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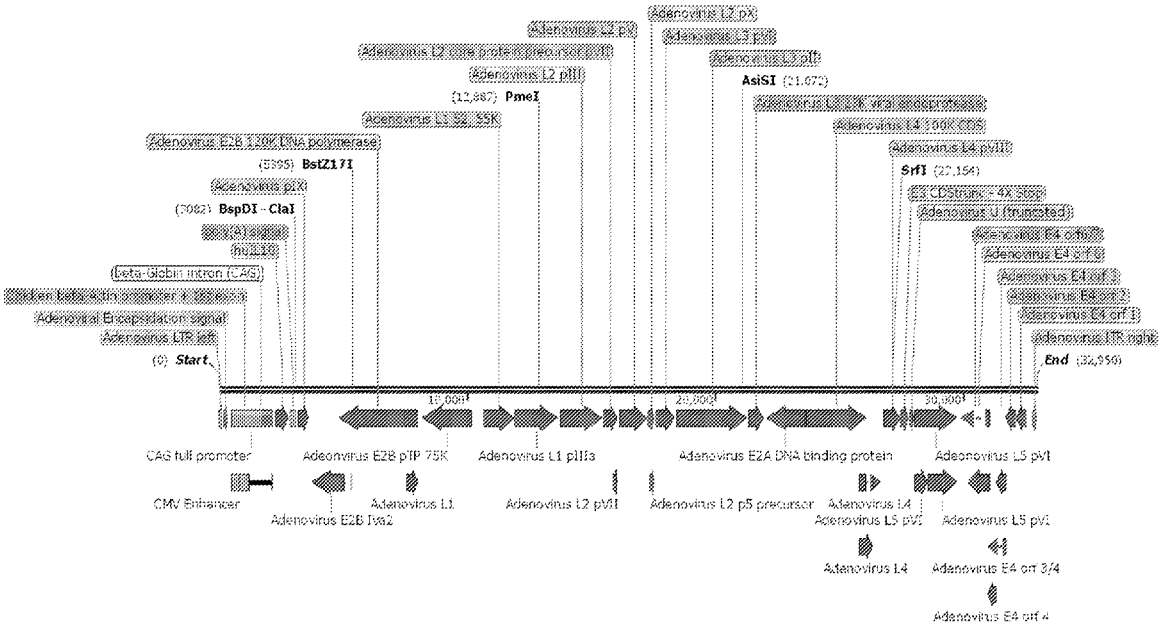


FIG. 1

(57) Abstract: An adenovirus expression vector is provided. The adenovirus expression vector may include: a) one or more mutations that render the adenovirus replication incompetent and b) at least one nucleotide sequence encoding a protein or an RNA is provided. A method of synthesizing an adenovirus vector is also provided. The synthesis may include: a) producing a plurality of overlapping adenovirus sub-fragments, each sub -fragment comprising a portion of the full genome of the adenovirus; b) circularizing the sub -fragments to form plasmid structures; and c) assembling the circularized sub-fragments into a linear structure, wherein the vector comprises a combination of two or more sub -fragments. A mammalian cell line configured to replicate adenoviral vectors, wherein the cell line comprises nucleotide sequences expressing El A and E1B gene products but is devoid of other adenovirus sequences is also provided.

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ADENOVIRAL EXPRESSION VECTOR AND METHODS AND CELL LINES FOR PRODUCTION

CROSS-REFERENCE TO RELATED APPLIATIONS

[0001] This application claims priority to U.S. Provisional Application No. 63/006,266 filed April 7, 2020 the entire contents of which are incorporated herein.

FIELD OF USE

[0002] This application relates generally to adenoviral vectors and gene therapy, and, more specifically, to non-replicating adenovirus expression vectors, methods of synthesis, and modes of replication.

BACKGROUND

[0003] Adenoviral vector nucleotide sequences are often many kilobases long, which makes *in vitro* manipulation less convenient and harder to achieve. Manipulation of adenoviral vectors, if done improperly, can also leaded to unchecked replication of the vector. Moreover, common mammalian cell lines used to produce adenovirus often possess endogenous copies of adenoviral genes, which can recombine with mutant adenoviral vectors and create infectious replicating viruses. Thus, there exists a need for safe, non-infectious, recombinant, non-replicating adenoviral vectors with effective expression cassettes for therapeutic use. It would also be desirable to provide a protocol for more efficient production of these recombinant vectors in bacteria, overcoming the difficulties in manipulating large adenoviral sequences. Finally, it would further be desirable to provide a mammalian cell line for replication of the engineered adenoviral vector to eliminate possible contamination of a replication competent adenovirus.

SUMMARY

[0004] The present disclosure provides an adenovirus vector possessing a) one or more mutations that render it replication incompetent, and b) carrying a nucleotide expression cassette

that allows expression of therapeutics when transduced into host cells. Sequences of the engineered adenovirus vectors are disclosed herein. Protocols and methods for more efficient production in bacteria of the synthesized adenovirus vectors are also provided herein. Finally, creation of an engineered mammalian cell line for replication of the engineered adenoviral vector to eliminate possible contamination of replication competent adenovirus is produced herewith.

[0005] The non-replicating adenovirus expression vector may include a) one or more mutations that render the adenovirus replication incompetent and b) at least one nucleotide sequence encoding a protein or an RNA. In some embodiments, the vector comprises a nucleotide sequence having at least 80% sequence identity to any of SEQ ID NOs: 1-2. SEQ ID NO: 1 encodes a full vector with plasmid backbone. SEQ ID NO: 2 encodes a full adenoviral vector of the present disclosure without a plasmid backbone. In certain preferred embodiments, the vector is an adenovirus serotype 5 vector. The mutations may comprise deletion of either an E1 gene or an E3 gene or both. At least one nucleotide sequence in the non-replicating adenovirus expression vector may be a transgene. In certain embodiments, the transgene expresses human IL10. In some embodiments, the transgene further comprises at least one of an enhancer/promoter region including CAG, human IL10 cDNA, or a polyadenylation signal, or any combination thereof. The polyadenylation signal further may be an SV40 region and/or a full polyA signal. The polyadenylation signal may be inserted, in some embodiments, between nucleotides 440 and 3515 in SEQ ID NO: 3. SEQ ID NO: 3 may be derived from accession number AY3339865 in the GenBank database.

