Patent Right	Date	November 30, 2022	Court	Intellectual Property High Court, Fourth Division
	Case number	2021 (Gyo-Ke) 10135		
- A case in which the court ruled that the JPO decision overlooked a difference from a cited invention but the difference is not a substantial difference, and determined that the JPO decision to the effect that the invention claimed in Claim 30 is not novel contains no error in its conclusion.				

Case type: Rescission of Trial Decision of Invalidation

Result: Dismissed

References: Article 29, paragraph (1), item (iii) and paragraph (2) of the Patent Act

Related rights, etc.: Appeal against Examiner's Decision of Refusal No. 2019-6665 and Patent Application No. 2016-502935

Summary of the Judgment

No. 1 Background

1. The Plaintiff filed an international application for an invention titled "Vector containing a stuffer/filler polynucleotide sequence and method of using the same" (Patent Application No. 2016-502935; priority date: March 15, 2013; priority country: United States: hereinafter referred to as the "Application") on March 14, 2014. However, the Plaintiff received an examiner's decision to reject the application and filed an appeal against a rejection (Appeal against Examiner's Decision of Refusal No. 2019-6665). However, the Plaintiff received a notice of grounds for rejection. Therefore, the Plaintiff made an amendment (the "Amendment") with regard to the claims.

On June 23, 2021, the JPO rendered a decision to the effect that the appeal is groundless (the "JPO Decision"). Therefore, the Plaintiff filed this lawsuit to seek the rescission of the JPO Decision.

2. Claims (those after the Amendment)

[Claim 1]

A recombinant vector plasmid comprising a vector genome containing a heterologous polynucleotide sequence and

a first inactive filler or stuffer polynucleotide sequence,

wherein said heterologous polynucleotide sequence has a length less than 4.7 Kb and is positioned within two adeno-associated virus (AAV) ITR sequences, said inactive filler or stuffer polynucleotide sequence is positioned outside two adeno-associated virus (AAV) ITR sequences, and said first inactive filler or stuffer polynucleotide

sequence has a length of 7.0 to 10.0 Kb.

[Claim 30]

An AAV particle comprising a vector genome of a recombinant vector plasmid claimed in any of Claims 1 to 27.

3. Summary of the JPO Decision

The JPO Decision determined that the invention claimed in Claim 30 ("Claimed Invention 30") is the invention stated in a cited document (Molecular Therapy. (2009) vol. 17, no. 1, p. 144-152) (the "Cited Invention") and that even if those inventions have a difference, Claimed Invention 30 does not involve an inventive step as it is an invention that a person skilled in the art would have easily been able to make based on the Cited Invention and well-known art, etc. In this determination, the part regarding novelty is as follows.

(1) Cited Invention as found in the JPO Decision

"A recombinant AAV that was prepared by transfecting 6,980 bp backbone containing an ampicillin resistance gene and an ITR-containing vector plasmid with a 4,297 nt transgene coding for human clotting factor IX into HEK 293 cells, wherein DNA impurities derived from said ITR-containing vector plasmid have been significantly reduced."

(2) The "vector genome" contained in an "AAV particle" pertaining to Claimed Invention 30 and the "vector genome" contained in a "recombinant AAV" pertaining to the Cited Invention do not differ in that both of them contain a tiny amount of residual plasmid DNA derived from two AAV ITR sequences, the area that is positioned within the two AAV ITR sequences and contains a heterologous polynucleotide sequence having a length less than 4.7 Kb, and the area positioned outside said two ITR sequences. Therefore, there is no difference between the "AAV particle" pertaining to Claimed Invention 30 and the "recombinant AAV" pertaining to the Cited Invention, both of which are AAV containing such "vector genome."

4. Grounds for rescission

Errors in determinations concerning involvement of novelty and an inventive step in Claimed Invention 30 (overlooking of a difference from the Cited Invention and error in the finding of common features)

No. 2 Summary of the court decision

1. In comprehensive consideration of matters disclosed in the description of the Application, the problem to be solved by Claimed Invention 30 is found to be the provision of a recombinant AAV particle containing less or no residual DNA impurities. The technical significance of Claimed Invention 30 can be considered to exist in solving

said problem through reducing the amount of residual DNA impurities derived from plasmid by ensuring that an AAV particle contains a vector genome of a vector plasmid that was prepared in a manner that when the length of a heterologous polynucleotide sequence positioned within two ITR sequences is less than the packaging limit of the AAV particle (about 4.7 Kb), the total of the length of an inactive filler or stuffer polynucleotide and that of a heterologous polynucleotide sequence, which is a transgene, exceeds at least the packaging limit.

Incidentally, even in consideration of the statements in the description of the Application, a unique technical significance cannot be found in the fact that a filler or stuffer polynucleotide sequence has a length of 7.0 to 10.0 Kb. However, said fact should be considered to be significant to the extent of constituting a length that contributes to the solution of the problem in light of the technical significance of the invention claimed in the Application.

According to the above, it cannot be considered reasonable to deal with only the structure wherein "said heterologous polynucleotide sequence has a length less than 4.7 Kb and is positioned within two adeno-associated virus (AAV) ITR sequences" (the structure of underlined part [i]) as the constituent feature of Claimed Invention 30. Although the structure wherein "said inactive filler or stuffer polynucleotide sequence is positioned outside two adeno-associated virus (AAV) ITR sequences, and said first inactive filler or stuffer polynucleotide sequence has a length of 7.0 to 10.0 Kb" (the structure of underlined part [ii]) is not packaged in the "AAV particle" of Claimed Invention 30 without any change, Claimed Invention 30 should be compared with the Cited Invention by taking into account this structure. Therefore, the determination in the JPO Decision contains an error in this regard.

2. (1) Claimed Invention 30 has a structure wherein "said heterologous polynucleotide sequence has a length less than 4.7 Kb and is positioned within two adeno-associated virus (AAV) ITR sequences." On the other hand, the Cited Invention has a structure of an "ITR-containing" vector plasmid "with a 4,297 nt transgene coding for human clotting factor IX." The "heterologous polynucleotide" of Claimed Invention 30 corresponds to the "human clotting factor IX" of the Cited Invention, and those inventions have a commonality in that they contain a vector plasmid having a length less than "4.7 Kb."

(2) Next, Claimed Invention 30 and the Cited Invention have a commonality in the problem to be solved thereby, specifically, to reduce the amount of DNA impurities derived from plasmid that are contained in an AAV particle in preparation by using a vector plasmid containing a transgene and a stuffer polynucleotide sequence. In

addition, in both of those inventions, the length of a transgene is less than 4.7 Kb, which is the packaging limit. However, the inventions differ in the length of an inactive filler or stuffer polynucleotide sequence of a vector plasmid.

However, as described above, it can be understood that the technical significance of Claimed Invention 30 does not exist in that the length of a filler or stuffer polynucleotide sequence of a vector plasmid is within a specific range, "7.0 to 10.0 Kb," but exists in ensuring that when the length of a transgene is less than the packaging limit of an AAV particle (about 4.7 Kb), the AAV particle contains a vector genome of a vector plasmid that was prepared in a manner that the total of the length of an inactive filler or stuffer polynucleotide and that of a heterologous polynucleotide sequence, which is a transgene, exceeds at least the packaging limit.

According to the above, in the Cited Invention, a stuffer sequence is also altered so that the backbone exceeds the packaging limit of an AAV, and it can be understood that the total of the length of a transgene (4,297 nt (about 4.297 Kb)) and the length of a stuffer sequence contained in this backbone with a length of 6,980 bp (about 6.980 Kb) exceeds at least the packaging limit, that is, 4.7 Kb. Therefore, Claimed Invention 30 and the Cited Invention are identical with each other in the technical significance, and setting of the length of a filler or stuffer polynucleotide sequence of a vector plasmid as a length within a specific range, "7.0 to 10.0 Kb," in Claimed Invention 30 cannot be considered to be a substantial difference.

In addition, the "6,980 bp backbone" of the Cited Invention is an "oversized backbone" (see [0173] to [0177]), and from the statements in the description of the Application, it can only be understood that an "inactive filler or stuffer polynucleotide sequence" having a "length of 7.0 to 10.0 Kb" of Claimed Invention 30 constitutes an oversized backbone. Therefore, from this perspective, those inventions can also not be considered to differ from each other.

Judgment rendered on November 30, 2022
2021 (Gyo-Ke) 10135 A Case of seeking rescission of the JPO decision
Date of conclusion of oral argument: September 28, 2022

Judgment

Plaintiff: The Children's Hospital of Philadelphia

Defendant: The Commissioner of the Japan Patent Office

Main text

1. The Plaintiff's claim shall be dismissed.

2. The Plaintiff shall bear the court costs.

3. The additional period for filing a final appeal and a petition for acceptance of final appeal against this judgment shall be 30 days.

Fact and reason

No. 1 Claim

The court shall rescind the decision made by the Japan Patent Office (JPO) on June 23, 2021 with regard to the case of Appeal against Examiner's Decision of Refusal No. 2019-6665.

No. 2 Outline of the case

1. Outline of procedures, etc. at the JPO (undisputed by the parties)

(1) The Plaintiff filed an international application (Japanese Patent Application No. 2016-502935, Priority date: March 15, 2013, Priority country: United States of America, hereinafter referred to as "the present application") with regard to an invention titled "VECTOR COMPRISING STUFFER/FILLER POLYNUCLEOTIDE SEQUENCE AND METHOD OF USING THE SAME" on March 14, 2014.

(2) The Plaintiff received an examiner's decision of refusal on January 17, 2019. Accordingly, the Plaintiff filed an appeal against the examiner's decision of refusal (Appeal against Examiner's Decision of Refusal No. 2019-6665) on May 22 of the same year, and also filed a procedure amendment for the scope of claims on the same day.

(3) The Plaintiff received a notice of reasons of refusal on September 2, 2020.

Accordingly, the Plaintiff filed a procedure amendment for the scope of claims on March 3, 2021, and also filed a procedure amendment for the scope of claims on the 4th of the same month (hereinafter, the amendment dated the same day is referred to as "the amendment of the present case"). However, on June 23 of the same year the Japan Patent Office made an appeal decision that "the request for the appeal is dismissed." (hereinafter referred to as "the appeal decision of the present case"), and a certified copy of the appeal decision was served on the Plaintiff on July 13 of the same year (Additional time frame: 90 days).

(4) The Plaintiff instituted an action of the present case for seeking a rescission of the appeal decision of the present case on November 9, 2021.

2. Recitation of the scope of claims

The scope of claims after the amendment of the present case consists of Claims 1 to 50, among which the recitation of Claims 1 and 30 is as follows (hereinafter an invention according to Claim 30 after the amendment of the present case is referred to as "Present Invention 30" and together with an invention according to Claim 1, may be referred to as "Present Invention").

[Claim 1]

A recombinant vector plasmid, comprising:

a vector genome comprising a heterologous polynucleotide sequence; and

a first inert filler or stuffer polynucleotide sequence,

wherein said heterologous polynucleotide sequence has a length less than 4.7 Kb and is positioned within two adeno-associated virus (AAV) ITR sequences, wherein said inert filler or stuffer polynucleotide sequence is positioned outside two adeno-associated virus (AAV) ITR sequences, and wherein said first inert filler or stuffer polynucleotide sequence has a length of 7.0 to 10.0 Kb.

[Claim 30]

An AAV particle, comprising the vector genome of the recombinant vector plasmid of any of Claims 1 to 27.

3. Abstract of the appeal decision of the present case

(1) Present Invention 30

Present Invention 30 is recited in the form of referring to Claims 1 to 27 of the present application, among which an invention according to a portion that refers to Claim 1 is as follows.

An AAV particle, comprising the vector genome

of the recombinant vector plasmid comprising:

a: a vector genome comprising a heterologous polynucleotide sequence; and

b: a first inert filler or stuffer polynucleotide sequence,

a-1: wherein said heterologous polynucleotide sequence has a length less than 4.7 Kb and is positioned within two adeno-associated virus (AAV) ITR sequences,

b-1: wherein said inert filler or stuffer polynucleotide sequence is positioned outside two adeno-associated virus (AAV) ITR sequences, and wherein said first inert filler or stuffer polynucleotide sequence has a length of 7.0 to 10.0 Kb.

