral particles harvested. In some embodiments, the cells are dissociated from the bioreactor before the cell lysis process is
10 initiated. In some embodiments, the cells are lysed in situ.

According to the present invention, for lysis, the cells are contacted with a composition comprising one or more compounds of Formula I and optionally a salt like sodium chloride. The salt like sodium chloride can be
15 added together with one or more compounds of Formula I or in a subsequent step. Preferably, the lysis solution is added to the bioreactor comprising the suspension of the cells so that a mixture of the cell suspension and the composition is generated.

Incubation of the mixture comprising the cells and the composition
20 comprising one or more compounds of Formula I and optionally a salt like sodium chloride is done typically for 30 to 180 minutes, preferably for an incubation time between 60 and 120 minutes. Shorter and longer times may also be appropriate.

25 The pH of the mixture during the incubation can vary in broad range. It can for example be between pH 4 and pH 10, typically it is between pH 6 and pH 9.

30 The temperature of the mixture during incubation can also vary in a broad range. It can for example be between 20 and 37°C.

To have an effective amount of a composition comprising one or more compounds of Formula I to promote cell lysis and release of the AAV particles from the cells the concentration of the one or more compounds of Formula I in the composition is such that it effectively induces cell lysis, that
5 means that after the incubation with the composition under suitable conditions as described above, at least 80%, preferably 100% of the cells are lysed. For this, the overall concentration of the one or more compounds of Formula I in the final mixture with the cells is preferably above its CMC. The concentration in the mixture with the cells is typically between 0.1%
10 and 4% (w/w), preferably between 0.25% (w/w) and 1% (w/w). Typically, the volume of the composition that is added is smaller than the volume of the cell culture.

Consequently, suitable concentrations of the one or more compounds of Formula I in the composition are between 10 and 30% (w/w). Suitable
15 concentrations of a salt like sodium chloride to be added to the cell suspension are between 1 and 6 mol/l, typically around 3 to 5 mol/l, so that the final concentration in the mixture with the cell suspension is between 0.05 and 1 mol/l. A solution of sodium chloride and/or salts like sodium chloride can be added to the cell suspension simultaneously with one or
20 more compounds of Formula I or it can be added subsequently, after a certain incubation time only with the detergent, preferably between 1 and 2 hours.

In a preferred embodiment, the detergent used is a C11-C15 secondary
25 alcohol ethoxylate according to CAS 68131-40-8 and/or a C12-C14 secondary alcohol ethoxylate according to CAS 84133-50-60, either as single component or as a mixture, e.g., with a salt like NaCl, preferably NaCl.

30 In a very preferred embodiment, the composition is an aqueous solution. It might also comprise one or more buffers. The preferred pH is between 6 and 9.

It has been found that the composition as defined above effectively induces lysis of the cells and that especially viral particles which tend to stick to the cells and the cellular debris can be effectively separated from the cells.

5

After incubation with the lysis composition the released AAVs can then be isolated and/or purified. This can be done with any methods. Typically, it is done by one or more method steps including filtration and/or centrifugation.

10

In one embodiment the mixture is filtered through a filter that removes large molecule contaminants and cellular debris but that permits AAVs to pass therethrough.

15

In one embodiment, the released viral particles can be separated and purified from the cell culture medium using clarification. Clarification can be a microfiltration process in which relatively larger components such as lysed cells and/or impurities are removed from a solution. Clarification filters include depth filtration, charged depth filtration and similar microfiltration techniques.

20

Tangential flow filtration can be used to concentrate the mixture of purified viral particles and to remove salts and proteins. Tangential flow filtration (TFF) refers to a generally rapid and efficient method for filtration or purification of a solution containing target product and/or impurities during which a solution or liquid stream flows parallel to a filtering membrane.

25

Centrifugation can for example be a low-speed centrifugation to remove larger particles like cellular debris. This can be for example done at 10000 to 12000 g for 10 to 30 minutes. The released viral particles can be found in the supernatant.

30

The isolation and/or purification of the AAVs typically includes one or more of the following process steps:

- Clarification
- filtration
- 5 - dialysis/ diafiltration
- tangential flow filtration
- treatment with nuclease, e.g. RNase and/or DNase
- treatment with chloroform
- ion exchange chromatography
- 10 - affinity chromatography
- hydrophobic interaction chromatography
- centrifugation
- PEG precipitation

- 15 In some embodiments, a nuclease, typically an endonuclease, is added, e.g., to reduce the amount of host cell DNA. It can be added directly to the mixture in the bioreactor before, while or after lysis. The nuclease may be one that degrades both DNA and RNA. In one embodiment, the endonuclease is a genetically engineered endonuclease from *Serratia*
- 20 *marcescens* that is sold under the name Benzonase® (EMD Millipore). In another embodiment the nuclease is a salt tolerant nuclease, preferably WT *Vibrio salmonicida* EndA nuclease. Said nuclease is for example described in Altermark B, et al., FEBS J. 274 (2007) 252-63. And Niiranen et al., FEBS J. 275 (2008) 1593–1605.

- 25 In various embodiments, ion exchange chromatography is applied for further purification. This can for example be an anion (AEX) or a cation exchange (CEX) capture chromatography step. Such a step is used, e.g., to separate the viral particles from host cell proteins, host cell DNA, host cell
- 30 lipids, detergents, and other process-related impurities. The principles of cation and anion exchange chromatography are well known in the art. The sample is loaded, and the column is washed with a loading buffer. Finally,

an elution buffer is used to elute the sample of interest off the column, and fractions containing the sample are collected.

5 Other suitable chromatographic methods are hydrophobic interaction chromatography, size exclusion chromatography or affinity chromatography. Instead of bind-elute mode, also flow-through mode can be suitable.

10 Precipitation of the AAVs with polyethylene glycol (PEG) is also possible. For this PEG is added to a viral sample. The molecular weight of the PEG is typically from about 3.000 to about 15.000 g/mol. The concentration of the PEG in the precipitation solution can be adjusted as needed, it can for example be around 5% (w/w).

15 Chloroform can be added to dissolve lipids and inactivate abundant proteins. The virus particles are not affected by the chloroform which can later be separated by phase separation and/or centrifugation.

20 Salts like sodium chloride can be removed from the virus particles by dialysis. The virus containing solution can be dialyzed against water or another solution depending on the impurity that shall be removed.

25 Typical AAV purification processes include clarification, concentration and diafiltration using tangential flow filtration, chromatography purification by using affinity chromatography and ion exchange chromatography. In some processes, ultracentrifugation and gradient ultracentrifugation are used instead of chromatography or in addition to chromatography. Final steps in AAV purification typically involves concentration and diafiltration into suitable excipient buffer composition and sterile filtration.