[0006] Insertion of the transgene in the adenoviral vector may replace the E1 gene but leaves the pIX gene intact. In some aspects of the disclosure, the recombinant vector can include a nucleic acid sequence having at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, or greater than 99% sequence identity to SEQ ID NOs: 1-2.

[0007] A method of synthesizing an adenovirus vector is also disclosed herein. The method may include: a) producing a plurality of overlapping adenovirus sub-fragments, each sub-fragment including a portion of the full genome of the adenovirus; b) circularizing the sub-fragments to form plasmid structures; c) assembling the circularized sub-fragments into a linear structure, wherein the vector includes a combination of two or more sub-fragments. In some

aspects of the method, the adenovirus vector may be adenovirus serotype 5. In certain embodiments, the vector may replicate at high efficiency.

[0008] A first sub-fragment may include at least one sequence as set forth in SEQ ID NOs: 4-5. In some embodiments, a second sub-fragment may include at least one sequence as set forth in SEQ ID NOs: 6-7.

[0009] In some embodiments, each sub-fragment may include about 50% of the full adenovirus genome. At least one fragment may include a transgene that expresses human IL-10. The transgene further may include at least one of an enhancer/promoter region, human IL10 cDNA, or a polyadenylation signal, or any combination thereof. The enhancer or promoter region may be a CAG promoter. The transgene further may include at least a polyadenylation signal comprising an SV40 promoter or a full poly(A) signal. The vector sequence also may have a linear cloning vector and or a backbone linear cloning vector. In certain embodiment, the linear cloning vector may be pJazz-OK. The human IL10 cDNA sequence may be optimized and encoded from SEQ ID NO: 8.

[0010] A mammalian cell configured to replicate adenoviral vectors, wherein the cell line comprises nucleotide sequences expressing E1A and E1B gene products but is devoid of other adenovirus sequences is also provided herein. The cell line further may comprise a lentiviral vector engineered to express the E1A and E1B gene products, wherein the lentiviral vector inhibits reconstitution of functional adenovirus when a mutant adenoviral viral vector is introduced into the cell line. In some embodiments, the cell line may comprise HeLa cells. In some embodiments, the cell line is produced by transfecting HEK293 cells with a lentiviral genome plasmid comprising at least an E1A encoding sequence and an E1B-encoding sequence.

[0011] The plasmid further may comprise at least an E1A encoding sequence and an E1B-encoding sequence contains E1A and E1B sequences arranged in a bidirectional expression unit with a bidirectional promoter to prevent recombination with a mutant adenoviral vector. In some aspects of the cell line, the bidirectional promoter may be a combination of truncated CMV and PGK promoters.

[0012] The cell line further can include a puromycin drug resistance gene in the lentiviral genome sequence to differentiate cells which have integrated the lentiviral sequence when the puromycin drug resistance gene is expressed. In some embodiments, a final vector of SEQ ID NO: 9 is used to produce lentivirus and HeLa cells, transduced and selected for puromycin resistance.

[0013] A method of producing and/or expanding the synthetic non-replicating adenoviral vector disclosed above is also provided herein. The method may include transforming a mammalian cell with an adenovirus vector comprising: a) one or more mutations that render the adenovirus replication incompetent and b) at least one nucleotide sequence encoding human IL-10, wherein the mammalian cell comprises nucleotide sequences expressing E1A and E1B gene products but is otherwise devoid of other adenovirus sequences, culturing the transformed mammalian cell in a cell line containing an inserted lentiviral E1A and E1B vector, and isolating the adenovirus vector.

[0014] In a further aspect of the disclosure, a method of treating a subject in need of a gene product is also provided herein. In some embodiments, the method may comprise, consist essentially of, or further, consist of administering to the subject a vector comprising (a) one or more mutations that render the adenovirus replication incompetent and (b) at least one nucleotide sequence encoding the gene product. In some embodiments of the method, the vector comprises a nucleotide sequence having at least 80% sequence identity over the entire sequence of SEQ ID NO: 1 or SEQ ID NO: 2, or a complementary sequence thereto. In certain embodiments, the vector may be an adenovirus serotype 5 vector. Additionally or alternatively, the one or more mutations may be a deletion of either an E1 gene or an E3 gene or both. The at least one nucleotide sequence further may include a transgene. In some embodiments, the transgene expresses human IL10.

[0015] In some embodiments of the method, the subject may be a human. In certain embodiments, the gene product is a peptide, including human peptides. In some embodiments, the gene product can be RNA.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] FIG. 1 is a schematic diagram of the genomic organization of a full non-replicating, adenoviral vector of the present disclosure, without a plasmid backbone.

[0017] FIG. 2 is a schematic diagram of the genome organization of a non-replicating, adenoviral vector of the present disclosure, with a plasmid backbone.

[0018] FIG. 3 is a schematic diagram of the genomic organization of an exemplary first sub-fragment in linear form.

[0019] FIG. 4 depicts a schematic diagram of the genomic organization of an exemplary second sub-fragment in linear form.

[0020] FIG. 5 shows a schematic diagram of the genomic organization of an exemplary first sub-fragment in circularized form.

[0021] FIG. 6 shows a schematic diagram of the genomic organization of an exemplary first second-fragment in circularized form.

[0022] FIG. 7 shows a schematic diagram of genomic organization of the final vector combining a first sub-fragment and a second sub-fragment.

[0023] FIG. 8 is a schematic diagram of genomic organization of a vector used to produce lentivirus and HeLa cell lines transduced and selected for puromycin resistance.

[0024] FIG. 9 is a flowchart of a disclosed production process of safe, replication incompetent adenoviral serotype 5 vectors.

[0025] FIG. 10 shows a schematic diagram of the full E1 cell creation vector with a plasmid backbone.

DETAILED DESCRIPTION

[0026] Definitions

[0027] As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the content clearly dictates otherwise. For example, reference to "a cell" includes a combination of two or more cells, and the like.