(2) Invention stated in a publication (Molecular Therapy. (2009) vol. 17, no. 1, pp. 144-152, hereinafter referred to as "Cited Document 1") distributed in Japan or a foreign country prior to the priority date of the present case (March 15, 2013) (hereinafter referred to as "Cited Invention")

"A recombinant AAV prepared by transfection of HEK293 cells using ITR-containing vector plasmids having a 6980 bp backbone containing ampicillin resistance gene and 4297 nt transgene encoding human coagulation factor IX, in which DNA impurities derived from said ITR-containing vector plasmids have been significantly reduced."

(3) A. Lack of novelty

The "AAV particle" according to Present Invention 30 comprises a "vector genome" comprising: two AAV ITR sequences; a region containing a heterologous polynucleotide sequence which is positioned within the two AAV ITR sequences and which has a length less than 4.7 Kb; and a very small amount (about 60 pg/10^9 vg) of residual plasmid DNA derived from a region which is positioned outside said region; i.e., outside the two AAV ITR sequences in the "recombinant vector plasmid" of Present Invention 30.

The "recombinant AAV" according to Cited Invention comprises a "vector genome" comprising: two AAV ITR sequences; a region which is positioned within the two AAV ITR sequences and which contains "4297 nt gene encoding human coagulation factor IX"; and a very small amount of DNA impurities derived from a 6980 bp region which is positioned outside said two ITR sequences in the "ITR-containing vector plasmid" of Cited Invention.

In this regard, the "4297 nt gene encoding human coagulation factor IX" in Cited Invention corresponds to the "heterologous polynucleotide sequence which has a length less than 4.7 Kb" in Present Invention 30.

In addition, since the "DNA impurities" in Cited Invention are derived from the 6980 bp region positioned outside the two AAV ITR sequences in the "ITR-containing vector plasmid" in Cited Invention, the "DNA impurities" in Cited Invention correspond to the "residual plasmid DNA" in Present Invention 30. Further, "five lots prepared

using a vector plasmid having an oversized (6980 bp) backbone (Lots 06002, 003A, NHP, 0802, 0803)" stated in Cited Document 1 contain 11 to 17.7 pg/10^9 vg (average: 14.2±2.6 pg/10^9 vg) of DNA impurities derived from the ITR-containing vector plasmid. This numerical value is equivalent to an amount of residual plasmid DNA (about 60 pg/10^9 vg) shown in the description of the present application. Thus, it can be deemed that the amount of "DNA impurities" contained in the vector genome of the "recombinant AAV" according to Cited Invention; that is, "residual plasmid DNA", is a very small amount of the same level as in Present Invention 30.

Then, there is no difference between the "vector genome" contained in the "AAV particle" according to Present Invention 30 and the "vector genome" contained in the "recombinant AAV" according to Cited Invention, in that both of the "vector genome" contain: two AAV ITR sequences; a region containing a heterologous polynucleotide sequence which is positioned within the two AAV ITR sequences and which has a length less than 4.7 Kb; and a very small amount of residual plasmid DNA derived from a region which is positioned outside said two ITR sequences. Thus, there is also no difference between the "AAV particle" according to Present Invention 30 and the "recombinant AAV" according to Cited Invention, since both are AAVs containing such a "vector genome."

Therefore, Present Invention 30 is an invention stated in Cited Document 1.

B. Lack of inventive step

Even if there is a difference between the "AAV particle" according to Present Invention 30 and the "recombinant AAV" according to Cited Invention, it can be deemed that such a difference is slight, and Present Invention 30 is an invention which could have been easily made by a person ordinarily skilled in the art on the basis of Cited Invention, the statement of Cited Document 1, and well-known art concerning AAV as of the priority date of the present application. Even taking into consideration the statement of the description attached to the written application of the present application (Exhibit Ko 2, hereinafter referred to as "the description of the present application" including the Drawings), it cannot be deemed that an effect achieved by Present Invention 30 has a remarkable point.

Therefore, Present Invention 30 is an invention which could have been easily made by a person ordinarily skilled in the art on the basis of Cited Invention, the statement of Cited Document 1, and well-known art concerning AAV at the time of the priority date of the present application.

(4) As mentioned in the foregoing, since Present Invention 30 falls under Article 29, paragraph (1), item (iii) and paragraph (2) of the Patent Act, a patent may not be granted.

Therefore, even without going so far as to discuss the inventions according to other claims, the present application shall be rejected.

4. Grounds for Rescission

Erroneous determination on novelty and inventive step of Present Invention 30 (Overlooking of differences from Cited Invention, Erroneous findings of common features)

(omitted)

No. 4 Judgment of this court

1. Statements in the description of the present application

(1) The description of the present application (Exhibit Ko 2) includes statements as shown in Attachment 1. According to these statements, it can be acknowledged that the Detailed Description of the Invention in the description of the present application discloses the following matters with regard to Present Invention 30.

A. Recombinant adeno-associated virus (AAV) vectors had a problem in which DNA fragments were packaged even when nuclease treatment was performed in order to remove DNA impurities present in the recombinant AAV vectors, and so on ([0002]). Most of encapsidated residual DNA in an AAV is derived from vector plasmid templates containing an inverted terminal repeat sequence (ITR) ([0003]).

The inventors of the present application revealed that levels of residual plasmid DNA impurities were increased in a recombinant adeno-associated virus (rAAV) vector plasmid containing a vector expression cassette which was shorter than the natural rAAV packaging capacity limit size (about 4.7 kb), and that the shorter the sequence in relation to the natural rAAV packaging capacity limit size, the greater the increase in the levels of the impurities ([0034]).

Present Invention provides a recombinant vector (e.g., AAV) plasmid having a sequence with a size close to the natural packaging capacity limit size of the virus (AAV) and a method of using said recombinant vector (e.g., AAV) plasmid; for example, in order to produce a recombinant virus particle with a reduced amount of or no residual DNA impurities. For example, optimizing the size of the vector genome sequence can mitigate potential risks associated with vector-mediated transfer of undesirable nucleic acid sequences such as bacterial genes causing antibiotic resistance ([0035]).

B. The recombinant vector plasmid of Present Invention is derived from the wild type genome of a virus such as AAV, in which the wild type genome is removed from

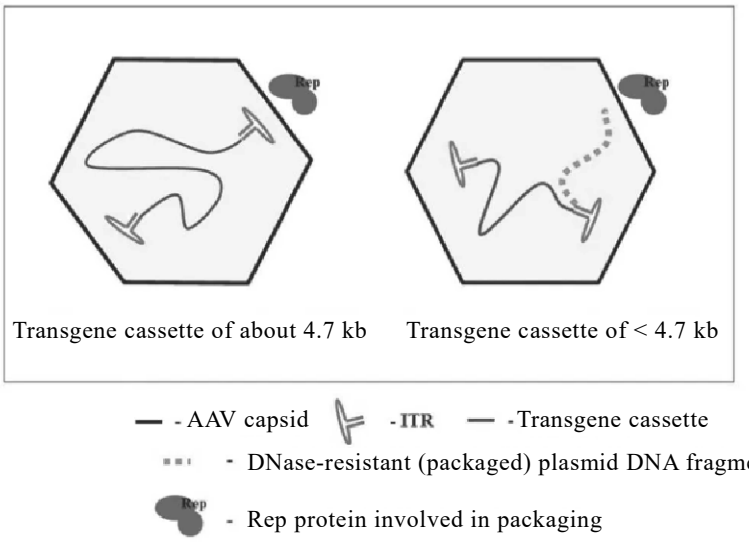
a virus (e.g., AAV) and replaced with a non-native nucleic acid such as a heterologous polynucleotide sequence (e.g., a therapeutic gene expression cassette) by means of molecular methods, and the "vector genome" refers to a portion of the recombinant vector plasmid which is packaged or encapsidated by a virus (e.g., AAV) and which comprises a heterologous polynucleotide sequence ([0040], [0080]).

C. The recombinant vector plasmid of Present Invention comprises an additional filler/stuffer nucleic acid sequence which alters or adjusts a length to the normal size or a size close to the normal size of a virus genome sequence to be packaged or encapsidated to form an infectious virus particle. In various embodiments, the filler/stuffer nucleic acid sequence is an untranslated (non-protein encoding) segment of nucleic acids. In a particular embodiment of the AAV vector, the heterologous polynucleotide sequence has a length less than 4.7 Kb, and the filler or stuffer polynucleotide sequence has a total length when combined with the heterologous polynucleotide sequence (e.g., inserted into a vector) of about 3.0 Kb to 5.5 Kb, 4.0 to 5.0 Kb, or 4.3 to 4.8 Kb. ([0082])

D. Figure 4 is a diagram showing encapsidation of plasmid DNA in a vector containing a short transgene cassette ([0031]).

[Figure 4]

Diagram showing encapsidation of plasmid DNA in vector containing a short transgene cassette



(2) Next, the Detailed Description of the Invention in the description of the present application includes the following disclosures as working examples.

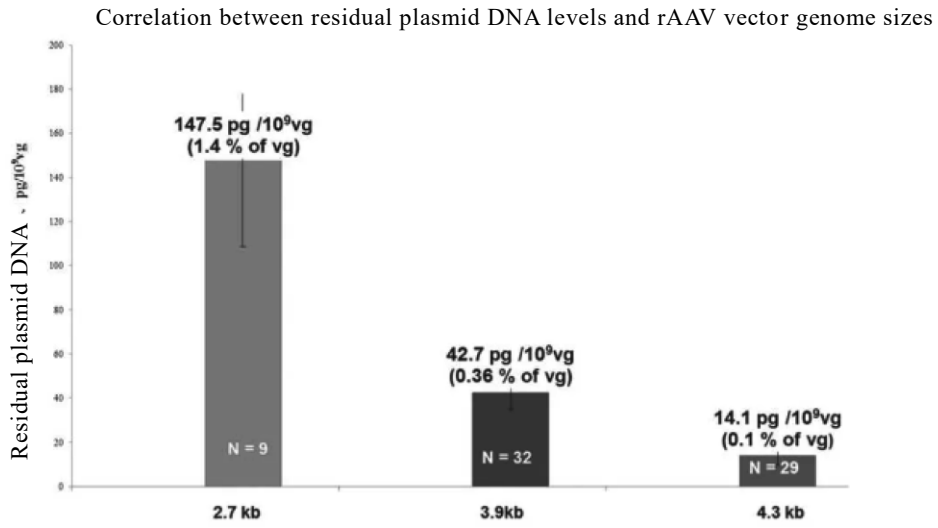
A. Example 1

It was indicated that the size of encapcidated DNA was comparable for vectors having a short transgene cassette (2716 bp) and vectors having a long transgene cassette (4297 bp), which approximately corresponded to the packaging capacity limit of AAV virus (4.5 Kb), and that DNA packaged in a vector having a short transgene cassette contained a plasmid sequence which flanks the genome ([0164], [0166], Figure 2B, Figure 2D).

B. Example 2

Levels of residual plasmid DNA measured using primers and probes specific to KanR or AmpR gene positioned close to the 5' ITR of the transgene cassette in the transgene plasmid backbone were evaluated in AAV2 vectors containing single-stranded standard transgene expression cassettes having sizes of 2.7 Kb, 3.7 Kb (note by this court: Figure 1 states "3.9 Kb", but this is recognized as a typographical error for "3.7 Kb"), and 4.3 Kb. As a result, the evaluation showed that rAAV A (size: 2.7 Kb) contained 164 pg/10^9 vg, rAAV B (size: 3.7 Kb) contained 42.7 pg/10^9 vg, and rAAV C (size: 4.3 Kb) contained 14.0 pg/10^9 vg of residual plasmid DNA ([0168] to [0171], Figure 1).