30

The present invention is further directed to a composition to be used in the method of the invention comprising an aqueous solution of one or more

compounds of Formula I and sodium chloride. The concentration of the one or more compounds of Formula I in the composition is typically above 1% (w/w), preferably between 10% (w/w) and 30% (w/w) so that when the composition is added to the suspension comprising the cells to be lysed, the final concentration of the one or more compounds of Formula I can be adjusted to being above 0.1 % (w/w), preferably between 0.25 and 1% (w/w).

In a preferred embodiment, the detergent used is a C11-C15 secondary alcohol ethoxylate according to CAS 68131-40-8 and/or a C12-C14 secondary alcohol ethoxylate according to CAS 84133-50-60, either as single component or as a mixture with salts like NaCl, preferably NaCl.

In a very preferred embodiment, the composition is an aqueous solution. It might also comprise one or more buffers.

The concentration of the salt like sodium chloride in the composition is typically between 1 and 6 mol/l, preferably around 3 to 5 mol/l, so that the final concentration in the mixture with the cell suspension is between 0.05 and 1 mol/l.

In a preferred embodiment, the composition is only made of water, one or more compounds of Formula I, sodium chloride and/or potassium chloride and optionally a buffer.

The present invention is also directed to a kit comprising the composition of the invention as described above and a nuclease, preferably an endonuclease, e.g., Benzonase®. The kit typically comprises two containers, e.g., bottles, with the two components but it may also comprise further components and thus further containers.

Another advantage of the present invention is that the detergent and optionally a salt like sodium chloride that is used for cell lysis and viral release can be removed using tangential flow filtration. In tangential flow filtration, ultrafiltration membranes can be used to purify and concentrate
5 viral particles and perform diafiltration to remove detergent.

The present invention is further illustrated by the following figures and examples, however, without being restricted thereto.

10 The entire disclosure of all applications, patents, and publications cited above and below are hereby incorporated by reference.

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Examples and Methods

Method 1. AAV2 release by cell lysis

AAV2 were produced via Ambr[®] 15 Advanced Microbioreactor system (Sartorius). HEK293 cells were seeded with 1.0×10^6 cells/mL.

5 The cells grew 24 hours until they reached 2.0×10^6 cells/mL. At this point the cells were transfected with a triple DNA plasmid polyethylenimine (PEI) co-precipitation.

After 72 hours of growth, the cells were lysate with the detergents and the nuclease, Benzonase[®] (Merck KGaA Darmstadt, Germany).

- 10
- Lysis time: 2 hours
 - Temperature: 37 °C
 - Detergent concentration: 0.5% wt.
 - Detergent types: Triton[™] X-100, Tween[™] 20 (benchmarks), secondary alcohol ethoxylate
- 15
- Nuclease amount: 100 U/mL Benzonase[®]

After 2 hours of lysis, a NaCl solution 0.5 M was added to the system for 1 hour.

The samples were centrifuged post lysis, and the supernatants were removed and stored at -70 °C for the essays.

20 Physical titers, capsid/mL, were measured by enzyme-linked immunosorbent assay (ELISA) using an AAV2 Titration ELISA kit (Progen) following manufacturer's protocol. Genome titers were measured by droplet digital PCR (ddPCR). All reagents and assay protocols are from Merck KGaA Darmstadt, Germany unless otherwise specified.

25

Method 2: Critical micelle concentration (CMC) determination

The critical micelle concentration of the C11-C15 secondary alcohol ethoxylate is determined at different temperatures and in different media.

30 The CMC is determined by measuring the static surface tension of several surfactant solutions at different concentrations. The static surface tension values are plotted in function of the logarithm of the surfactant concentration. The CMC corresponds to the concentration point (or narrow

range) at which a sharp change of the slope occurs. Further details of the method are provided in the guideline ISO 4311(ISO 1979).

The static surface tension is measured by using a Force Tensiometer K100C (Krüss GmbH). The Wilhelmy plate was employed for the measurement.

The following parameters were adopted.

Measurement temperature adjusted by a Peltier temperature controller.

Measurement time: 300 s

Data points: 50

Frequency: 1 Hz

The data are acquired until the standard deviation of the surface tension measured is less than 0.1 mN/m from its average value for 50 data points.

The solution is poured in the glass vessel of the force tensiometer K100C.

The solution is allowed to reach the selected temperature. Just before

starting the measurement, the Wilhelmy plate is flamed until glowing red to eliminate contaminations and assure a contact angle equal to zero.

This device is equipped with two automatic dispensers. One dispenses the liquid and the other extracts the same volume. Instead of preparing

manually several solutions, only a highly concentrated solution is required

as starting point. The device proceeds to execute progressive dilutions and measures the static surface tension of each one, plotting the data in a graph static surface tension vs concentration.

Method 3: AAV2 and AAV5 release from HEK 293 and Sf-RVN cells by cell lysis

HEK293 suspension cells were selected to produce AAV5, HEK293 adhesion cells for AAV2 and Sf-RVN[®] cells for AAV2. Each cell line was cultivated according to their type with the following procedures.

Suspension HEK 293 cells (clone AC2) were seeded on Day 0 in a 500 mL baffled vented Erlenmeyer flask with 1.5×10^6 vc/mL in 125 mL of proprietary cell culture media (Virus Express Excell HEK293 CD Viral

Vector Medium, Merck KGaA Darmstadt Germany). On Day 1 the cells reached a VCD $\geq 3 \times 10^6$ vc/mL and they were transfected with a triple DNA plasmid and polyethylenimine (PEI). The cells were monitored and let grow for two days. Harvest took place on Day 4.

5

Adhesion HEK293T cells at passage 7 with a viable cell density of 2.72×10^4 vc/cm² and cultured in DMEM medium (D6171 Merck KGaA Darmstadt Germany) with 10% FBS and 2 mM of L-Glutamine, were used to seed the required number of viable cells into two T225 flasks. On Day 1 the cells reached a VCD $\sim 1.5 \times 10^5$ vc/cm² and they were transfected with a double DNA plasmid and PEI. The cells were monitored and let grow for two days. Harvest took place on Day 4. The transfected cells were detached with Trypsin and centrifuged at 250 x g, 10 minutes to recover them. 2.0×10^6 transfected cells were transferred per condition.

10

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Sf-RVN[®] cells were cultivated with the proprietary cell culture media (EXCELL CD Insect Cell Culture Medium, Merck KGaA Darmstadt Germany) in 1 L bioreactor (Applikon). The seeding density was 2×10^6 vc/mL in 750 mL medium. The cells were infected with Baculovirus vectors at the time of seeding. Cells were cultivated for 4 days and harvested.

20

After the harvest all the three kinds of cells were lysed with the following procedure:

- Detergent concentration: 0.5 % wt/vol.
- Detergents: Tween[™] 20 and Triton[™] X-100 (benchmarks), secondary alcohol ethoxylate
- Nuclease: 25 U/mL Benzonase[®] endonuclease (Merck KGaA Darmstadt Germany) or Salt tolerant Benzonase[®] endonuclease (Merck KGaA Darmstadt Germany) with 2 mM MgCl₂
- NaCl content: NaCl already present in the cell culture media (125 mM) in the experiments with Benzonase[®] endonuclease (Merck KGaA Darmstadt Germany; in the experiments with Salt tolerant Benzonase[®] endonuclease

25

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(Merck KGaA Darmstadt Germany) NaCl was added to reach a concentration of 500 mM.