[0028] As used herein, the term "about" encompasses the range of experimental error that may occur in a measurement and will be clear to the skilled artisan.

[0029] As used herein, "adenovirus" means a medium-sized (90-100 nm), nonenveloped polyhedral virus that may include a capsid and a double-stranded linear DNA genome. The adenovirus can be a naturally occurring, but isolated, adenovirus or a recombinant adenovirus, or a chimeric variant thereof.

[0030] As used herein, "administering" means a method of giving a dosage of a pharmaceutical composition (*e.g.*, a recombinant adenovirus of the invention) to a subject. The compositions utilized in the methods described herein can be administered, for example, intramuscularly, intravenously, intradermally, percutaneously, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostatically, intrapleurally, intratracheally, intranasally, intravitreally, intravaginally, intrarectally, topically, intratumorally, peritoneally, subcutaneously, subconjunctivally, intravesicularlly, mucosally, intrapericardially, intraocularly, orally, topically, locally, by inhalation, by injection, by infusion, by continuous infusion, by localized perfusion bathing target cells directly, by catheter, by lavage, by gavage, in crèmes, or in lipid compositions. The preferred method of administration can vary depending on various factors (*e.g.*, the components of the composition being administered and the severity of the condition being treated).

[0031] Throughout this specification and claims, the word "comprise," or variations such as "comprises" or "comprising," will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

As used herein, "protein" includes peptides, polypeptides, or proteins of any length and any secondary or tertiary structure.

[0032] As used herein, "gene product" is meant to include mRNAs, other nucleic acids (*e.g.*, microRNAs) transcribed from a gene as well as proteins, including polypeptides, translated from

those mRNAs. A gene product can be a soluble protein, chemokine, cytokine, soluble receptor, an antibody, antibody fragments, an antibody-like molecule, or enzyme. For example, the gene product can be an antigen-binding fragment of an antibody, or the gene product can be some or all of the variable portion of IgG or IgM. In some embodiments, the gene product can be an antibody fragment lacking the Fc domain. The gene product can comprise or consist of a complementarity-determining region (CDR). In some embodiments, the gene product can be an anti-IL6 antibody or antibody fragment. In some embodiments, the gene product is from a virus. In some embodiments, the gene product is a therapeutic gene product, including, but not limited to, the interleukins (*e.g.*, human IL-10), interferon proteins, Factor VIII, Factor IX, erythropoietin, alpha-1 antitrypsin, calcitonin, glucocerebrosidase, growth hormone, low density lipoprotein (LDL), receptor IL-2 receptor and its antagonists, insulin, globin, immunoglobulins, catalytic antibodies, insulin-like growth factors, superoxide dismutase, immune responder modifiers, parathyroid hormone and interferon, nerve growth factors, tissue plasminogen activators, and colony stimulating factors.

[0033] By "portion" or "fragment" is meant a part of a whole. A portion may comprise at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% of the entire length of an polynucleotide or polypeptide sequence region. For polynucleotides, for example, a portion may include at least 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 15000, 20000, 25000, 30000, 35000 or more contiguous nucleotides of a reference polynucleotide molecule. For polypeptides, for example, a portion may include at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 50, 75, 90, 100, 125, 150, 175, 200, 225, 250, 275, 300, or 350 or more contiguous amino acids of a reference polypeptide molecule.

[0034] As used herein, "sequence identity" or "sequence similarity" means that the identity or similarity between two or more amino acid sequences, or two or more nucleotide sequences, is expressed in terms of the identity or similarity between the sequences. Sequence identity can be measured in terms of "percentage (%) identity," wherein the higher the percentage, the more identity shared between the sequences. Sequence similarity can be measured in terms of percentage similarity (which takes into account conservative amino acid substitutions); the higher the percentage, the more similarity shared between the sequences. Homologs or orthologs

of nucleic acid or amino acid sequences possess a relatively high degree of sequence identity/similarity when aligned using standard methods. Sequence identity may be measured using sequence analysis software on the default setting. Such software may match similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications.

[0035] A "subject" is a vertebrate, such as a mammal (*e.g.*, primates and humans). Mammals also include, but are not limited to, farm animals (such as cows), sport animals (*e.g.*, horses), pets (such as cats and dogs), mice, and rats. A subject to be treated according to the methods described herein (*e.g.*, a subject having a disease such as cancer and/or a disease caused by an infective agent, *e.g.*, a bacterium, virus, fungus, or parasite) may be one who has been diagnosed by a medical practitioner as having such a condition. Diagnosis may be performed by any suitable means. A subject in whom the development of an infection or disease (or disease symptoms) is being prevented may or may not have received such a diagnosis. One skilled in the art will understand that a subject to be treated according to the present invention may have been subjected to standard tests or may have been identified, without examination, as one at high risk due to the presence of one or more risk factors (*e.g.*, exposure to a biological agent, such as a virus).

[0036] As used herein, "vector" refers to a composition that includes one or more genes (non-structural or structural), or fragments thereof, from a viral species, such as an adenoviral species (*e.g.*, sAd5, sAd1, etc.), that may be used to transmit one or more heterologous genes from a viral or non-viral source to a host or subject. The nucleic acid material of the viral vector may be encapsulated, *e.g.*, in a lipid membrane or by structural proteins (*e.g.*, capsid proteins), that may include one or more viral polypeptides (*e.g.*, a glycoprotein). The viral vector can be used to infect cells of a subject, which, in turn, promotes the translation of the heterologous gene(s) of the viral vector into a protein product.

[0037] The term "virus," as used herein, is defined as an infectious agent that is unable to grow or reproduce outside a host cell and that infects mammals (*e.g.*, humans) or birds.

[0038] Other features and advantages of the invention will be apparent from the following Detailed Description, the drawings, and the claims.

Adenoviral Polynucleotides of the Disclosure

[0039] The complete genome sequences of novel adenoviral vectors are disclosed below. The adenovirus 5 genome is over thirty-five kilobases in size, making in vitro manipulation less convenient. Thus, synthetic DNA methods were used to generate two or more small fragments to allow for higher efficiency molecular cloning using bacterial hosts. The final, non-replicating adenovirus expression vector may include a) one or more mutations that render the adenovirus replication incompetent and b) at least one nucleotide sequence encoding a protein or an RNA.