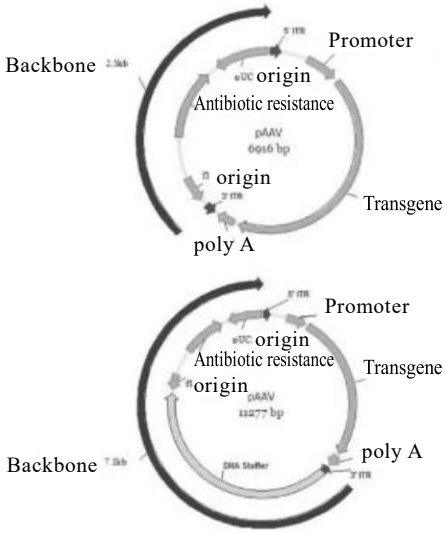
[Figure 1]



Further, packaging of vector plasmid "backbone" DNA occurs to a substantial degree through "reverse packaging" from ITR, which is greatly reduced using an oversized (> 4.7 Kb) (note by this court: [0172] states "> 4.7 bp", but this is recognized as a typographical error for "> 4.7 Kb") backbone ([0172], Figure 5).

[Figure 5]

Oversized plasmid backbone (7.1 Kb) exceeding the AAV packaging capacity limit markedly reduces non-vector DNA packaging.



Using various production systems:
Transfection using pRC plasmid
Transfection using pRG plasmid
rAd infection using production cell lines

In all methods, marked packaging of cis plasmid backbone was observed. In vectors purified by the density gradient centrifugation method, these product-related impurities are 3 to 7% of vg-containing particles.
(It is likely to be higher in vectors purified without a step of density-based separation.)

(Chadeuf et al., (2005), MoL Ther, 12:744)

Using an oversized backbone reduces residual plasmid DNA by about 40-fold (0.1 to 1.0%).

C. Example 3

DNA impurities in vectors prepared without using an oversized backbone were compared to DNA impurities in vectors prepared using an oversized backbone. As a result, when a vector plasmid backbone has a length of 3.8 Kb, DNA impurities were 301 pg/10^9 vg on average, and in contrast, and when a vector plasmid backbone has a length of 6.9 Kb, DNA impurities were 60 pg/10^9 vg on average. An oversized backbone in a vector plasmid can be used to reduce impurities in the preparation of viral vectors ([0173] to [0177], Figure 6).

(3) According to the disclosure of the description of the present application as mentioned in (1) and (2) above, it can be acknowledged that Present Invention 30 makes it its problem to provide a recombinant AAV particle with a reduced amount of or no residual DNA impurities ((1)A above) and that the above problem is solved by using a vector plasmid comprising a heterologous polynucleotide sequence having a length less than 4.7 Kb (positioned within two AAV ITR sequences) and an inert filler or stuffer polynucleotide sequence ([0166], Figure 1, Figure 2, and Figure 4. Note that this point will be discussed in detail in 3(1)D below.).

2. Statements in Cited Document 1

(1) Cited Document 1 includes statements as shown in Attachment 2 (Note that its translation text is based on Exhibit Otsu 1). According to these statements, it can be acknowledged that Cited Document 1 discloses the following matters.

A. Previous studies have reported that encapsidated plasmid DNA impurities are

primarily derived from the backbone of a plasmid for ITR-containing vector production. In this regard, an influence of vector plasmid backbone size on the amount of residual plasmid DNA impurities in recombinant AAV2 and AAV6 was assessed (1-2 in Attachment 2).

Eight lots of AAV2 vectors and two lots of AAV6 vectors which express human coagulation factor IX were produced using a vector plasmid containing a 6980 bp backbone exceeding the AAV packaging limit of about 4700 nt. As comparison subjects, one lot of AAV2 vectors and four lots of AAV6 vectors were prepared using vector plasmids having backbones of 2620 bp or 2638 bp (1-3 and 1-5 in Attachment 2).

B. Residual plasmid DNA levels were measured by Q-PCR using primers and probes specific to Amp^R (ampicillin resistance gene), which is a sequence common to all three production plasmids used for vector generation. As a result, average residual plasmid DNA levels measured in five lots produced using the vector plasmid having an oversized (6980 bp) backbone were 14.2±2.6 pg/10^9 vg, which was 7.6-fold lower than an average value of 107.6±27.6 pg/10^9 vg measured in five lots prepared using the vector plasmid having smaller (2620 bp or 2638 bp) backbones. Therefore, using the oversized backbone in the plasmid for vector production achieved a great reduction in plasmid-derived DNA impurities (1-2 and 1-3 (Table 1) in Attachment 2).

C. In the current study, when AAV vectors were produced by gene transfer (transfection) of HEK293 cells using a vector plasmid having a backbone smaller than the packaging limit of AAV, nucleotide-resistant plasmid DNA impurities contained in the resulting vectors were 2.9 to 5.7%. An effect of using a vector plasmid in which a stuffer sequence was modified in such a way that the backbone exceeded the packaging capacity of AAV2 was investigated, and it was found that the levels of encapsidated plasmid DNA impurities were greatly reduced (7.6-fold). (1-4 in Attachment 2).

(2) According to the disclosure mentioned in (1) above, it can be acknowledged that Cited Document 1 states Cited Invention as found by the appeal decision of the present case (No. 2, 3(2) above).

3. Grounds for rescission (Erroneous determination on novelty and inventive step of Present Invention 30 (Overlooking of differences from Cited Invention, Erroneous findings of common features))

(1) Technical significance of Present Invention 30

A. The Plaintiff asserts, as mentioned in No. 3, 1(1) above, the following: it can be deemed that when contrasting Present Invention 30 with Cited Invention, there is a

difference in the length of the inert filler or stuffer polynucleotide sequence, and that a difference in the amount of residual DNA impurities contained in each AAV particle in Present Invention 30 and Cited Invention is due to this difference in length; and thus, it can be deemed that this difference in length is a substantial difference. Accordingly, in the first place, the technical significance of Present Invention 30 will be discussed.

B. Claim 30 according to Present Invention 30 is recited in the form of referring to the "recombinant vector plasmid" of any of Claims 1 to 27. Among these claims, an invention according to a portion referring to Claim 1 (Present Invention 30) is identified as follows (Underlined portions are added by this court.).

"An AAV particle, comprising a vector genome of

a recombinant vector plasmid comprising:

a vector genome comprising a heterologous polynucleotide sequence; and

a first inert filler or stuffer polynucleotide sequence,

wherein said heterologous polynucleotide sequence has a length less than 4.7 Kb and is positioned within two adeno-associated virus (AAV) ITR sequences [i], wherein said inert filler or stuffer polynucleotide sequence is positioned outside two adeno-associated virus (AAV) ITR sequences, and wherein said first inert filler or stuffer polynucleotide sequence has a length of 7.0 to 10.0 Kb [ii]"

C. As mentioned in B above, Present Invention 30 has a constituent feature of the "filler or stuffer polynucleotide sequence has a length of 7.0 to 10.0 Kb," whereas it is clear that Cited Invention does not include a statement with regard to this point. The Plaintiff asserts, as explained above, that it can be deemed that the difference between Present Invention 30 and Cited Invention is due to this difference in length, and thus, this difference in length is a substantial difference. Accordingly, this point will be discussed below.

Present Invention 30 is the AAV particle comprising the vector genome of the recombinant vector plasmid constituted by underlined portions [i] and [ii], which is not constituted as comprising the recombinant vector plasmid itself. AAV particles are generally made by transfection of 293 cells with three types of plasmid vectors: a vector plasmid in which a target transgene is inserted between two ITR, an AAV helper plasmid, and an adenovirus helper plasmid, in which the transgene between the two ITR is encapsidated into the AAV (Figure 3 in Exhibit Otsu 5). Thus, referring also to Figure 4 in the description of the present application, it can be deemed that the "AAV particle comprising the vector genome" of Present Invention 30 comprises the heterologous polynucleotide sequence which is positioned within two adeno-associated virus (AAV) ITR and which has a length less than 4.7 Kb (the constituent feature of

underlined portion [i]). However, it cannot be naturally deemed that the "AAV particle comprising the vector genome" of Present Invention 30 comprises the filler or stuffer polynucleotide sequence according to the constituent feature of underlined portion [ii]. The Defendant places importance on this point and asserts, as mentioned in No. 3, 2(1) above, that: the AAV vector (AAV particle) comprises a "genome in which two ITR sequences and a target gene (therapeutic gene) sandwiched between the two ITR sequences" and does not comprise a whole vector plasmid itself comprising the above genome and backbone; thus, the backbone sequence outside ITR is not substantially contained in the AAV vector; and that Present Invention and Cited Invention do not differ in the constituent features in terms of comprising "two ITR sequences" and the "vector genome" that is the "heterologous polynucleotide sequence" which is "positioned within" the two ITR sequences and which "has a length less than 4.7 Kb." However, the appeal decision of the present case found, as mentioned in No. 2, 3(3)A above, that the vector genome is not limited to a region comprising the heterologous polynucleotide sequence, but also comprises a very small amount of residual plasmid DNA derived from a region which is positioned outside said region; i.e., outside the two AAV ITR sequences. It is hard to deny the possibility that the vector genome comprises at least a portion of the backbone sequence outside the ITR. In either case, if the technical significance of Present Invention 30 is not limited to the constituent feature of underlined portion [i], but also exists in the constituent feature of underlined portion [ii], it cannot be deemed to be reasonable to take up only the constituent feature of underlined portion [i] as the constituent features of Present Invention 30. Thus, the technical significance of Present Invention 30 will be further discussed.

D. (a) The description of the present application states that "Studies disclosed herein show that levels of residual plasmid DNA impurities were increased in a recombinant adeno-associated virus (rAAV) vector plasmid containing a vector expression cassette which was shorter than the natural rAAV packaging capacity limit size (about 4.7 kb), and that the shorter the sequence in relation to the natural rAAV packaging capacity limit size, the greater the increase in the levels of the impurities. ..."([0034]). In addition, Figure 2 shows experimental results of packaging vector plasmids containing a short transgene cassette (2.7 Kb) and a long transgene cassette (4.3 Kb. [0164] states "4.2 kb", but Figure 2 states "4297 bp", thus this is recognized as a typographical error for "4.3 Kb.") ([0164]), and in addition thereto, states that "The size of encapcidated DNA (e.g., DNase-resistant plasmid backbone DNA in conjunction with vector genome size) was comparable between vectors having

a short genome and vectors having a long genome, which approximately corresponded to the packaging capacity limit of AAV virus (4.5 Kb) (note by this court: based on the statement [0034], etc., this is recognized as a typographical error for 4.7 Kb). DNA packaged in the vector having short genome contained a plasmid sequence which flanks the genome, indicating that a vector having a short genome packaged a plasmid sequence with a size up to the maximum packaging capacity limit of AAV virus"([0166]). Further, Figure 4 discloses a diagram illustrating that DNase-resistant plasmid DNA fragments are also encapsidated when the length of the transgene cassette of the AAV particle packaging capacity limit size (about 4.7 Kb) is shorter (< 4.7 Kb) as compared to when the length of the transgene cassette of the AAV particle packaging capacity limit size is the AAV particle packaging capacity limit size (about 4.7 Kb). In addition thereto, taking into overall consideration the disclosure of Example 2 in 1(2)B above, it can be understood that the AAV particle which encapsidated the heterologous polynucleotide using the vector plasmid also packages a plasmid sequence which flanks the transgene (heterologous polynucleotide) up to the packaging capacity limit size (4.7 Kb) and that the shorter the size of the transgene in relation to the packaging capacity limit size, the greater the increase in the amount of plasmid DNA impurities contained in a purified AAV vector (AAV particle).

Furthermore, the description of the present application states that "In one aspect, the filler or stuffer polynucleotide sequence is inert or innocuous and has no function or activity. ... the filler or stuffer polynucleotide sequence is not a sequence which encodes a protein or a peptide, and the filler or stuffer polynucleotide sequence is a sequence different from any of: the heterologous polynucleotide sequence; an AAV inverted terminal repeat sequence (ITR); an expression control element; an origin of replication; a selectable marker; and a poly-Adenine (poly-A) sequence"([0012]), "The recombinant vector plasmid as defined herein comprises an additional filler/stuffer nucleic acid sequence which alters or adjusts a length to the normal size or a size close to the normal size of a virus genome sequence to be packaged or encapsidated to form an infectious virus particle. In various embodiments, the filler/stuffer nucleic acid sequence is an untranslated (non-protein encoding) segment of nucleic acids. ..."([0082]), "... In ... another aspect, the filler or stuffer polynucleotide sequence is located next to 5' side and/or 3' side ITR sequences which flank outside the respective 5' end and/or 3' end of a heterologous polynucleotide sequence. ..."([0007]). Taking into overall consideration these statements, it can be understood that the inert filler or stuffer polynucleotide sequence outside ITR is a non-functional or non-active nucleic acid fragment to be inserted in order to prevent packaging or encapsidation of a

recombinant plasmid vector sequence evaluated as residual plasmid DNA.