- Lysis time: 2 h
- Temperature: 37 °C
- 5 % CO₂

5

Following incubation, total cell count and viability were determined with the Vi-CELL (Beckman Coulter).

After cell viability quantification, virus-containing supernatant was clarified by centrifugation.

10

The supernatant was analyzed to determine genome titer (viral genome copies/mL) by digital PCR.

The infectivity of AAV2 produced by HEK293 adhesion cells was tested with the transduction unit assay. Before the infectivity evaluation, the residual detergent was removed from the samples using a detergent removal column (Pierce).

15

20 **Examples**

Example 1: AAV2 titer determination after cell lysis

The AAV2 genome titers were measured with ddPCR (droplet digital PCR) after the lysis steps and the centrifugation. In Figure 1 the genome titers after the lysis with the different detergents are reported. The AAV2 recovered from the cells by using the three different detergents, our secondary alcohol ethoxylate and the two benchmarks, is comparable within the error range.

25

By measuring the physical titer, capsid/mL, by using the ELISA it is possible to calculate the ratio full/empty capsid. This ratio is obtained dividing the viral genome titer by the capsid titer. As shown in the table, the ratio is comparable for all three detergents.

30

Detergent	Empty/full capsid ratio (%)
0.5% Secondary Alcohol Ethoxylate	14
0.5% Tween™ 20	11
0.5% Triton™ X-100	31

5

Example 2: Critical micelle concentration (CMC) determination

The critical micelle concentration is determined according to Method 2. The surface tension values measured for each dilution are plotted in a graph static surface tension vs concentration. The concentration axis is in logarithmic base. The critical micelle concentration is the concentration (or the narrow range of concentrations) corresponding to the beginning of the micelle self-assembly. From this concentration (or narrow range) on, an increase of surfactant concentration does not affect the surface tension and the plot of surface tension vs concentration is a horizontal line. The CMC corresponds to the concentration point (or narrow range) at which a sharp change of the slope occurs. In Figure 2 the CMC of the secondary alcohol ethoxylate in Milli-Q® water at 23.9 °C is equal to 49 mg/L.

10

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Example 3: AAV2 and AAV5 release from HEK 293 and Sf-RVN cells by cell lysis

The cell lysis efficacy of the detergents was evaluated by counting the total and living cells in each sample. This determination was performed automatically with the Vi-CELL for both HEK293 cell lines and manually evaluating microscope images for the Sf-RVN® cells. With all the cell lines, the lysis of most of the cells was evident from the microscope images, confirming the effective lysis performance of the secondary alcohol ethoxylate, comparable to or better than the benchmarks, Triton™ X-100 and Tween™ 20.

25

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The viability of HEK293 cells (adherent and suspension) after lysis are shown in Figure 3, 4, 5, 6. The percentage is calculated based on the automated counting performed by the software, so should be considered as

a trend with the actual viable percentage likely to be lower because the software may count lysed cells as still viable.

The percentage cell viability data confirmed as well that the secondary alcohol ethoxylate lyses the cells comparably well if not better than the benchmarks, Triton™ X-100 and Tween™ 20. Comparing the experiments with different NaCl concentration, the increase of NaCl concentration increased the lysis efficiency also without detergent due to the osmosis.

The genome titer was determined with digital PCR. Figures 7, 8, 9, 10,11,12 display that the secondary alcohol ethoxylate was as efficient as the benchmarks in releasing capsids (data not shown) and the viral genome titers were comparably high, at all NaCl concentrations.

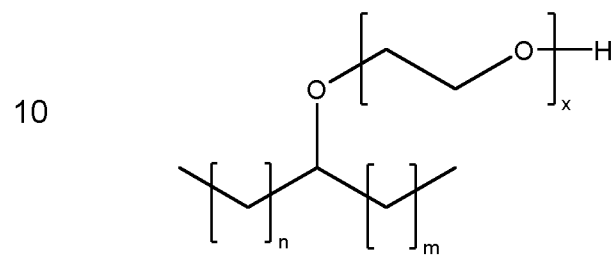
Following cellular release and purification, the AAV must be functional to be an effective product. The infectivity of the AAV is a critical quality requirement. The infectivity was determined with a transduction unit assay and the results demonstrate that the secondary alcohol ethoxylate preserves the infectivity of the AAV. (Figure 13 and 14)

These results demonstrate that the secondary alcohol ethoxylate do not cause harm to non-enveloped target virus vector product. The data shows that there is no difference in functional virus titer between the secondary alcohol ethoxylate and the benchmark detergents.

The increase of NaCl concentration led to the increase of the infectious particles, as a consequence of a more efficient lysis and higher release of capsids.

Claims

1. A method for lysing cells enclosing adeno-associated virus (AAV) particles by contacting a suspension of said cells with an effective amount of a composition comprising one or more compounds of Formula I to promote cell lysis and release of the AAV particles from the cells with



with $x = 7$ to 15

15 and $n + m = 8$ to 12 .