[0040] FIG. 1 is a schematic diagram of the genome organization of a non-replicating, adenoviral vector of the present disclosure, without a plasmid backbone. FIG. 2 is a schematic diagram of the genome organization of a non-replicating, adenoviral vector of the present disclosure, with a plasmid backbone. In some aspects of the disclosure, the vector comprises a nucleotide sequence having at least 80% sequence identity to SEQ ID NOs: 1-2. In some aspects of the disclosure, the recombinant vector can include a nucleic acid sequence having at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, greater than 99%, or any value therebetween sequence identity to SEQ ID NOs: 1-2.

[0041] In certain preferred embodiments, the vector is an adenovirus serotype 5 vector. The mutations may comprise deletion of either an E1 gene or an E3 gene or both, to prevent adenoviral replication *in vivo*. At least one nucleotide sequence in the non-replicating adenovirus expression vector may be a transgene. In certain embodiments, the transgene expresses human IL10. In some embodiments, the transgene further comprises at least one of an enhancer/promoter region including CAG, human IL10 cDNA, or a polyadenylation signal, or any combination thereof. The polyadenylation signal further may be an SV40 region and/or a full polyA signal. The polyadenylation signal may be inserted, in some embodiments, between nucleotides 440 and 3515 in SEQ ID NO: 3. SEQ ID NO: 3 may be derived from accession number AY3339865 in the GenBank database. In certain preferred embodiments, a transgene for expression of human IL10 is included in the adenoviral sequence, which further includes an enhancer/promoter region (CAG), human IL10 cDNA, and a polyadenylation signal (SV40, full polyA signal), which is inserted between nucleotide 440 and 3515 relative to Adenovirus

sequence (GenBank accession number AY339865), replacing the E1 genes but leaving the pIX gene intact.

[0042] Sequences encoding an inserted transgene in the adenoviral vector may include a variety of gene products, and RNA or DNA transcribed therapeutics, including, but not limited to, interferon (IFN) proteins, Factor VIII, Factor IX, erythropoietin, alpha-1 antitrypsin, calcitonin, glucocerebrosidase, growth hormone, low density lipoprotein (LDL), receptor IL-2 receptor and its antagonists, insulin, globin, immunoglobulins, catalytic antibodies, the interleukins, insulin-like growth factors, superoxide dismutase, immune responder modifiers, parathyroid hormone and interferon, nerve growth factors, tissue plasminogen activators, and/or colony stimulating factors, or fragments thereof.

[0043] The engineered adenoviral vector may include any nucleotide (DNA or RNA) sequence configured for inclusion with the expression cassette. Exemplary transgenes that may be inserted into the adenoviral construct include, but are not limited to, Angiotensin-converting enzyme 2 precursor (ACE2) antibodies (SEQ ID NO: 14 [no plasmid backbone], SEQ ID NO: 15 [with plasmid backbone]), chimeric monoclonal antibodies such as Unitixin scFv (SEQ ID NO: 16 [no plasmid backbone], SEQ ID NO: 17 [with plasmid backbone]), transforming growth factor (TGF) beta res IL-10 (SEQ ID NO: 18 [with plasmid backbone], SEQ ID NO: 19 [no plasmid backbone]), interleukin 13 (SEQ ID NO: 20 [no plasmid backbone], SEQ ID NO: 21 [with plasmid backbone]), interleukin 8 with short hairpin RNA (shRNA) (SEQ ID NO: 22 [with plasmid backbone], SEQ ID NO: 23 [no plasmid backbone]), IL-1 receptor agonist 2 and IL-10 (SEQ ID NO: 24 [with plasmid backbone], SEQ ID NO: 25 [no plasmid backbone]), IL-1 receptor agonist (SEQ ID NO: 26 [with plasmid backbone], SEQ ID NO: 27 [no plasmid backbone]), endothelial nitric oxide synthase (eNOS) (SEQ ID NO: 28 [with plasmid backbone], SEQ ID NO: 29 [no plasmid backbone]), cystic fibrosis transmembrane conductance regulator (CFTR) (SEQ ID NO: 30 [with plasmid backbone], SEQ ID NO: 31 [no plasmid backbone]), Beta-1,4-galactosyltransferase 1 (B4GALT1) enzymes (SEQ ID NO: 32 [with plasmid backbone], SEQ ID NO: 33 [no plasmid backbone]).

Protocol for More Efficient Adenoviral Production in Bacteria

[0044] A method of synthesizing an adenovirus vector is also disclosed herein. The method may include: a) producing a plurality of overlapping adenovirus sub-fragments, each sub-fragment including a portion of the full genome of the adenovirus; b) circularizing the sub-fragments to form plasmid structures; c) assembling the circularized sub-fragments into a linear structure, wherein the vector includes a combination of two or more sub-fragments. In some aspects of the method, the adenovirus vector may be adenovirus serotype 5. In certain embodiments, the vector may replicate at high efficiency.

[0045] A first sub-fragment may include at least one sequence as set forth in SEQ ID NOs: 4-5. In some embodiments, a second sub-fragment may include at least one sequence as set forth in SEQ ID NOs: 6-7. FIG. 3 depicts a schematic diagram of the genomic organization of an exemplary first sub-fragment in linear form. FIG. 4 depicts a schematic diagram of the genomic organization of an exemplary second sub-fragment in linear form. FIG. 5 shows a schematic diagram of the genomic organization of an exemplary first sub-fragment in circularized form and FIG. 6 shows a schematic diagram of the genomic organization of an exemplary first second-fragment in circularized form.

[0046] FIG. 7 shows a schematic diagram of genomic organization of the final vector combining a first sub-fragment and a second sub-fragment. Here it is shown that the final adenovirus serotype 5 genome may be assembled from the plurality of circularized fragments, which creates a linear structure for high level DNA replication in bacteria used in viral production. The high level of DNA replication and design allow for greater amounts of DNA to use for transfection and first round viral production. This is particularly useful when manipulating larger adenoviral vectors, such as the serotype 5 adenoviral vector, which is over 35 kilobases in size.