(b) Taking into overall consideration the disclosure in the description of the present application as pointed out in (a) above, it can be acknowledged that Present Invention 30 makes it its problem to provide a recombinant AAV particle with a reduced amount of or no residual DNA impurities, as mentioned in 1(3) above. In this regard, it can be understood that the technical significance of Present Invention 30 exists in that when the heterologous polynucleotide sequence positioned within two ITR has a length less than the packaging capacity limit of an AAV particle (about 4.7 Kb), the AAV particle is made to comprise a vector genome of a vector plasmid prepared in such a way that the combined length of an inert filler or stuffer polynucleotide with the heterologous polynucleotide sequence of transgene exceeds at least the packaging capacity limit, to thereby reduce an amount of residual DNA impurities derived from the plasmid and to solve the above problem.

E. The Plaintiff asserts, as mentioned in No. 3, 1(1) above, that the amount of residual DNA impurities contained in the AAV particle will vary depending on the length of the first inert filler or stuffer polynucleotide sequence (7.0 to 10.0 Kb in Present Invention 30) in the backbone contained in the recombinant vector plasmid. In this regard, the description of the present application does not disclose working examples showing residual DNA impurities contained in an AAV particle produced using a vector plasmid prepared so as to satisfy the constituent feature of the "inert filler or stuffer polynucleotide sequence is positioned outside two adeno-associated virus (AAV) ITR sequences, and wherein said first inert filler or stuffer polynucleotide sequence has a length of 7.0 to 10.0 Kb" (the constituent feature of underlined portion [ii]). However, the description of the present application discloses that using a vector plasmid having an oversized (it is interpreted to mean exceeding the packaging capacity limit of an AAV particle) backbone (7.1 Kb) reduces an amount of residual plasmid DNA impurities by about 40-fold (0.1 to 1.0%) (Figure 5), and that using a vector plasmid having an oversized backbone of 6.9 Kb reduced an amount of residual plasmid DNA impurities by about five-fold as compared to the case of using a vector plasmid having a non-oversized backbone (3.8 Kb) (Example 3). The "backbone" as used herein is interpreted to be a region outside two ITR sequences in a vector plasmid and to include an inert filler or stuffer polynucleotide sequence (Figure 5 in the description of the present application also states that a portion outside two ITR within which a transgene is positioned in a vector plasmid is a backbone.).

On the other hand, it can be acknowledged that the lengths of transgenes contained in the vector plasmids differ between the upper panel and the lower panel of

Figure 5 in the description of the present application, but there is no statement on the specific length in Figure 5. In addition, also in Figure 6, there is no statement on the lengths of transgenes contained in the vector plasmids with backbone sizes of 3.8 Kb and 6.9 Kb. Further, according to each of the disclosures in 1(1)A, 1(2)A, and B, Figure 1, and Figure 4 above, the description of the present application merely discloses that adopting a transgene cassette with a size closer to the packaging capacity limit size (4.7 Kb) reduced an amount of DNA impurities and that the shorter a transgene cassette in relation to the packaging capacity limit size, the greater the increase in an amount of DNA impurities. Thus, it cannot be understood that regardless of transgene length, the longer the length of the backbone containing the filler or stuffer polynucleotide, the more an amount of DNA impurities will be markedly reduced.

Then, even though Figure 5 and Example 3 above state that an amount of DNA impurities was reduced, this cannot be immediately understood to mean that this reduction is because the backbone containing the filler or stuffer polynucleotide has a long length. Further, with regard to the filler or stuffer polynucleotide sequence having a length of 7.0 to 10.0 Kb (underlined portion [ii]), no unique technical significance can be found in its length itself, but it should be understood that such fact is significant to the extent of constituting the length which is conducive to the solution of the problem in light of the technical significance mentioned in D(b) above.

F. According to the foregoing, it cannot be deemed that it is reasonable to take up only the constituent feature of underlined portion [i] as constituent features of Present Invention 30. Further, even though the "AAV particle" of Present Invention 30 packages the constituent feature of underlined portion [ii] as is, Present Invention 30 including this constituent feature should be contrasted with Cited Invention. Thus, in this regard, the determination by the appeal decision of the present case is erroneous.

(2) Contrast between Present Invention 30 and Cited Invention

A. The Plaintiff asserts that the constituent feature of underlined portion [ii] of Present Invention 30 is a substantial difference between Present Invention 30 and Cited Invention. Accordingly, hereinafter, Present Invention 30 will be contrasted with Cited Invention on the premise of the findings in (1) above.

As mentioned in (1)B above, Present Invention 30 has the constituent feature of "said heterologous polynucleotide sequence has a length less than 4.7 Kb and is positioned within two adeno-associated virus (AAV) ITR sequences." In contrast to this, Cited Invention has the constituent feature of the vector plasmid "containing ITR having 4297 nt transgene encoding human coagulation factor IX." The "heterologous polynucleotide" of Present Invention 30 corresponds to "human coagulation factor IX"

of Cited Invention, which are common in the feature of containing a vector plasmid having a length less than "4.7 Kb."

B. Next, Present Invention 30 has the constituent feature of "said inert filler or stuffer polynucleotide sequence is positioned outside two adeno-associated virus (AAV) ITR sequences, and wherein said first inert filler or stuffer polynucleotide sequence has a length of 7.0 to 10.0 Kb."

In contrast to this, Cited Invention has the constituent feature of a vector plasmid of "a 6980 bp backbone containing ampicillin resistance gene." In addition, Cited Document 1 states that "Eight lots of AAV2 vectors and two lots of AAV6 vectors which express human coagulation factor IX (AAV-hFIX) were produced using a vector plasmid containing a 6980 bp backbone exceeding the AAV packaging limit of about 4700 nt." (1-5 in Attachment 2), "An effect of using a vector plasmid in which a stuffer sequence was modified in such a way that the backbone exceeded the packaging capacity of AAV2 was investigated, and it was found that this strategy greatly reduced the levels of encapsidated plasmid DNA impurities (7.6-fold, $P < 0.001$)"(1-4 in Attachment 2). Thus, it can be understood that Cited Invention is an invention which solved the problem of reducing an amount of DNA impurities derived from a plasmid contained in an AAV particle by using a vector plasmid in which a stuffer sequence was modified in such a way that a backbone exceeded the packaging capacity limit of AAV.

Then, Present Invention 30 and Cited Invention have commonality in the problem of reducing an amount of DNA impurities derived from a plasmid contained in an AAV particle when prepared using a vector plasmid containing a transgene and a stuffer polynucleotide sequence. In addition, as mentioned in A above, both transgenes have a length less than a packaging capacity limit of 4.7 Kb, but differ in a length of an inert filler or stuffer polynucleotide sequence of a vector plasmid.

However, in Present Invention 30, the technical significance does not exist in that the length of the filler or stuffer polynucleotide sequence of the vector plasmid is made to be in the specific range of length that is a "length of 7.0 to 10.0 Kb." Further, it can be understood that its technical significance exists in that when the transgene has a length less than the packaging capacity limit of an AAV particle (about 4.7 Kb), the AAV particle is made to comprise a vector genome of a vector plasmid prepared in such a way that the combined length of an inert filler or stuffer polynucleotide with the heterologous polynucleotide sequence of transgene exceeds at least the packaging capacity limit, which is as mentioned in (1)D(b) above. Then, also in Cited Invention, it can be understood that a stuffer sequence is modified in such a way that a backbone

exceeds the packaging capacity limit of AAV, and that the length of the transgene (4297 nt (about 4.297 Kb)) and the length of the stuffer sequence contained in this backbone (6980 bp (about 6.980 Kb)) are greater than at least a packaging capacity limit of 4.7 Kb. Thus, Present Invention 30 and Cited Invention are identical in their technical significance, and it cannot be deemed to be a substantial difference that the length of the filler or stuffer polynucleotide sequence of the vector plasmid was made to be in the specific range of length that is "a length of 7.0 to 10.0 Kb" in Present Invention 30. In addition, the "6980 bp backbone" in Cited Invention is an "oversized backbone"(see [0173] to [0177]). In this regard, as mentioned in (1)E above, it can be merely understood from the statement in the description of the present application that the "inert filler or stuffer polynucleotide sequence" having a "length of 7.0 to 10.0 Kb" in Present Invention 30 constitutes an oversized backbone. Thus, also from this point of view, it cannot be deemed that the inventions differ.

(3) The Plaintiff's assertion

A. The Plaintiff asserts, as mentioned in No. 3, 1(2)A(b) above, the following: [i] the description of the present application discloses in Figure 5 that an amount of DNA impurities contained in AAV prepared using a 7.1 Kb backbone is reduced by about 40-fold as compared to the case of using a vector plasmid containing a 2.5 Kb backbone; [ii] since the length of the backbone in Comparative Example of Figure 5 (2.5 Kb) is close to the 2620 bp or 2638 bp backbone as shown in Cited Document 1, the amount of residual DNA in the AAV particle prepared using the vector plasmid having the 2.5 Kb backbone in Comparative Example of Figure 5 is comparable to the amount of residual DNA contained in the AAV particle prepared using the vector plasmid having backbones of 2620 bp or 2638 bp as disclosed in Cited Document 1, and on the premise of this value, the Plaintiff estimates an amount of DNA impurities having a backbone length of 7.1 Kb in Example 2 (reduction by 40-fold); and [iii] by referring to each of the statements in [0177], Figure 5, Figure 6, and [0172] of the description of the present application, the description of the present application discloses that a range of reduction in an amount of residual DNA impurities increases by increasing the length of an oversized backbone, and on the premise of this disclosure, it can be understood that since a recombinant vector plasmid of Present Invention 30 exceeds a backbone length of 7.1 Kb, the amount of residual DNA contained in the AAV particle of Present Invention 30 is even less than the value estimated in [ii].

However, as mentioned in (1)E above, although the description of the present application shows in the upper panel and the lower panel of Figure 5 that the length of the backbone in the vector plasmid is 2.5 Kb and 7.1 Kb, respectively, there is no

statement on the length of the transgene contained in the vector plasmid, and it can be acknowledged that the length of the transgene differs between the upper panel and the lower panel of Figure 5. Thus, it cannot be interpreted that an amount of residual DNA contained in the AAV particle after being encapsidated as a result of adjusting only the length of the backbone using the same transgene was reduced by "about 40-fold." In addition to this, with regard to the vector plasmid in the lower panel, it is stated that "Using an oversized backbone reduces residual plasmid DNA by about 40-fold (0.1 to 1.0%)." However, it is not stated together with its experimental conditions and the specific experimental results, and it is also unclear how the value was calculated. Thus, it cannot be deemed that the description of the present application discloses the matter as asserted in [i] above. Further, it is not clear whether the transgene of the vector plasmid "having a backbone length of 2.5 Kb" in Figure 5 is the same as the transgene of the vector plasmid having a backbone length of "2620 bp or 2638 bp" in Cited Document 1. Nevertheless, it is also not reasonable to estimate the amount of DNA impurities contained in the AAV particle prepared using the vector plasmid having a backbone length of 7.1 Kb shown in the lower panel of Figure 5 in the description of the present application by referring to the amount of DNA impurities contained in the AAV particle disclosed in Cited Document 1.

In addition, with regard to the statement in the description of the present application asserted in [iii] by the Plaintiff, under the circumstance where the length of a transgene contained in the vector plasmid is unclear in the first place, it is merely a comparison between the amount of DNA impurities derived from the plasmid in the AAV particle prepared using the vector plasmid without an oversized backbone and the amount of DNA impurities derived from the plasmid in the AAV particle prepared using a vector plasmid containing an oversized backbone. This is not an experimental result showing a relationship between a backbone length and an amount of plasmid DNA impurities among vector plasmids having oversized backbones, and these statements do not indicate that the longer an oversized backbone in relation to a packaging capacity limit, the greater a reduction in an amount of DNA impurities. Thus, it cannot be deemed that the description of the present application discloses that a range of reduction in an amount of residual DNA impurities is increased by increasing a length of an oversized backbone.