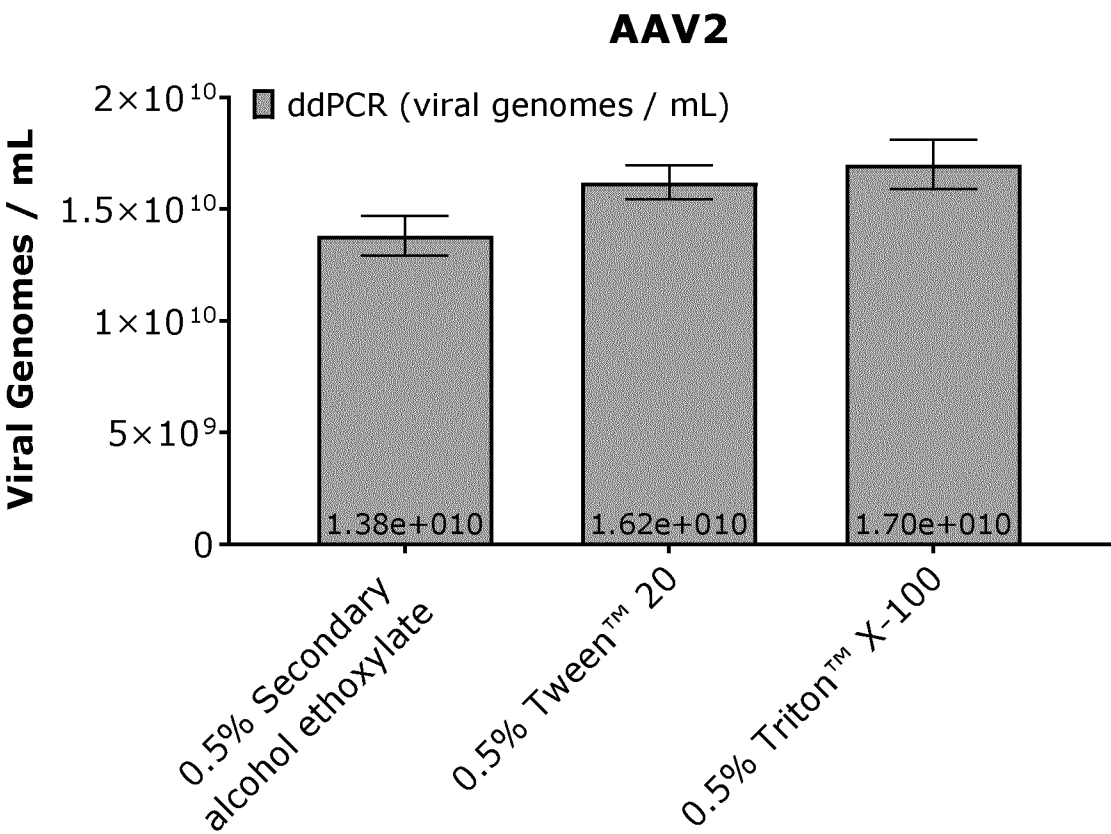
2. A method for purifying viral particles of adeno-associated virus (AAV) from a sample comprising cells enclosing the viral particles by
- a) contacting a suspension of the cells and the viral particles with an effective amount of a composition comprising one or more compounds of Formula I to promote cell lysis and release of the viral particles from the cells
- b) isolating and/or purifying the viral particles.
3. Method according to claim 2 characterized in that the composition comprises one or more compounds of Formula I and one or more salts selected from salts comprising a metal cation selected from K^+ , Na^+ , Li^+ , Mg^{2+} , Ca^{2+} and an anionic component selected from F^- , SO_4^{2-} , HPO_4^{2-} , acetate, Cl^- and/or that said salts are added to the suspension of the cells subsequent to said composition.

4. Method according to claim 2 or claim 3, characterized in that in step a) the cells are contacted with the composition for 30 to 180 minutes.
5. Method according to one or more of claims 2 to 4, characterized in that the composition comprises a C11-C15 secondary alcohol ethoxylate according to CAS 68131-40-8 and/or a C12-C14 secondary alcohol ethoxylate according to CAS 84133-50-60.
6. Method according to one or more of claims 2 to 5, characterized in that the overall concentration of the one or more compounds of formula I in the mixture obtained in step a) is between 0.1 and 4% (w/w).
7. Method according to one or more of claims 2 to 6, characterized in that the overall concentration of the one or more salts comprising a metal cation selected from K^+ , Na^+ , Li^+ , Mg^{2+} , Ca^{2+} and an anionic component selected from F^- , SO_4^{2-} , HPO_4^{2-} , acetate, Cl^- in the mixture obtained in step a) is between 0.05 and 1 mol/l.
8. Method according to one or more of claims 2 to 7, characterized in that the composition is an aqueous solution comprising one or more compounds according to Formula I and sodium chloride.
9. Method according to one or more of claims 2 to 8, characterized in that the overall concentration of the one or more compounds according to Formula I oxide in the composition is between 10% and 30% (w/w).
10. Method according to one or more of claims 2 to 9, characterized in that the concentration of sodium chloride in the composition is between 1 and 6 mol/l.
11. Method according to one or more of claims 2 to 10, characterized in that step b) comprises a filtration and/or centrifugation step.

12. Method according to one or more of claims 2 to 11, characterized in that the method of the present invention comprises one or more of the following steps:
- 5 - Clarification and filtration
 - dialysis and diafiltration
 - treatment with nuclease, e.g., RNase and/or DNase
 - treatment with chloroform
 - ion exchange chromatography
- 10 - affinity chromatography
 - hydrophobic interaction chromatography
 - centrifugation
 - PEG precipitation
- 15 13. A composition comprising one or more compounds according to Formula I and a salt comprising a metal cation selected from K^+ , Na^+ , Li^+ , Mg^{2+} , Ca^{2+} and an anionic component selected from F^- , SO_4^{2-} , HPO_4^{2-} , acetate, Cl^- .
- 20 14. A composition according to claim 13, characterized in that the composition is an aqueous solution comprising 10 to 30 % (w/w) of a C11-C15 secondary alcohol ethoxylate according to CAS 68131-40-8 and/or a C12-C14 secondary alcohol ethoxylate according to CAS 84133-50-60 and 1 to 6 mol/l NaCl.
- 25 15. A kit comprising the composition according to claim 13 or claim 14 as well as a nuclease.
- 30