[0047] In some embodiments, each sub-fragment may include about 50% of the full adenovirus genome. At least one fragment may include a transgene that expresses human IL-10. The transgene further may include at least one of an enhancer/promoter region, human IL10 cDNA, or a polyadenylation signal, or any combination thereof. The enhancer or promoter region may be a CAG promoter. The transgene further may include at least a polyadenylation signal comprising an SV40 promoter or a full poly(A) signal. The vector sequence also may have a

linear cloning vector and or a backbone linear cloning vector. In certain embodiments, the linear cloning vector may be pJazz-OK (*see, e.g., BigEasy® v2.0: Linear Cloning Kits*, LUCIGEN CORPORATION, Jan. 1, 2018), hereby incorporated by reference). The teachings of the human IL10 cDNA sequence may be optimized and encoded from SEQ ID NO: 8.

Engineered Mammalian Cell Line for Replication of the Engineered Adenoviral Vector

[0048] A mammalian cell configured to replicate adenoviral vectors, wherein the cell line comprises nucleotide sequences expressing E1A and E1B gene products but is devoid of other adenovirus sequences is also provided herein.

[0049] The adenoviral vectors disclosed above may have been mutated to remove the viral E1 and E3 genes in order to prevent the viruses from replicating and thus being infectious. Mammalian cell lines supplied with the two E1 gene products, E1A and E1B, can support replication of the mutant virus. However, a common mammalian cell line used to produce adenovirus, HEK293, possesses an endogenous copy of the adenoviral E1 gene, which can recombine with mutant adenoviral vector and recreate infectious replicating virus. Therefore, a novel cell line, devoid of adenoviral sequences, is provided herein to express the E1A and E1B gene products using a vector which does not allow reconstitution of functional adenovirus when the mutant viral vector is introduced.

[0050] The cell line may comprise a lentiviral vector engineered to express the E1A and E1B gene products, wherein the lentiviral vector inhibits reconstitution of functional adenovirus when a mutant adenoviral viral vector is introduced into the cell line. In some embodiments, the cell line may comprise HeLa cells. In some embodiments, the cell line is produced by transfecting HEK293 cells with a lentiviral genome plasmid comprising at least an E1A encoding sequence and an E1B-encoding sequence.

[0051] The plasmid further may comprise at least an E1A encoding sequence and an E1B-encoding sequence contains E1A and E1B sequences arranged in a bidirectional expression unit with a bidirectional promoter to prevent recombination with a mutant adenoviral vector. In some aspects of the cell line, the bidirectional promoter may be a combination of truncated CMV and PGK promoters.

[0052] The cell line further can include a puromycin drug resistance gene in the lentiviral genome sequence to differentiate cells which have integrated the lentiviral sequence when the puromycin drug resistance gene is expressed. In some embodiments, a final E1 cell creation vector of SEQ ID NO: 9 is used to produce lentivirus and HeLa cells, transduced and selected for puromycin resistance. FIG. 8 is a schematic diagram of genomic organization of a vector used to produce lentivirus and HeLa cell lines transduced and selected for puromycin resistance.

[0053] The polynucleotide sequence of SEQ ID NO: 9 does not include a plasmid backbone. SEQ ID NO: 10, however, encodes a full E1 cell creation vector with a plasmid backbone, as depicted in the schematic diagram of the plasmid genome in FIG. 10.

[0054] A method of producing and/or expanding the synthetic non-replicating adenoviral vector disclosed above is also provided herein. The method may include transforming a mammalian cell with an adenovirus vector comprising: a) one or more mutations that render the adenovirus replication incompetent and b) at least one nucleotide sequence encoding human IL-10, wherein the mammalian cell comprises nucleotide sequences expressing E1A and E1B gene products but is otherwise devoid of other adenovirus sequences, culturing the transformed mammalian cell in a cell line containing an inserted lentiviral E1A and E1B vector, and isolating the adenovirus vector.

[0055] In some embodiments, the engineered adenoviral serotype 5 vector of the disclosure is introduced into engineered cells in order to generate mutant adenovirus which expresses human IL-10. FIG. 9 is a flowchart, wherein it can be seen that in this mode of production a lentiviral E1A/E1B vector is first introduced into HeLa cells. The modified HeLa cell lines may then be tested for vector production to be sure there is no contamination of the cell line. Assuming there is no infection contamination or unexpected replication, one can then proceed with small scale vector production and amplification.

[0056] Together, the mutant adenoviral vector of the present disclosure and the associated production cell line allow for the expansion of non-infectious therapeutic agents expressing adenovirus without the risk of recombination and restoration of replication competence.

[0057] Potential applications may include, but are not limited to, adenoviral expression of human IL-10, which in turn can inhibit immune activity in autoimmune disorders as well as reduce rejection risk in allo-transplant or xenotransplant.

[0058] In a further aspect of the disclosure, a method of treating a subject in need of a gene product is also provided herein. In some embodiments, the method may comprise, consist essentially of, or further, consist of administering to the subject a vector comprising (a) one or more mutations that render the adenovirus replication incompetent and (b) at least one nucleotide sequence encoding the gene product. In some embodiments of the method, the vector comprises a nucleotide sequence having at least 80% sequence identity over the entire sequence of SEQ ID NO: 1 or SEQ ID NO: 2, or a complementary sequence thereto. In certain embodiments, the vector may be an adenovirus serotype 5 vector. Additionally or alternatively, the one or more mutations may be a deletion of either an E1 gene or an E3 gene or both. The at least one nucleotide sequence further may include a transgene. In some embodiments, the transgene expresses human IL10.

[0059] In some embodiments of the method, the subject may be a human. In certain embodiments, the gene product is a peptide, including human peptides. In some embodiments, the gene product can be RNA.

EXAMPLES

[0060] The following examples are to illustrate the invention. They are not meant to limit the invention in any way.

[0061] The practice of this invention may employ, unless otherwise indicated, conventional techniques of molecular biology, cell biology, and recombinant DNA, which are within the skill of the person skilled in the art (*see*, *e.g.*, Green and Sambrook. *Molecular Cloning: A Laboratory Manuel*, 4th edition, 2012; Ausubel, et al. *Current Protocols in Molecular Biology*, 1987.