According to the foregoing, based on [i] to [iii] in A above, it cannot be immediately acknowledged that the amount of residual DNA contained in the AAV particle of Present Invention 30 is even less than 2.69 ± 0.69 pg/10^9 vg, and in addition, even if reduction in the amount of residual DNA occurs, it cannot also be immediately

acknowledged that the reduction is caused by increasing a length of an oversized backbone. Therefore, the Plaintiff's assertion mentioned above is erroneous in its premise.

B. Incidentally, the appeal decision of the present case contrasts, as mentioned in No. 2, 3(3)A above, Present Invention 30 with Cited Invention to determine a difference therebetween, based on the amount of DNA impurities contained in Present Invention 30 being "about 60 pg/10^9 vg." However, this value is the amount of DNA impurities derived from the plasmid contained in the AAV particle prepared using the plasmid having a backbone length of 6.9 Kb in Example 3 ([0177], Figure 6). In this regard, the vector plasmid comprising the "filler or stuffer polynucleotide sequence" having a length of 7.0 to 10.0 Kb of Present Invention 30 has a length greater than the backbone length of this Example. Therefore, as the Plaintiff points out in No. 3, 1(2)A(d) above, some parts of the determination by the appeal decision of the present case, which made contrast and determination by referring to the value in Example 3 without any explanation, are not reasonable. However, according to what this court has explained so far, they do not affect the conclusion.

(4) Summary

According to the foregoing, when contrasting Present Invention 30 with Cited Invention, it cannot be immediately acknowledged that an amount of residual DNA impurities and its degree contained in each AAV particle in Present Invention 30 and Cited Invention differ depending on the length of the inert filler or stuffer polynucleotide sequence. In addition, although the length of the inert filler or stuffer polynucleotide sequence differs, it cannot be acknowledged that such difference is significant in light of the technical significance of the invention. Thus, it cannot be deemed that this difference in length is a substantial difference. Then, it can be deemed that Present Invention 30 is an invention stated in Cited Document 1. Therefore, it should be deemed that Present Invention 30 lacks novelty. The Plaintiff also asserts other matters at great length, but none of them can affect the above conclusion.

4. Conclusion

According to the foregoing, grounds for rescission asserted by the Plaintiff are unfounded. In addition, since Present Invention 30 falls under Article 29, paragraph (1), item (iii) of the Patent Act, a patent may not be granted. Thus, even without going so far as to discuss the invention according to other claims, the appeal decision of the present case to determine that the present application shall be rejected is reasonable in the conclusion. Therefore, the Plaintiff's claim should be dismissed.

For the foregoing reasons, the judgment is rendered as mentioned in the main text.

Intellectual Property High Court, Fourth Division

Presiding judge: KANNO Masayuki

Judge: NAKAMURA Kyo

Judge: OKAYAMA Tadahiro

(Attachment 1)

Description of the Present Application

[Detailed Description of the Invention]

[0002]

Recombinant adeno-associated virus (AAV) vectors have been shown to be extremely promising for therapeutic applications in several early clinical trials to date, as reported by multiple groups. In order to advance this new class of biologic product towards late phase clinical trials and to obtain eventual approval as medicines, it is necessary to further improve vector characterization and quality control methods. For example, it is necessary to further understand how vector design and manufacturing process parameters affect impurity profiles in purified clinical grade vectors. Removal of DNA impurities present in AAV vectors is made complicated by the fact that DNA fragments may be packaged even when nuclease treatment which can efficiently remove treatable nucleic acids during vector purification is performed, and thus become resistant to nuclease treatment performed in such a way as to allow maintenance of vector particle integrity.

[0003]

An important objective in the design of rAAV production systems is to analyze the characteristics of vector-related impurities including wild-type/pseudo wild-type AAV species (wtAAV), residual DNA impurities encapsidated in AAV, and empty capsids, and to implement strategies which can minimize/control their generation. These product-related impurities closely resemble the vector itself, and cannot be easily separated from bona fide vectors during the purification process. DNA impurities other than vectors have been reported to account for 1 to 8% of the total DNA amount in purified vector particles (Smith PH Wright JF. Qu G. et al 2003, Mo. Therapy, 7:8348; Chadeuf G. Ciron C. Moullier P. Salvetti A., Mo. Therapy 2005, 12:744. Report from the Committee for Medicinal Products for Human Use (CHMP) gene therapy expert group meeting. European Medicines Agency EMEA/CHMP 2005, 183989/2004). Most of encapsidated residual DNA is derived from vector plasmid templates containing an inverted terminal repeat sequence (ITR).

[Summary of the Invention]

[0004]

The present invention provides a recombinant vector plasmid and a virus particle which comprises (encapsidates or packages) a vector genome. In one embodiment, a

recombinant vector plasmid comprises a heterologous polynucleotide sequence, and a filler polynucleotide sequence or stuffer polynucleotide sequence.

[0005]

The present invention also provides an AAV particle which comprises (encapsidates or packages) an AAV vector plasmid and an AAV vector genome. In one embodiment, the recombinant AAV vector plasmid comprises a heterologous polynucleotide sequence, and a filler polynucleotide sequence or a stuffer polynucleotide sequence.

[0006]

In various embodiments, the heterologous polynucleotide sequence has a length less than about 4.7 Kb. In a particular aspect, the heterologous polynucleotide sequence has a length less than 4.7 Kb and is positioned within two adeno-associated virus (AAV) ITR sequences. In a particular aspect, the total combined length of the heterologous polynucleotide sequence with the filler or stuffer polynucleotide sequence is about 3.0 to 5.5 Kb, or about 4.0 to 5.0 Kb, or about 4.3 to 4.8 Kb.

[0007]

The filler or stuffer polynucleotide sequence can be located at any desired position in a vector, unless a function or activity of the vector is impaired. In one aspect, the filler or stuffer polynucleotide sequence is not located between 5' side and/or 3' side ITR sequences which flank outside the respective 5' end and/or 3' end of the heterologous polynucleotide sequence. In another aspect, the filler or stuffer polynucleotide sequence is located between 5' side and/or 3' side ITR sequences which flank outside the respective 5' end and/or 3' end of the heterologous polynucleotide sequence. In yet another aspect, the filler or stuffer polynucleotide sequence is located next to 5' side and/or 3' side ITR sequences which flank outside the respective 5' end and/or 3' end of the heterologous polynucleotide sequence. And in yet another aspect, the filler or stuffer polynucleotide sequence is located within the heterologous polynucleotide sequence such as an intron in a genomic nucleic acid.

[0008]

Thus, in various embodiments, one filler or stuffer polynucleotide sequence is positioned within two adeno-associated virus (AAV) ITR sequences, or one filler or stuffer polynucleotide sequence is positioned outside two adeno-associated virus (AAV) ITR sequences, or there are two fillers or stuffer polynucleotide sequences, wherein a first filler or stuffer polynucleotide sequence is positioned within two adeno-associated virus (AAV) ITR sequences, and a second filler or stuffer polynucleotide sequence is positioned outside two adeno-associated virus (AAV) ITR sequences.

[0009]

In yet various other embodiments, the filler or stuffer polynucleotide sequence is a sequence having a length of 1 to 10, 10 to 20, 20 to 30, 30 to 40, 40 to 50, 50 to 60, 60 to 75, 75 to 100, 100 to 150, 150 to 200, 200 to 250, 250 to 300, 300 to 400, 400 to 500, 500 to 750, 750 to 1,000, 1,000 to 1,500, 1,500 to 2,000, 2,000 to 2,500, 2,500 to 3,000, 3,000 to 3,500, 3,500 to 4,000, 4,000 to 4,500, 4,500 to 5,000, 5,500 to 6,000, 6,000 to 7,000, 7,000 to 8,000, or 8,000 to 9,000 nucleotides.

[0010]

In a further particular aspect, when the filler or stuffer polynucleotide sequence is positioned between two adeno-associated virus (AAV) ITR sequences, the total combined length of the heterologous polynucleotide sequence with the filler or stuffer polynucleotide sequence is about 3.0 to 5.5 Kb, or about 4.0 to 5.0 Kb, or about 4.3 to 4.8 Kb. In yet another particular aspect, when the filler or stuffer polynucleotide sequence is positioned outside two adeno-associated virus (AAV) ITR sequences, the filler or stuffer polynucleotide sequence has a length greater than 4.7 Kb, about 5.0 to 10.0 Kb, or about 6.0 to 8.0 Kb.

[0012]

In one aspect, the filler or stuffer polynucleotide sequence is inert or innocuous and has no function or activity. In various particular embodiments, the filler or stuffer polynucleotide sequence is not a bacterial polynucleotide sequence, the filler or stuffer polynucleotide sequence is not a sequence which encodes a protein or a peptide, and the filler or stuffer polynucleotide sequence is a sequence different from any of: the heterologous polynucleotide sequence; an AAV inverted terminal repeat sequence (ITR); an expression control element; an origin of replication; a selectable marker; and a poly-Adenine (poly-A) sequence.

[0014]

According to the present invention, in a recombinant vector (e.g., AAV) plasmid and a virus (e.g., AAV) particle which comprises (encapsidates or packages) a recombinant vector (e.g., AAV) genome, the heterologous polynucleotide sequence can be transcribed and subsequently translated into a protein, or can be transcribed into a transcript having a function or activity in itself. In one embodiment, a heterologous polynucleotide sequence encodes a protein having therapeutic effects. In a particular embodiment, said protein is a blood coagulation factor (e.g., Factor XIII ...), ...

[0018]

In the present invention, a recombinant vector (e.g., AAV) plasmid and a virus (e.g., AAV) particle which comprises (encapsidates or packages) a recombinant vector

(e.g., AAV) genome comprises an additional element which functions in cis or trans. In a particular embodiment, a virus (e.g., AAV) particle which comprises (encapsidates or packages) a recombinant vector (e.g., AAV) plasmid and/or a recombinant vector (e.g., AAV) genome also comprises one or more inverted terminal repeat sequences (ITR) which flank 5' end or 3' end of a heterologous polynucleotide sequence, an expression control sequence which drives transcription of a heterologous polynucleotide sequence (e.g., a promoter or enhancer which contributes to transcription of a heterologous polynucleotide sequence, such as a constitutive or regulatable control sequence, or a tissue-specific expression control element), a poly-Adenine sequence positioned on 3' of a heterologous polynucleotide sequence, a selectable marker (e.g., a protein which provides antibiotic resistance such as Kanamycin resistance), and/or an origin of replication.

[Brief Description of Drawings]

[0027]

[Figure 1] Figure 1 shows that levels of plasmid DNA impurities contained in purified AAV vector preparations depend on the size of a transgene cassette.

[0028]

[Figure 2] Figure 2 shows mapping of 5' end of vector genome performed by using PCR and a set of primers covering a region spanning a transgene cassette and upstream plasmid backbone sequences, before and after DNase 1 treatment. A single primer positioned within the transgene cassette (circled) was used in combination with primers spanning sequences in the transgene cassette and flanking segments of plasmid backbones containing antibiotic resistance genes (KanR and AmpR). PCR reaction was analyzed by 1% agarose gel electrophoresis.

[0029]

Figures 2A to 2D show PCR on vectors containing a short or long transgene cassette before and after DNase treatment.

[Figure 2A] Figure 2A shows PCR on a vector containing a short transgene cassette (2.7 kb) before DNase treatment.

[Figure 2B] Figure 2B shows PCR on a vector containing a short transgene cassette (2.7 kb) after DNase treatment and DNA purification.

[Figure 2C] Figure 2C shows PCR on a vector containing a long transgene cassette (4.3 kb) before DNase treatment.

[Figure 2D] Figure 2D shows PCR on a vector containing a long transgene cassette (4.2 kb) after DNase treatment and DNA purification.

[0030]

Figures 3A to 3B show plasmid control PCR which was performed on production plasmid DNA containing a transgene cassette using the above-mentioned set of primers, before and after treatment with DNase 1.