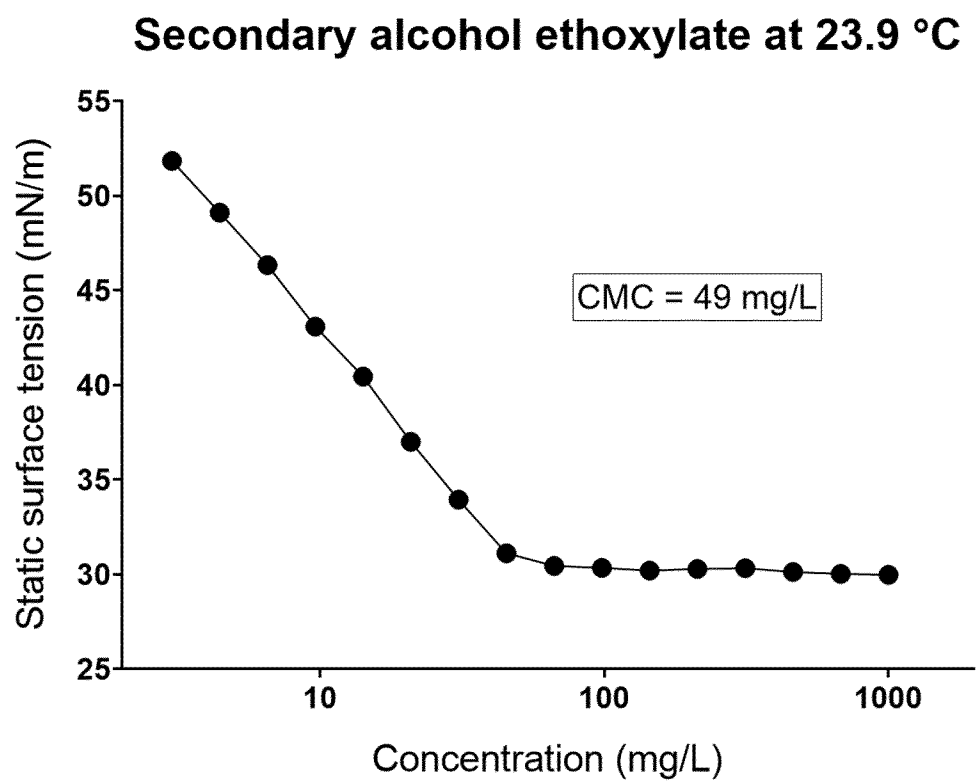
1/14

Fig. 1



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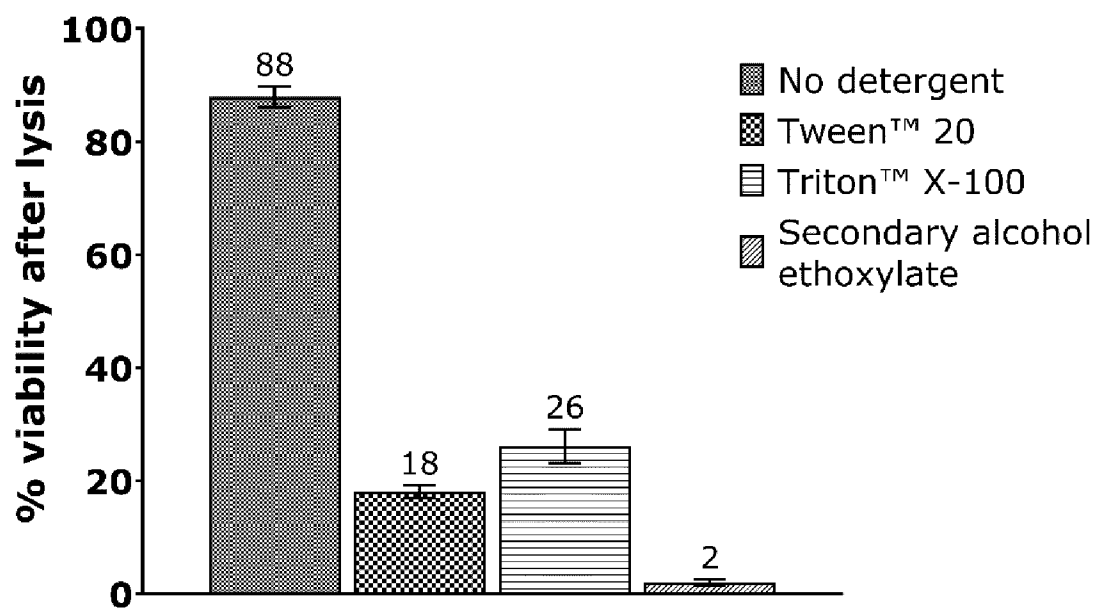
Fig. 2



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Fig.3

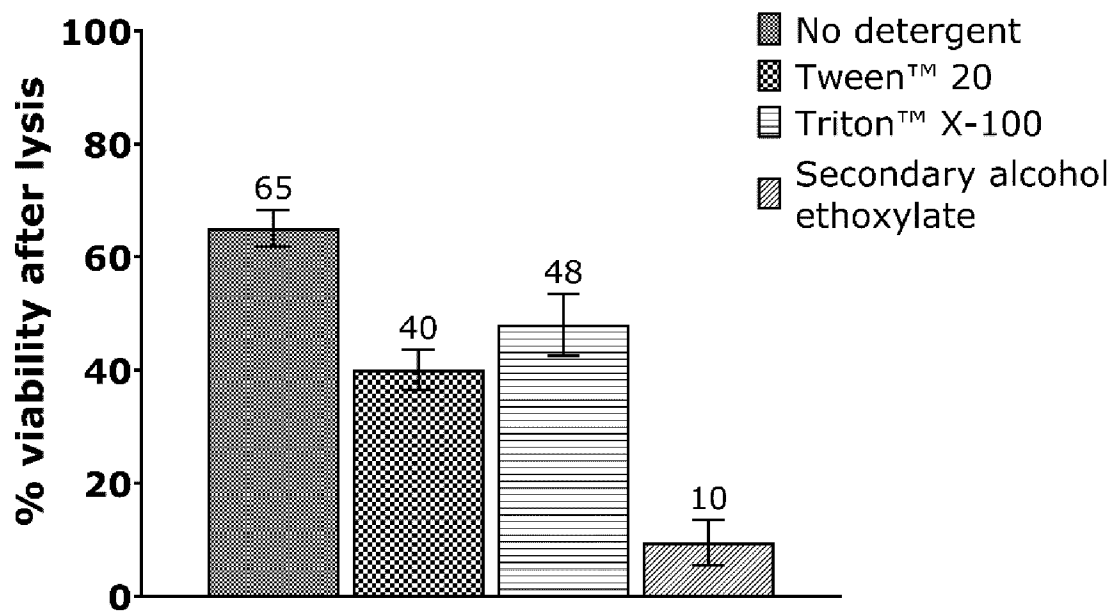
HEK293 Adherent Cells - AAV2
Benzonase[®] Endonuclease, no NaCl added



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Fig. 4

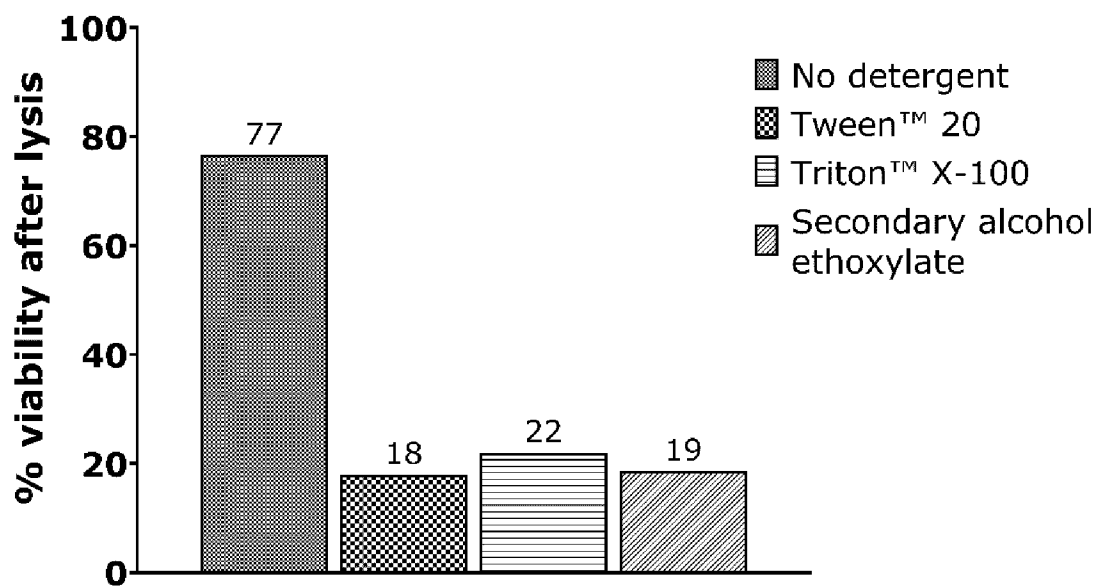
HEK293 Adherent Cells - AAV2
Benzonase[®] Salt Tolerant Endonuclease,
0.5 M NaCl