Example 1: Sequence of Engineered Adenovirus Serotype 5 Vector

[0062] Synthesis and description of an engineered adenovirus serotype 5 vector is described in Example 1. The adenovirus sequence used for synthesis was derived from public domain sources, notably sequence AY339865, available in GenBank,

worldwideweb.ncbi.nlm.nih.gov/genbank/. Two smaller fragments were generated using synthetic DNA methods to allow for higher efficiency cloning using bacterial hosts. The synthetic fragments were designed to include deletions of the E1 and E3 genes to prevent adenoviral replication *in vivo*. A transgene for expression of human IL-10 was included in the adenoviral sequence, which includes an enhancer/promoter region (CAG), human IL10 cDNA, and a polyadenylation signal (SV40, full polyA signal), which was inserted between nucleotides 440 and 3515 relative to Adenovirus sequence AY339865, which replaces the E1 gene, but leaves the pIX gene intact.

Example 2: Protocol for More Efficient Production of AdVector in Bacteria

[0063] Here, the construction of a plasmid-based system to generate recombinant sAd5 vectors and protocol for more production in bacteria is described. In a first step of an aspect of the disclosure, adenovirus 5 genome was synthesized as several overlapping DNA fragments. The overlapping DNA fragments were assembled into two linear sub-fragments, each bearing approximately one half of the full genome.

[0064] A first sub-fragment may include at least one sequence as set forth in SEQ ID NOs: 4-5. In some embodiments, a second sub-fragment may include at least one sequence as set forth in SEQ ID NOs: 6-7. FIG. 3 depicts a schematic diagram of the genomic organization of an exemplary first sub-fragment in linear form. FIG. 4 depicts a schematic diagram of the genomic organization of an exemplary second sub-fragment in linear form.

[0065] In a second step of the protocol, adenovirus serotype 5 genomic sub-fragments were circularized to form traditional plasmid structures to enable efficient replication in bacterial hosts.

[0066] FIG. 5 shows a schematic diagram of the genomic organization of an exemplary first sub-fragment in circularized form and FIG. 6 shows a schematic diagram of the genomic organization of an exemplary first second-fragment in circularized form.

[0067] In a third step of the protocol, the final adenovirus serotype 5 genome was assembled from the two circularized fragments, creating a linear structure for high level DNA replication in bacteria use in viral production. The high level of DNA replication and design allow for greater

amounts of DNA to use for transfection and first round viral production. The final vector may comprise additive combination of the first sub-fragment and the second sub-fragment.

[0068] Creation of the DNA sequences encoding the Adenovirus 5 vector for replication at high efficiency in bacteria allows for better initial vector creation. Vector sequences to allow for high efficiency bacterial replication can include pJazz-OK from Lucigen Catalog No. 43036. pJazz-OK allows for less DNA loss due to replication stress and much more convenient cloning. *See*, *e.g.*, training manual available at worldwideweb.lucigen.com/docs/manuals/MA033-BigEasy-v20-Linear-Cloning-Kit.pdf. The sequence also may include human IL10 complementary DNA sequences, as a non-limiting example. The human IL-10 cDNA sequence may be sequence codon optimized from NM_00572.3 using the codon optimization tool in Geneious version 9.0, as disclosed in SEQ ID NO: 8.

[0069] Other adenoviral genome sequence listings may be derived from public sources.

Example 3: Creation of Engineered Mammalian Cell Line for Replication of the Engineered Adenoviral Vector

[0070] Here, the creation of an engineered mammalian cell line for replication of the engineered adenoviral vector to eliminate possible contamination of the replication competent adenovirus is described. Described above, the adenoviral vector was mutated to remove the viral E1 and E3 genes in order to prevent the virus from replicating and thus being infectious. Mammalian cells supplied with the two E1 gene products, E1A and E1B, can support replication of the mutant virus. A common mammalian cell line used to produce adenovirus, HEK293, possesses an endogenous copy of the adenoviral E1 gene, which can recombine with mutant adenoviral vectors and recreate infectious replicating virus. Thus, a novel cell line devoid of adenoviral sequences is engineered here to express the E1A and E1B gene products using a lentiviral vector which does not allow reconstitution of functional adenovirus when the mutant adenoviral vector is introduced.

[0071] The adenoviral E1 gene products, E1A and E1B, were inserted into a plasmid backbone comprising the backbone viral elements and Puromycin resistance from Systems Biosciences Catalog Number CD510B-1 and the bidirectional promoter from ABM Catalog Number LV039.

The bidirectional promoter was situated such that the E1A and E1B were in different orientations and driven by separate parts of the promoter element.

[0072] The lentiviral genome plasmid and accessory plasmids from CellBioLabs Catalog Number VPK-206 were transfected into HEK293T (ATCC Catalog Number CRL-3216) cells to create a lentiviral vector. The E1A and E1B sequences were arranged in a bidirectional expression unit, to prevent recombination with the mutant adenoviral vector created in Examples 1 and 2, discussed above. The bidirectional promoter is a combination of truncated CMV and PGK promoters. A puromycin drug resistance gene is included in the lentiviral sequence to allow selection of cells which have integrated the lentiviral sequence. The final vector is diagrammed and mapped in FIG. 8, and was used to produce lentivirus, and HeLa cells (ATCC Catalog Number CCL2) transduced and selected for puromycin resistance.

[0073] The vector described in Examples 1 and 2 was introduced into the cells created in this Example 3 in order to generate mutant adenovirus capable of expressing human IL-10. FIG. 9 is a brief flowchart of the process of this Example. As illustrated, the lentiviral E1A/E1B vector is inserted into the HeLa cells. The modified cells are then tested for vector production. Finally, a user may proceed with vector production and amplification of the cell line. SEQ ID NO: 9 SEQ encodes the full E1 cell creation vector without the plasmid backbone. SEQ ID NO: 10 encodes the full E1 cell creation vector and includes the plasmid backbone. FIG. 10 shows a schematic diagram of the full E1 cell creation vector with the plasmid backbone.