[Figure 3A] Figure 3A shows a plasmid containing a transgene cassette (2.7 kb).

[Figure 3B] Figure 3B shows a plasmid containing a transgene cassette (4.3 kb)

[Figure 3C] Figure 3C shows plasmid samples after DNase treatment.

[0031]

[Figure 4] Figure 4 shows a diagram of encapsidation of plasmid DNA into a vector containing a short transgene cassette.

[0032]

[Figure 5] Figure 5 shows that an oversized plasmid backbone (7.1 Kb) which is present in trans and exceeds the AAV packaging capacity limit size markedly reduces non-vector DNA packaging.

[0033]

[Figure 6] Figure 6 shows residual plasmid DNA contained in purified AAV vectors produced using vector plasmids containing normal size (circle) versus oversized (triangle) backbones.

[Description of Embodiments]

[0034]

Studies disclosed herein show that levels of residual plasmid DNA impurities were increased in a recombinant adeno-associated virus (rAAV) vector plasmid containing a vector expression cassette which was shorter than the natural rAAV packaging capacity limit size (about 4.7 kb), and that the shorter the sequence was in relation to the natural rAAV packaging capacity limit size, the greater the increase in the levels of the impurities. To give a specific example, rAAV A (2.7 kb size) contained 164 pg/10^9 vg of residual plasmid DNA (n = 9). rAAV B (3.7 kb size) contained 42.7 pg/10^9 vg of residual plasmid DNA (n = 32). rAAV C (4.3 kb size) contained 14.0 pg/10^9 vg of residual plasmid DNA (n = 29). Consequently, these studies show that adjusting a length of an expression cassette during vector design such that the length will be the same as or close to the (natural) packaging capacity limit size of a virus (AAV) capsid can reduce or prevent encapsidation of contaminating nucleic acid, and thus reduces virus (AAV) particles containing encapsidated nucleic acid impurities.

[0035]

Therefore, the present invention provides a recombinant vector (e.g., AAV) plasmid having a sequence with a size close to the natural packaging capacity limit size

of the virus (AAV), and a method of using said recombinant vector (e.g., AAV) plasmid, for example, to produce a recombinant virus particle with a reduced or no amount of residual DNA impurities. For example, optimizing the size of the vector genome sequence can mitigate potential risks associated with vector-mediated transfer of undesirable nucleic acid sequences such as bacterial genes causing antibiotic resistance.
[0036]

A recombinant vector (e.g., AAV) plasmid of the present invention in which a packaged (encapsidated) region (referred to as "vector" or "vector genome") with a size close to the natural packaging capacity limit size of a virus (e.g., AAV) can be used to transfer/deliver heterologous polynucleotide sequences such as sequences (genes) encoding proteins which provide desirable or therapeutic effects, as well as inhibitory (e.g., anti-sense) nucleic acids which reduce or inhibit expression of undesirable or defective (e.g., pathologic) genes, and thus can treat various diseases. For example, a recombinant vector (e.g., AAV) plasmid in which a packaged (encapsidated) region (vector genome) has a sequence with a size close to the natural packaging capacity limit size of the virus (AAV) can be used to transfer/deliver therapeutic genes for the treatment of genetic deficiency diseases such as hemophilia A or B and other metabolic or plasma protein deficiencies as well as for other therapeutic purposes.
[0040]

The recombinant "vector plasmid" and the "AAV vector plasmid" are derived from the wild type genome of a virus such as AAV, in which the wild type genome is removed from the virus (e.g., AAV) and replaced with a non-native nucleic acid such as a heterologous polynucleotide sequence (e.g., therapeutic gene expression cassette) by using molecular methods. Usually, in case of AAV, one or both inverted terminal repeat (ITR) sequences of the wild type AAV genome are retained in the AAV vector plasmid. The whole or a portion of the virus genome is replaced with a heterologous polynucleotide sequence, and said heterologous polynucleotide sequence is usually a non-native nucleic acid with respect to the virus (e.g., AAV) genome nucleic acid. Thus, a viral vector (e.g., AAV) is distinguished from a virus (e.g., AAV) genome.
[0078]

The term "vector" refers to a plasmid, virus (e.g., AAV vector), cosmid, or other vehicle which can be manipulated by insertion or incorporation of a polynucleotide. Such vectors can be used for genetic manipulation (i.e., "cloning vector") to transfer/import polynucleotide into cells and to transcribe or translate the polynucleotide inserted into cells. A vector plasmid generally comprises at least one origin of replication for propagation in a cell and optionally additional elements, such

as a heterologous polynucleotide sequence, an expression control element (e.g., a promoter, an enhancer), a selectable marker (e.g., antibiotic resistance), or a poly-Adenine sequence.

[0080]

Regarding the recombinant vector plasmid, a vector genome refers to a portion of the vector plasmid which is packaged or encapsidated by a virus (e.g., AAV) and which comprises a heterologous polynucleotide sequence. The plasmid portion of the recombinant vector plasmid comprises a backbone sequence used for helper cell transfection and for cell production of virus which packages/encapsidates the vector genome, but is not itself packaged/encapsidated by a virus (e.g., AAV).

[0082]

The recombinant vector plasmid as defined herein comprises an additional filler/stuffer nucleic acid sequence which alters or adjusts a length to the normal size or a size close to the normal size of a virus genome sequence to be packaged or encapsidated to form an infectious virus particle. In various embodiments, the filler/stuffer nucleic acid sequence is an untranslated (non-protein encoding) segment of nucleic acids. In a particular embodiment of the AAV vector, the heterologous polynucleotide sequence has a length less than 4.7 Kb, and the filler or stuffer polynucleotide sequence has a total length when combined with the heterologous polynucleotide sequence (e.g., inserted into a vector) of about 3.0 to 5.5 Kb, or 4.0 to 5.0 kb, or 4.3 to 4.8 kb. ...

[0083]

As disclosed herein, the filler or stuffer polynucleotide sequence may be in any position compatible with vector function within the recombinant vector plasmid, relative to other sequences such as the heterologous polynucleotide sequence, control elements, ITR, origin of replication, selectable markers, etc. In a particular embodiment, the filler or stuffer polynucleotide sequence is positioned between a 3' ITR and 5' ITR which flank the respective 3' end and 5' end of the heterologous polynucleotide sequence. For example, in the AAV vector plasmid, the filler or stuffer polynucleotide sequence is present in the vector genome portion of the recombinant vector plasmid and is therefore used for virus packaging/encapsidation. In another particular embodiment, the filler or stuffer polynucleotide sequence is positioned outside a 3' ITR and 5' ITR which flank the respective 3' end and 5' end of the heterologous polynucleotide sequence. For example, in the AAV vector plasmid, the filler or stuffer polynucleotide sequence is present in the backbone or plasmid portion of the recombinant vector plasmid. In yet another particular embodiment, the filler or

stuffer polynucleotide sequence is positioned within the heterologous polynucleotide sequence. For example, in the AAV vector plasmid, the filler or stuffer polynucleotide sequence positioned within the heterologous polynucleotide sequence is present in the vector genome portion of the recombinant vector plasmid and is therefore used for virus packaging/encapsidation.

Examples

Example 1

[0162]

Standard PCR was performed on plasmid DNA containing an AAV vector genome sequence as a positive control for PCR amplification (Figure 3, Panels A and B), and on vector genome DNA extracted from the purified vector preparation (Figure 2, Panels A and C, vector genome sizes are 2.7 Kb and 4.2 Kb, respectively) and on vector genome DNA extracted after treating the vector with DNase I (Figure 2, Panels B and D, vector genome sizes are 2.7 Kb and 4.2 Kb, respectively). A primer positioned within a transgene cassette (red circle in the Figure) was paired with a set of primers covering the sequence in the transgene cassette and the sequence in the flanking segment of the plasmid backbone containing the antibiotic resistance gene (KanR or AmpR), and was used for each PCR. PCR reactants were analyzed by 1% agarose/EtBr gel electrophoresis.

[0163]

Data for Figure 3, controls for PCR amplification, and plasmid DNA. When plasmid DNA was used as a template, PCR fragments were generated for all primer pairs (nine primer pairs, Panels A and B) suggesting that all primer pairs used in this study generate PCR products. All PCR products showed expected sizes as predicted based on plasmid sequences and primer positions. As expected, when the plasmid sample was treated with DNase I before PCR, PCR amplification was not observed (Panel C).

[0164]

Data for Figure 2. When PCR was performed on DNA extracted from a purified vector, only one set of primer pairs gave a PCR product in the experiment (six primer pairs for vectors having a short genome of 2.7 Kb (Panels A and B), and four primer pairs for vectors having a long genome of 4.2 Kb (Panels C and D)). The maximum size of PCR products (combined with the vector genome sequence outside PCR amplification) showed that the maximum size of the amplified DNA corresponded to the packaging capacity limit of AAV virus (4.5 Kb).

[0165]

Even when the vector preparation was treated with DNase I before extracting DNA from the vector (Figure 2, Panels B and D), no change in the PCR amplification pattern was observed (Figure 2, Panels A and C, compared to Panels B and D, respectively). That is, there was no change in the number of primer pairs generating PCR products and the size of PCR products, suggesting that sequences amplified in PCR (vector genome and flanking sequences) were protected from DNase, and encapcidated.

[0166]

The size of encapcidated DNA (e.g., DNase-resistant plasmid backbone DNA combined with vector genome size) was comparable between vectors having a short genome and vectors having a long genome, which approximately corresponded to the packaging capacity limit of AAV virus (4.5 Kb). DNA packaged in the vector having a short genome contained a plasmid sequence which flanks the genome, indicating that a vector having a short genome packaged a plasmid sequence with a size up to the maximum packaging capacity limit of AAV virus.

Example 2

[0167]

This working example includes a description on the generation and purification of recombinant AAV viruses by using a GMP-compliant manufacturing process.

[0168]

Residual plasmid DNA levels were evaluated in a series of AAV2 vectors containing single-stranded standard transgene expression cassettes in the following size ranges. Namely, rAAV A is 2.7 kb (57% of wild type size), rAAV B is 3.7 kb (83% of wild type), and rAAV C is 4.3 kb (91% of wild type). For each construct, multiple lots were generated and purified by using the same process. Vectors were generated by helper virus-free transfection of HEK293 cells and purified by combination of cation exchange chromatography (foros SOHS) and isOQVCnic cesium chloride ultracentrifugation.

[0169]

Concentration of residual plasmid DNA was measured as the number of KanR gene copies per ml of vector preparation and expressed as a % of vector genome (vg) or pg per 10^9 vg based on the assumption that the number of KanR (AmpR) copies represents the number of full-length plasmid copies (even in the worst case). Vector titer and concentration of residual plasmid DNA were measured by real-time quantitative PCR (qPCR) with TaqMan technology in accordance with the manufacturer's protocol using primers and probes specific to KanR or AmpR gene

positioned close to the 5' ITR of the transgene cassette in the transgene plasmid backbone.

[0170]

In DNase experiments, 1×10^6 copies of the vector genome or plasmid were digested with 5U of DNase 1. Amplification was performed by standard PCR using 500 copies of the vector genome or plasmid per PCR reaction. Plasmid DNA impurities in AAV vectors having a short transgene cassette were resistant to DNase, indicating that plasmid DNA fragments close to vector plasmid ITR were encapsidated.

[0171]

Residual plasmid DNA was quantified by a conservative method based on measured copy number values for target qPCR amplified products in vector samples. Namely, the copy number was multiplied by the Mr of the plasmid to calculate the residual plasmid DNA. The level of impurities as a function of transgene cassette size is summarized in Figure 1. The evaluation showed that rAAV A (size: 2.7 kb) contained 164 pg/10^9 vg (n = 9), rAAV B (size: 3.7 kb) contained 42.7 pg/10^9 vg (n = 32), and rAAV C (size: 4.3 kb) contained 14.0 pg/10^9 vg (n = 29) of residual plasmid DNA.

[0172]

Further, packaging of vector plasmid "backbone" DNA occurs to a substantial degree through "reverse packaging" from ITR, which is greatly reduced using an oversized (> 4.7 bp) backbone (Figure 5).

Example 3

[0173]

This working example includes a description on the study in which DNA impurities in vectors prepared without using an oversized backbone were compared to vectors prepared using an oversized backbone.