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Fig.5

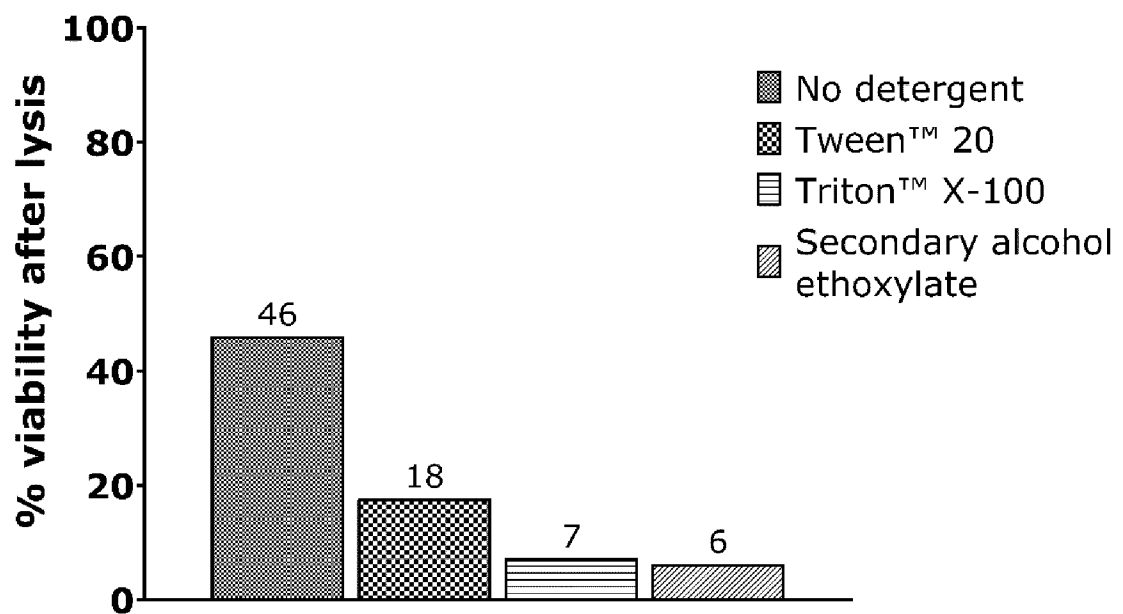
HEK293 Suspension Cells - AAV5
Benzonase[®] Endonuclease, no NaCl added



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Fig.6

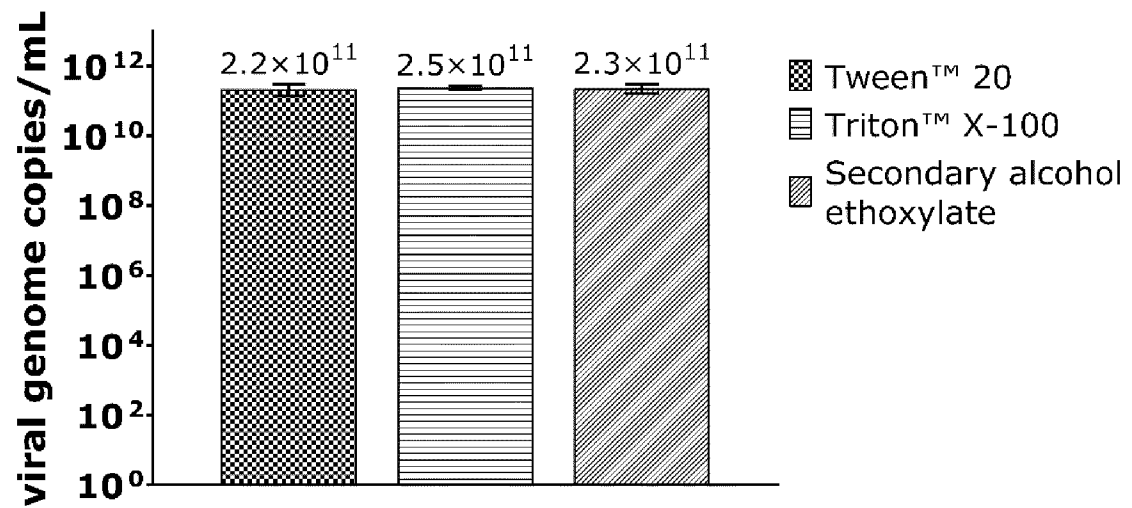
**HEK293 Suspension Cells - AAV5
Benzonase[®] Salt Tolerant Endonuclease,
0.5 M NaCl**



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Fig.7

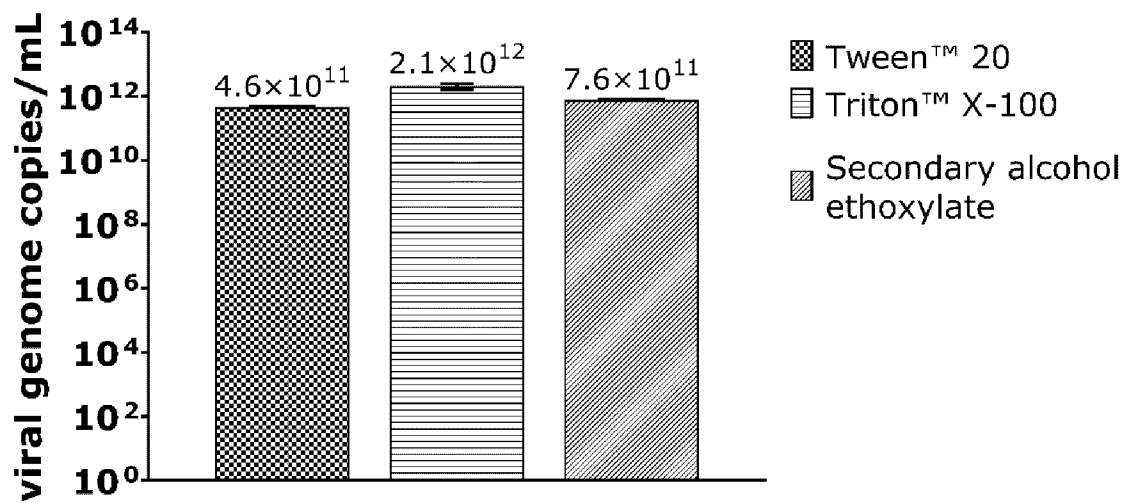
HEK293 Adherent Cells - AAV2
Benzonase[®] Endonuclease, no NaCl added