[0174]

A series of 12 batches of recombinant AAV vectors were prepared by using the same method, based on vector production by transient transfection of human embryonic kidney (HEK) 293 cells, in accordance with the following vector production and purification method.

Vector production in HEK293 cell culture:

1. Initiation of HEK293 Master Cell Bank cell culture in a T75 flask
2. Passage of cells into about two T225 flasks
3. Passage of cells into about two roller bottles
4. Passage of cells into about ten roller bottles

5. Passage of cells into about 102 roller bottles

6. Transfection of cells with plasmid DNA containing a vector plasmid having an oversized vector backbone or a vector plasmid without an oversized vector backbone

7. Exchange to serum-free medium

Vector purification (downstream process):

8. Harvest of vector-containing cells and culture fluid

9. Concentration and diafiltration of harvest by Tangential Flow Filtration (TFF) (100 kDa)

10. Microfluidization of concentrated harvest

11. Filtration of microfluidized intermediate product (0.65 μm/0.2 μm serial pore size)

12. Purification by ion exchange chromatography

13. Purification by cesium chloride isopycnic gradient ultracentrifugation

14. Buffer exchange by TFF (100 kDa)

15. Preparation of purified bulk vectors by formulation and 0.2 μm filtration

16. Preparation of vial-filled purified vectors by final 0.2 μm filtration, filling into vial, and finishing

[0175]

Samples of purified vectors from nine batches of vectors prepared using production plasmid vectors containing an oversized backbone and three batches of vectors prepared using production plasmid vectors without an oversized backbone were subjected to measurement of residual host cell plasmid DNA as determined by qPCR measurement of residual levels of ampicillin and kanamycin resistant genes which are not intended to be part of the purified vector product (and are hence impurities). The method used for measurement of these impurities is mentioned as follows.

Residual plasmid DNA by real-time qPCR

[0176]

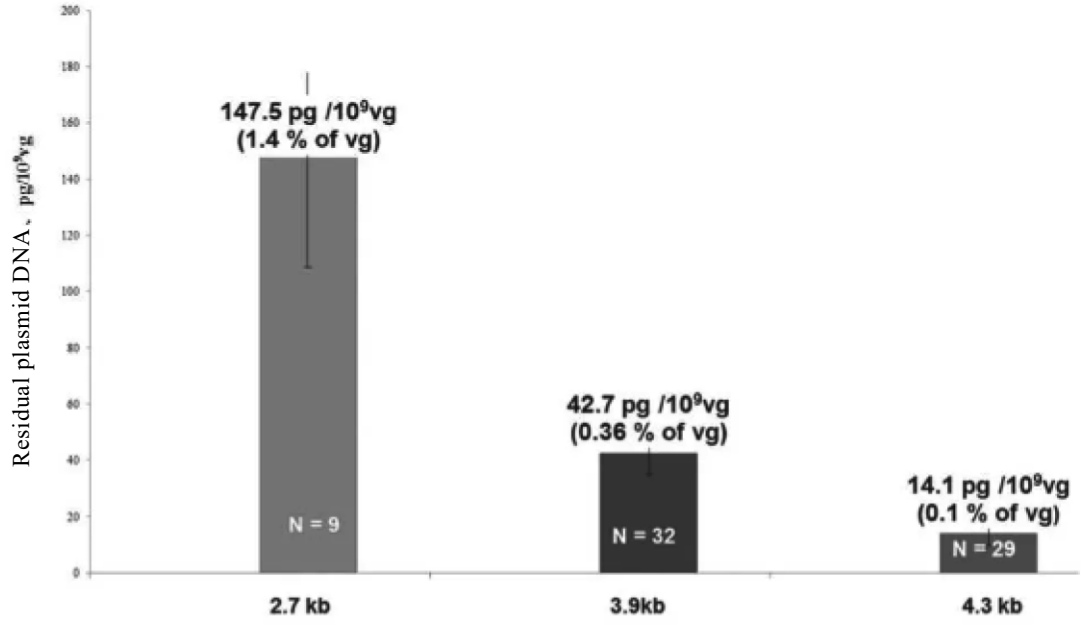
The mentioned TaqManR real-time qPCR procedure uses target-specific Q-PCR primers and probes to detect the specific sequences in the production plasmids (Amp^R or Kan^R) used for vector generation. In the case where one target was common for all plasmids used in vector manufacture, total residual plasmid was determined in a single qPCR assay. In the case where both Amp^R and Kan^R were present in one or more production plasmids for a given batch, the total residual plasmid was calculated as the sum of Amp^R residual DNA and Kan^R residual DNA, each of which was determined in a separate test. ...

[0177]

In the levels of residual plasmid DNA impurities obtained in the 12 quantification results shown in Figure 6, the average of plasmid DNA impurities per 10^9 vector genomes in vectors prepared using a vector plasmid without (lacking) an oversized backbone was 301 pg. This was five-fold higher than 60 pg, which was the average of plasmid DNA impurities per 10^9 vg measured in vectors prepared using an oversized backbone. Therefore, an oversized backbone in a vector plasmid can be used to reduce impurities in the preparation of viral vectors.

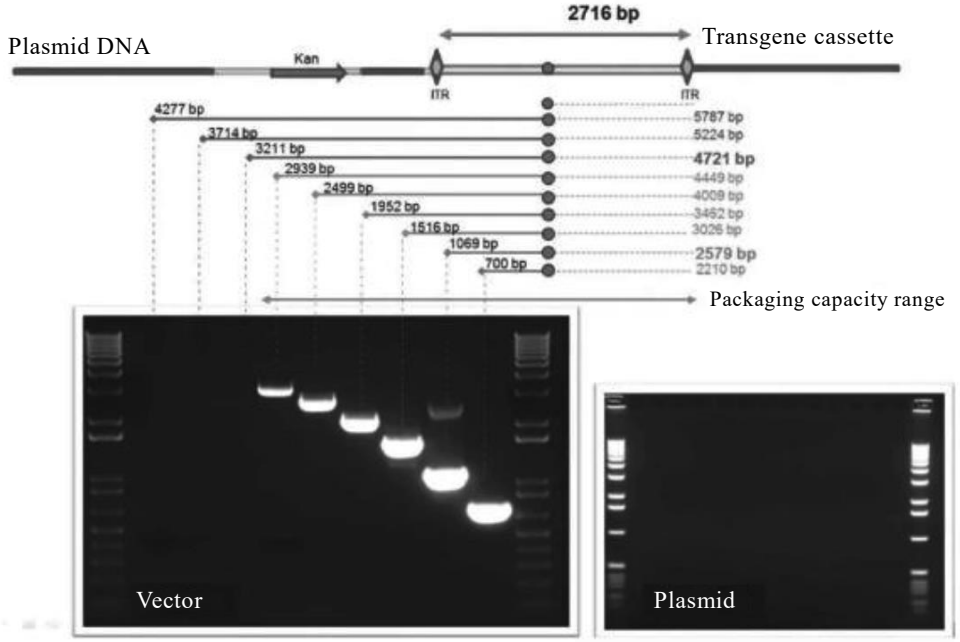
[Figure 1]

Correlation between residual plasmid DNA levels and rAAV vector genome sizes



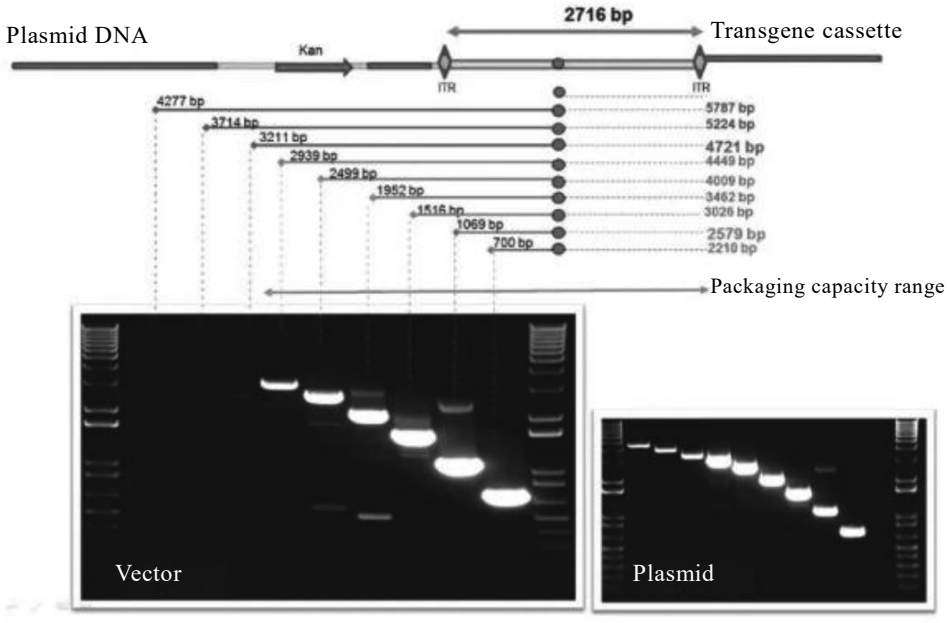
[Figure 2A]

PCR on a vector containing short (2.7 kb) transgene cassette before DNase treatment



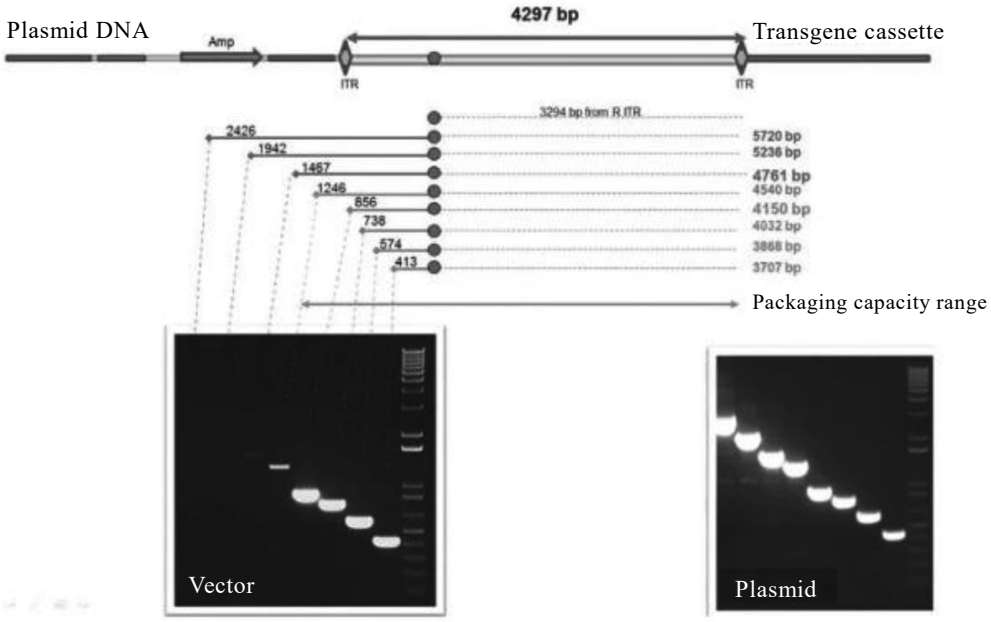
[Figure 2B]

PCR on a vector containing short (2.7 kb) transgene cassette after DNase treatment and DNA purification with High Pure Viral Nucleic Acid Kit (Roche)