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Fig.8

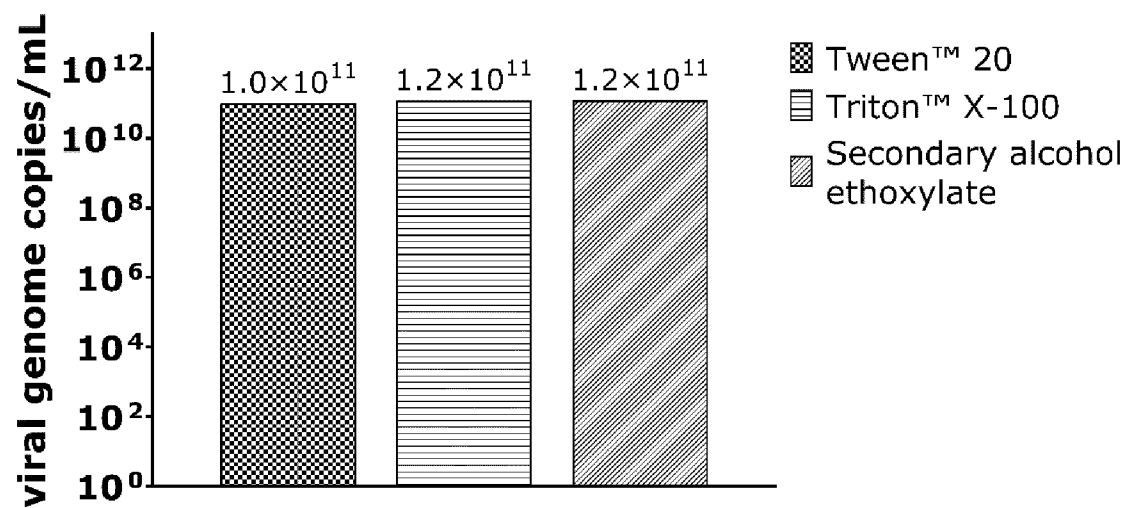
HEK293 Adherent Cells - AAV2
Benzonase[®] Salt Tolerant Endonuclease,
0.5 M NaCl



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Fig.9

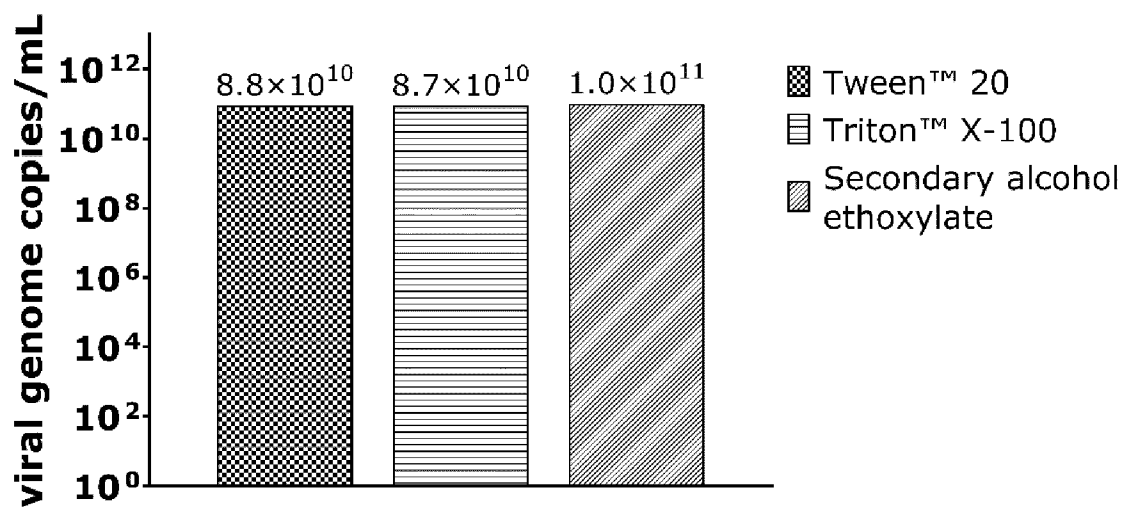
HEK293 Suspension Cells - AAV5
Benzonase[®] Endonuclease, no NaCl added



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Fig.10

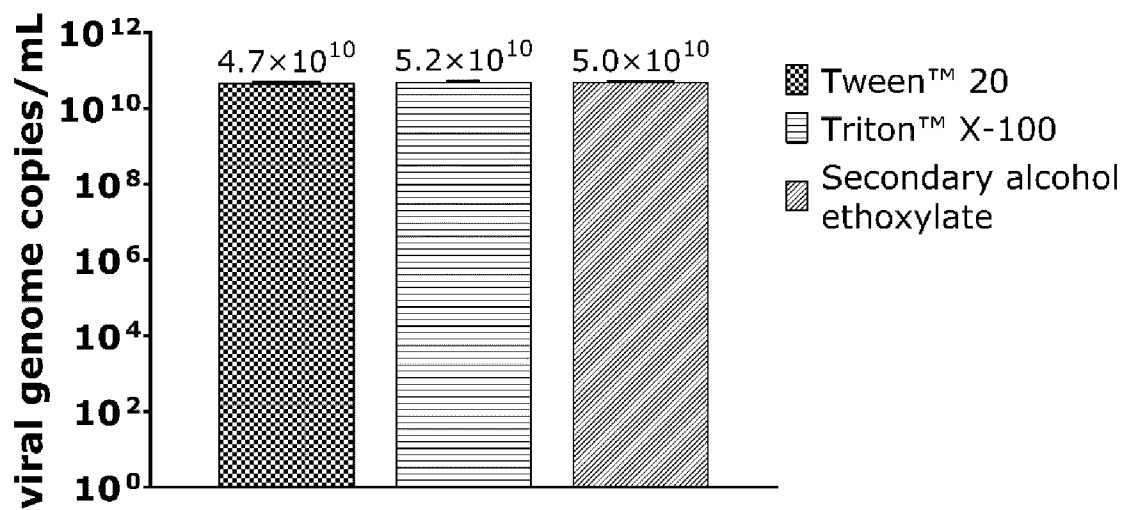
HEK293 Suspension Cells - AAV5
Benzonase[®] Salt Tolerant Endonuclease,
0.5 M NaCl



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Fig.11

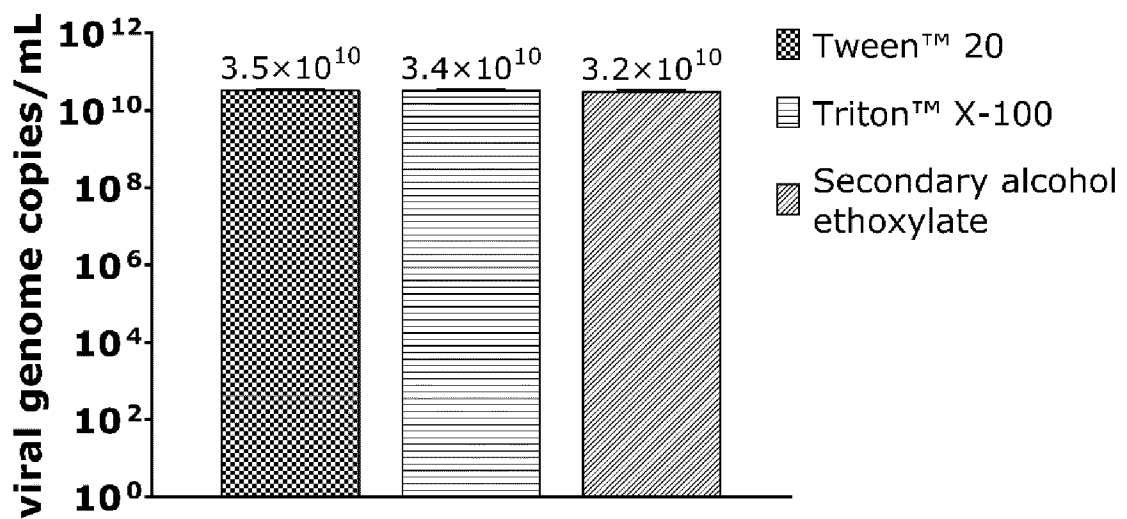
Sf-RVN[®] Suspension Cells - AAV2
Benzonase[®] Endonuclease, no NaCl added



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Fig.12

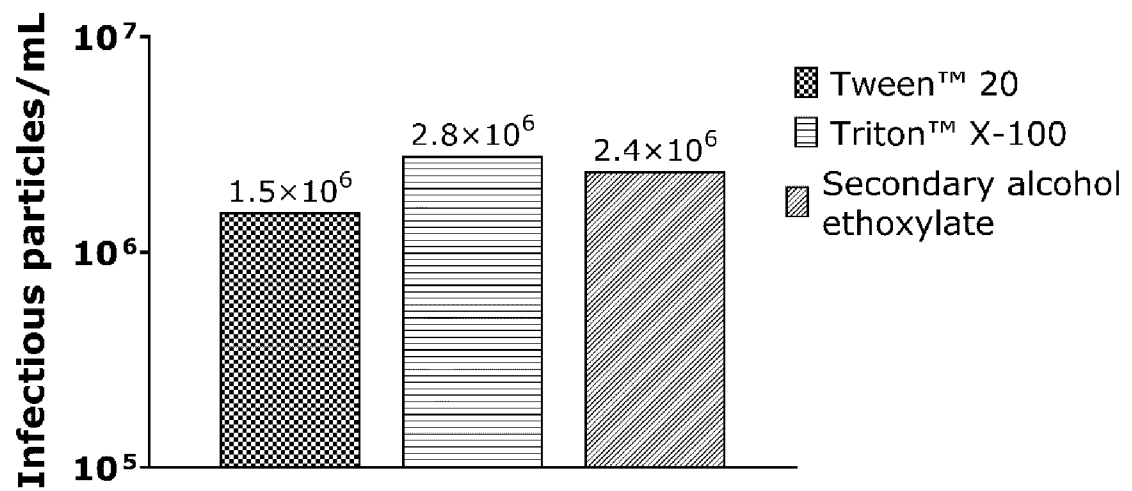
Sf-RVN[®] Suspension Cells - AAV2
Benzonase[®] Salt tolerant Endonuclease,
0.5 M NaCl



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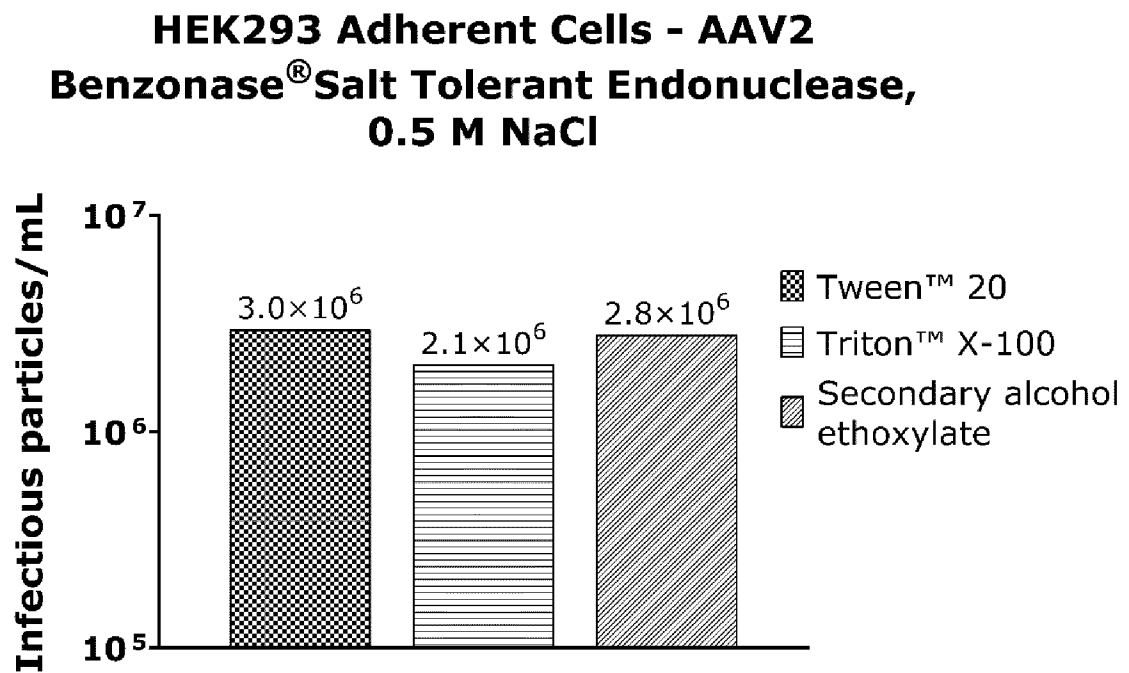
Fig.13

HEK293 Adherent Cells - AAV2
Benzonase[®] Endonuclease, no NaCl added



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Fig.14



INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2024/068725

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N15/86 C11D1/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2020/023612 A1 (VOYAGER THERAPEUTICS INC [US]) 30 January 2020 (2020-01-30) claims; examples -----	1 - 15
X	BERA A ET AL: "Enhanced oil recovery by nonionic surfactants considering micellization, surface, and foaming properties", PETROLEUM SCIENCE, vol. 14, no. 2, 8 April 2017 (2017-04-08), pages 362-371, XP036242329, ISSN: 1672-5107, DOI: 10.1007/S12182-017-0156-3 [retrieved on 2017-04-08] Experimental, Fig. 8-10; -----	13



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

30 September 2024

Date of mailing of the international search report

10/10/2024

Name and mailing address of the ISA/

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Authorized officer

Sommer, Birgit

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2024/068725

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2020023612 A1	30-01-2020	AU 2019310459 A1	18-02-2021
		CA 3107462 A1	30-01-2020
		CN 112770812 A	07-05-2021
		EP 3826719 A1	02-06-2021
		IL 280350 A	01-03-2021
		JP 2021530548 A	11-11-2021
		SG 11202100704P A	25-02-2021
		US 2021355454 A1	18-11-2021
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