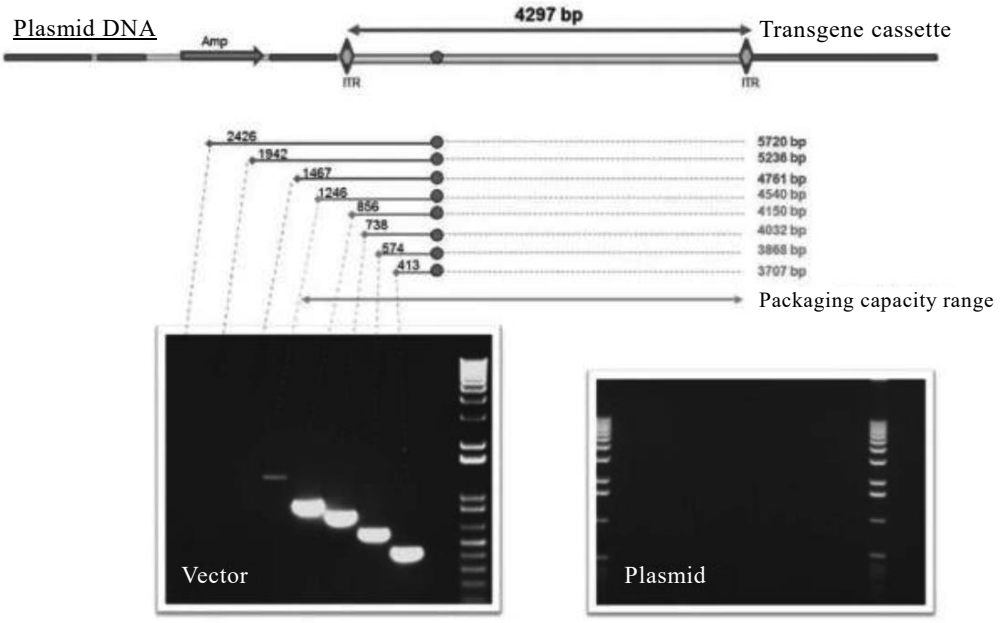
[Figure 2C]

PCR on a vector containing long (4.3 kb) transgene cassette before DNase treatment



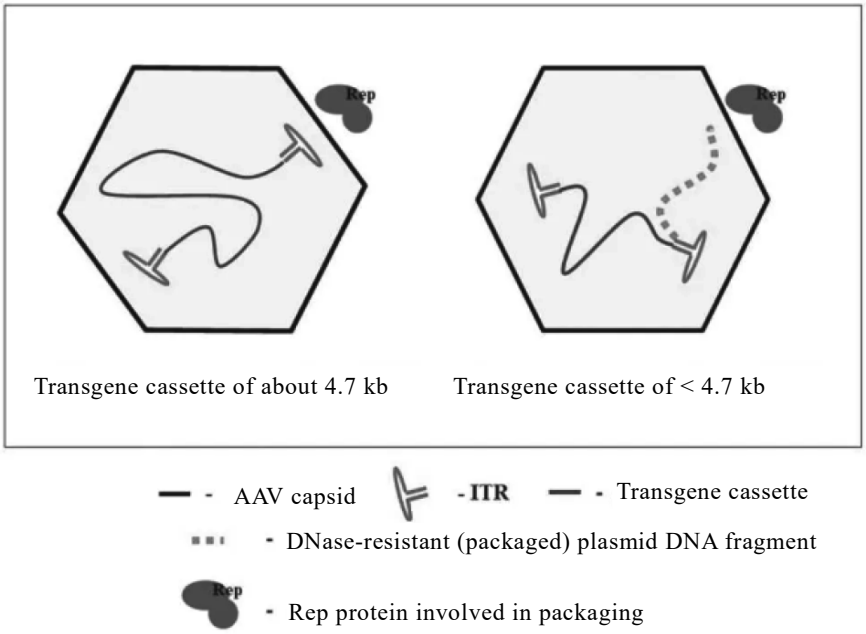
[Figure 2D]

PCR on a vector containing long (4.2 kb) transgene cassette after DNase treatment and DNA purification with High Pure Viral Nucleic Acid Kit (Roche)



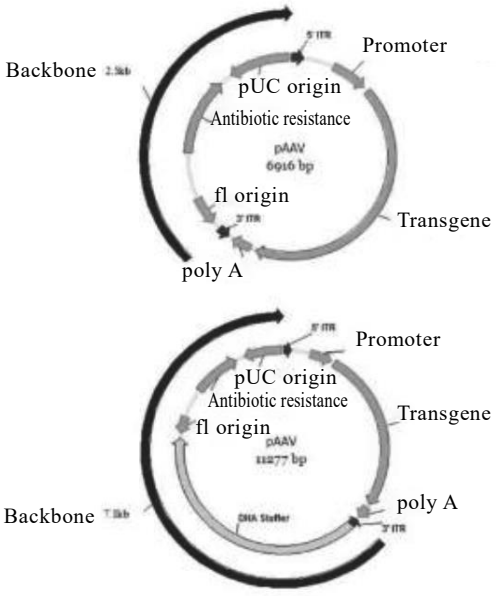
[Figure 4]

Diagram showing encapsidation of plasmid DNA in a vector containing short transgene cassette



[Figure 5]

Oversized plasmid backbone (7.1 Kb) exceeding the AAV packaging capacity limit markedly reduces non-vector DNA packaging.



Using various production systems:
- Transfection using pRC plasmid
- Transfection using pRG plasmid
- rAd infection using production cell lines

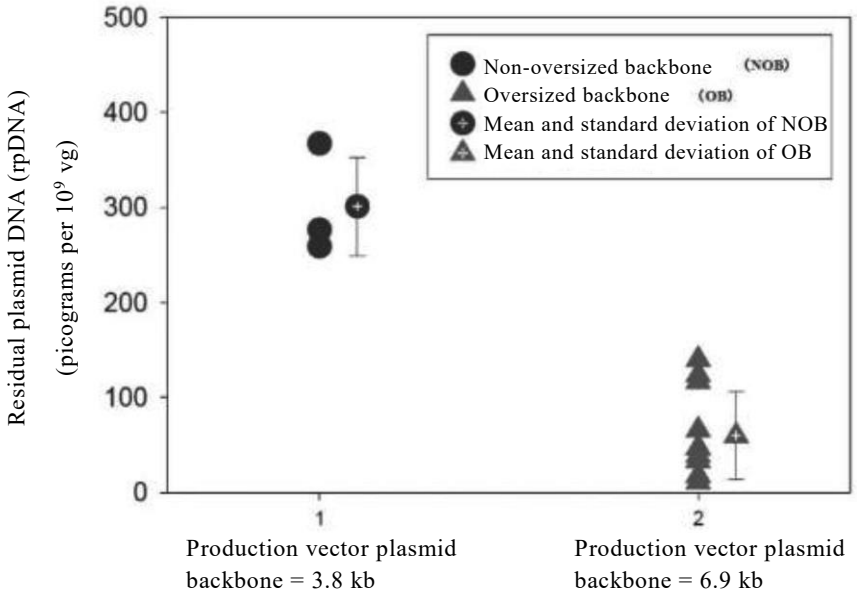
In all methods, marked packaging of cis plasmid backbone was observed. In vectors purified by the density gradient centrifugation method, these product-related impurities are 3 to 7% of vg-containing particles.
(It is likely to be higher in vectors purified without a step of density-based separation.)

(Chadeuf et al., (2005), MoL Ther, 12:744)

Using an oversized backbone reduces residual plasmid DNA by about 40-fold (0.1 to 1.0%).

[Figure 6]

Residual plasmid DNA in purified AAV vectors produced using vector plasmids containing non-oversized[1] (red) versus oversized (green) backbones



[1] Oversized; i.e., a size exceeding the packaging capacity limit of adeno-associated virus (about 4.7 kb).

(Attachment 2)

(1-1)

"... In order to minimize encapsidated DNA impurities in clinical vectors, the following two methods were used: (i) a plasmid for vector (cis) production having a backbone exceeding the packaging limit of AAV; and (ii) a vector purification step which achieves separation of the vector from vector-related impurities (example: empty capsids). In conclusion, residual cap expression was not detected after transfer with AAV2-hFIX clinical vectors."(page 1, left column, lines 14 to 20)

(1-2)

"Influence of vector plasmid backbone size on levels of DNA impurities

Previous studies have reported that encapsidated plasmid DNA impurities are primarily derived from the backbone of the plasmid for ITR-containing vector (cis) production. An influence of vector plasmid backbone size on an amount of residual plasmid DNA impurities in recombinant AAV2 and AAV6 was assessed. Residual plasmid DNA levels were measured by Q-PCR using primers and probes specific to Amp^R, which is a sequence common to all three production plasmids used for vector generation (Table 1). The vectors compared in this experiment were each generated and purified by using a common method (chromatography-gradient). Thus, vector purity was high, empty capsids were removed, and an efficient nuclease digestion step which removed non-encapsidated nucleic acid impurities was included. Average residual plasmid DNA levels were measured in five lots produced using vector plasmids. As a result, the plasmid having an oversized (6980 bp) backbone (Lots 06002, 003A, NHP, 0802, 0803) was 14.2±2.6 pg/10^9 vg, which was 7.6-fold lower ($P < 0.001$) than an average value of 107.6±27.6 pg/10^9 vg measured in five lots produced using the vector plasmid having smaller (2620 bp or 2638 bp) backbones (Lots N0701, 0701, 0702, 0703, 0801). Therefore, using the oversized backbone in the plasmid for vector production achieved a great reduction in plasmid-derived DNA impurities." (page 146, right column, lines 17 to 40)

(1-3)

Table 1 Levels of DNA impurities contained in purified AAV vectors

Vector Lot #	Purification method	Serotype	Transgene/size (nt)	Backbone/size (nt) [a]	293DNA (pg/10^9vg)	Plasmid DNA (pg/10^9vg)
1053	grad-only[b]	2	hFIX/4297	6,980	–	8.6
DCL 1	gen1-Chr[c]	2	hFIX/4297	6,980	208	44.8
DCL 2	gen1-Chr	2	hFIX/4297	6,980	127	24.2
DCL 3	gen1-Chr	2	hFIX/4297	6,980	122	30.1
DCL 4	gen1-Chr	2	hFIX/4297	6,980	89	37.9
06002	chr-grad[d]	2	hFIX/4297	6,980	27	11
003A	chr-grad	2	hFIX/4297	6,980	20.9	13.0
NHP	chr-grad	2	hFIX/4297	6,980	38.5	13.3
0802	chr-grad	6	hFIX/4297	6,980	7.5	15.8
0803	chr-grad	6	hFIX/4297	6,980	6.3	17.7
N0701	chr-grad	6	U/4679	2,638	16.4	122
0701	chr-grad	6	U/4679	2,638	10.1	77.6
0702	chr-grad	6	D/4811	2,620	12.8	103
0703	chr-grad	6	D/4811	2,620	8.7	147
0801	chr-grad	2	cFIX/4406	2,620	16.9	88.3

Abbreviations: AAV, adeno-associated virus; vg, vector genome.
[a] backbone size of plasmids for vector (cis) production. [b] gradient-only purification method.
[c] gen1-chromatography purification method. [d] chromatography-gradient purification method.

(Page 147, Table 1)

(1-4)

"In the current study, when AAV vectors were produced by gene transfer (transfection) of HEK293 cells using a vector plasmid having a backbone smaller than the packaging limit of AAV, nuclease-resistant plasmid DNA impurities contained in the resulting vectors were 2.9 to 5.7%, which was consistent with previous reports by others. The major source of the encapsidated plasmid DNA was the backbone of ITR-containing vector plasmid.

...

An effect of using a vector plasmid in which a stuffer sequence was modified in such a way that the backbone exceeded the packaging capacity of AAV2 was investigated, and it was found that this strategy greatly reduced the levels of encapsidated plasmid DNA impurities (7.6-fold, $P < 0.001$). For vector serotypes with packaging capacities higher than AAV2, the size of the backbone required to prevent reverse packaging is predicted to be correspondingly larger." (page 149, right column, line 10 from the bottom to page 150, left column, line 8).

(1-5)

"Materials and Methods

AAV generation and purification. Vector generation was performed by helper

virus-free gene transfer of HEK293 cells using three modified plasmids. The clinical AAV2-hFIX vector Lot 1053 was purified by the twice cesium chloride gradient ultracentrifugation method (gradient-only method). In this study, 14 lots of additional AAV vectors were produced and analyzed. Eight lots of AAV2 vectors and two lots of AAV6 vectors which express human coagulation factor IX (AAV-hFIX) were produced using a vector plasmid containing a 6980 bp backbone exceeding the AAV packaging limit of about 4700 nt. One lot of AAV2 and four lots of AAV6 were produced using vector plasmids having backbones of 2620 bp or 2638 bp." (page 150, left column, line 11 from the bottom to line 1 from the bottom)