[0074] In summary, a method of producing a vector as in Examples 1 and 2 is disclosed herein. The method may include transforming a mammalian cell with an adenovirus vector comprising: a) one or more mutations that render the adenovirus replication incompetent and b) at least one nucleotide sequence encoding a transgene, wherein the mammalian cell comprises nucleotide sequences expressing E1A and E1B gene products but is otherwise devoid of other adenovirus sequences, culturing the transformed mammalian cell in a cell line containing an inserted lentiviral E1A and E1B vector, and isolating the adenovirus vector.

[0075] One advantage of the methods, vectors, and cell lines described above is that the mutant adenoviral vector and associated production cells allow the production of non-infectious human IL-10 expressing adenovirus without the risk of recombination and restoration of replication

competence. A potential application of the disclosed vectors, methods, and cells include adenoviral expression of human IL-10 to inhibit immune activity in autoimmune disorders. The aspects of the disclosure may also be used to reduce rejection risk in allotransplant and xerotransplant procedures.

EQUIVALENTS

[0076] The present technology is not to be limited in terms of the particular embodiments described in this application, which are intended as single illustrations of individual aspects of the present technology. Many modifications and variations of this present technology can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. Functionally equivalent methods and apparatuses within the scope of the present technology, in addition to those enumerated herein, will be apparent to those skilled in the art from the foregoing descriptions. Such modifications and variations are intended to fall within the scope of the appended claims. The present technology is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled. It is to be understood that this present technology is not limited to particular methods, reagents, compounds, compositions or biological systems, which can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

[0077] In addition, where features or aspects of the disclosure are described in terms of Markush groups, those skilled in the art will recognize that the disclosure is also thereby described in terms of any individual member or subgroup of members of the Markush group.

[0078] As will be understood by one skilled in the art, for any and all purposes, particularly in terms of providing a written description, all ranges disclosed herein also encompass any and all possible subranges and combinations of subranges thereof. Any listed range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, tenths, *etc.* As a non-limiting example, each range discussed herein can be readily broken down into a lower third, middle third and upper third, *etc.* As will also be understood by one skilled in the art all language such as "up to," "at least," "greater than," "less than," and the like, include the number recited and refer to ranges which can

be subsequently broken down into subranges as discussed above. Finally, as will be understood by one skilled in the art, a range includes each individual member. Thus, for example, a group having 1-3 cells refers to groups having 1, 2, or 3 cells. Similarly, a group having 1-5 cells refers to groups having 1, 2, 3, 4, or 5 cells, and so forth.

[0079] All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

[0080] Other embodiments are set forth within the following claims.

SEQUENCE LISTINGS

Table 1. Adenovector no plasmid backbone (SEQ ID NO: 1)

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Table 2. Full Vector with Plasmid Backbone (SEQ ID NO: 2)

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Table 4. 3' End Fragment 1 (SEQ ID NO: 4)

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Table 5. 5' End Fragment 1 (SEQ ID NO: 5)

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Table 6. 3' End Fragment 2 (SEQ ID NO: 6)

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Table 7. 5' End Fragment 2 (SEQ ID NO: 7)

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Table 8. Codon Optimized Human IL10 cDNA (SEQ ID NO: 8)

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Table 9. E1 Cell Creation Vector (SEQ ID NO: 9)

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Table 10. Full E1 Cell Creation Vector with Plasmid Backbone (SEQ ID NO: 10)

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Table 11. Adenovirus 3' End (Right Side Second Product) SEQ ID NO: 11

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Table 12. Adenovirus 5' End (Left Side Second Product) (SEQ ID NO: 12)

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Table 13. 3' End Fragment (SEQ ID NO: 13)

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Table 14. Ad5ACE2 No Plasmid (SEQ ID NO: 14)

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Table 15. Ad5ACE2 with Plasmid Backbone (SEQ ID NO: 15)

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Table 16. Ad5UnituxinscFv with Plasmid Backbone (SEQ ID NO: 16)

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Table 18. Ad5-TGFβ-RES-IL10 with Plasmid Backbone (SEQ ID NO: 18)

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Table 19. Ad5-TGFβ-RES-IL10 No Plasmid Backbone (SEQ ID NO: 19)

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Table 20. Ad5-IL13 with Plasmid Backbone (SEQ ID NO: 20)

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Table 21. AD5-IL13 No Plasmid Backbone (SEQ ID NO: 21)

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Table 22. AD5-IL8-shRNA With Plasmid Backbone (SEQ ID NO: 22)

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Table 23. Ad5-IL8-shRNA with Plasmid Backbone (SEQ ID NO: 23)

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Table 24. Ad5-IL1-RA2A-IL10 with plasmid backbone (SEQ ID NO: 24)

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Table 25. Ad5-IL1-RA2A-IL10 No Plasmid Backbone (SEQ ID NO: 25)

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Table 26. Ad5-IL1-RA with Plasmid Backbone (SEQ ID NO: 26)

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Table 27. Ad5-IL1-RA No Plasmid Backbone (SEQ ID NO: 27)

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Table 28. Ad5eNOS with Plasmid Backbone (SEQ ID NO: 28)

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Table 29. Ad5eNOS No Plasmid Backbone (SEQ ID NO: 29)

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Table 30. Ad5-CFTR with Plasmid Backbone (SEQ ID NO: 30)

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Table 31. Ad5-CFTR No Plasmid Backbone (SEQ ID NO: 31)

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Table 32. Ad5-B4Gal-Cas9 with Plasmid Backbone (SEQ ID NO: 32)

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Table 33. Ad5-B4Gal-Cas9 No Plasmid Backbone (SEQ ID NO: 33)

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WHAT IS CLAIMED IS:

1. A non-replicating adenovirus expression vector comprising: a) one or more mutations that render the adenovirus replication incompetent and b) at least one nucleotide sequence encoding a gene product.
2. The vector of claim 1, wherein the vector comprises a nucleotide sequence having at least 80% sequence identity over the entire sequence of SEQ ID NO: 1 or SEQ ID NO: 2, or a complementary sequence thereto.
3. The vector of claim 2, wherein the vector comprises a nucleic acid sequence having at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, or greater than 99% sequence identity to SEQ ID NOs: 1-2.
4. The vector of claim 1, wherein the vector comprises an adenovirus serotype 5 vector.
5. The vector of claim 1, wherein the one or more mutations comprise deletion of either an E1 gene or an E3 gene or both.
6. The vector of claim 1, wherein the at least one nucleotide sequence comprises a transgene.
7. The vector of claim 6, wherein the transgene expresses human IL10.
8. The vector of claim 6, wherein the transgene further comprises at least one of an enhancer/promoter region comprising a CAG region, human IL10 cDNA, or a polyadenylation signal, or any combination thereof.
9. The vector of claim 8, wherein the polyadenylation signal comprises an SV40 region and/or a full polyA signal.
10. The vector of claim 8, wherein the polyadenylation signal is first inserted between nucleotides 440 and 3515 in SEQ ID NO: 3.
11. The vector of claim 8, wherein insertion of the transgene replaces the E1 gene but leaves the pIX gene intact.
12. A method of synthesizing an adenovirus vector comprising:

    a) producing a plurality of overlapping adenovirus sub-fragments, each sub-fragment comprising a portion of the full genome of the adenovirus;

    b) circularizing the sub-fragments to form plasmid structures; and

    c) assembling the circularized sub-fragments into a linear structure, wherein the vector comprises a combination of two or more sub-fragments.
13. The method of claim 12, wherein the adenovirus vector comprises adenovirus serotype 5.

14. The method of claim 13, wherein the vector replicates at high efficiency in bacteria.
15. The method of claim 12, wherein a first sub-fragment comprises a sequence as set forth from the 3' end in SEQ ID NO: 4 or from the 5' end in SEQ ID NO: 5.
16. The method of claim 12, wherein a second sub-fragment comprises a sequence as set forth from the 3' end in SEQ ID NO: 6 or from the 5' end in SEQ ID NO: 7.
17. The method of claim 12, wherein a right end of a second sub-fragment is encoded by SEQ ID NO: 11.
18. The method of claim 12, wherein a left end of a second sub-fragment is encoded by SEQ ID NO: 12.
19. The method of claim 12, wherein a 3' end of a second sub-fragment is encoded by SEQ ID NO: 13.
20. The method of claim 12, wherein each sub-fragment comprises about 50% of the full adenovirus genome.
21. The method of claim 20, wherein at least one fragment includes a transgene that expresses human IL-10.
22. The method of claim 21, wherein the transgene further comprises at least one of an enhancer or a promoter region, a human IL10 cDNA gene, or a polyadenylation signal, or any combination thereof.
23. The method of claim 22, wherein the enhancer/promoter region comprises a CAG promoter.
24. The method of claim 22, wherein the transgene includes at least a polyadenylation signal comprising an SV40 promoter or a full poly(A) signal.
25. The method of claim 12, wherein the vector sequence comprises a linear cloning vector backbone that is removed from final synthesis of the adenovirus vector.
26. The method of claim 22, wherein the human IL10 cDNA sequence is optimized and encoded from SEQ ID NO: 8.
27. A mammalian cell line configured to replicate adenoviral vectors, wherein the cell line comprises nucleotide sequences expressing E1A and E1B gene products but is devoid of other adenovirus sequences.
28. The cell line of claim 24, further comprising a lentiviral vector engineered to express the E1A and E1B gene products, wherein the lentiviral vector inhibits reconstitution of functional adenovirus when a mutant adenoviral viral vector is introduced into the cell line.

29. The cell line of claim 25, wherein the cell line comprises HeLa cells.

30. The cell line of claim 28, wherein the cell line is produced by transfecting HEK293 cells with a lentiviral genome plasmid comprising at least an E1A encoding sequence and an E1B-encoding sequence.

31. The cell line of claim 27, wherein the plasmid comprising at least an E1A encoding sequence and an E1B-encoding sequence contains E1A and E1B sequences arranged in a bidirectional expression unit with a bidirectional promoter to prevent recombination with a mutant adenoviral vector.

32. The cell line of claim 28, wherein the bidirectional promoter is a combination of truncated CMV and PGK promoters.

33. The cell line of claim 27, further comprising a puromycin drug resistance gene in the lentiviral genome sequence to differentiate cells which have integrated the lentiviral sequence when the puromycin drug resistance gene is expressed.

34. The cell line of claim 27, wherein a final vector encoded by SEQ ID NO: 9 is used to produce lentivirus and HeLa cells, transduced and selected for puromycin resistance.

35. A method of producing a vector as in claim 1, comprising transforming a mammalian cell with an adenovirus vector comprising: a) one or more mutations that render the adenovirus replication incompetent and b) at least one nucleotide sequence encoding a transgene, wherein the mammalian cell comprises nucleotide sequences expressing E1A and E1B gene products but is otherwise devoid of other adenovirus sequences, culturing the transformed mammalian cell in a cell line containing an inserted lentiviral E1A and E1B vector, and isolating the adenovirus vector.

36. The method of claim 35, wherein the transgene expresses human IL-10.

37. A method of treating a subject in need of a gene product comprising administering to the subject a vector comprising (a) one or more mutations that render the adenovirus replication incompetent and (b) at least one nucleotide sequence encoding the gene product.

38. The method of claim 37, wherein the vector comprises a nucleotide sequence having at least 80% sequence identity over the entire sequence of SEQ ID NO: 1 or SEQ ID NO: 2, or a complementary sequence thereto.

39. The method of claim 37, wherein the vector comprises an adenovirus serotype 5 vector.

40. The method of claim 37, wherein the one or more mutations comprise deletion of either an E1 gene or an E3 gene or both.
41. The method of claim 37, wherein the at least one nucleotide sequence comprises a transgene.
42. The method of claim 42, wherein the transgene expresses human IL10.
43. The method of claim 42, wherein the subject is a human.
44. The method of claim 42, wherein the gene product is a peptide.
45. The method of claim 44, wherein the peptide is a human peptide.
46. The method of claim 42, wherein the gene product is RNA.

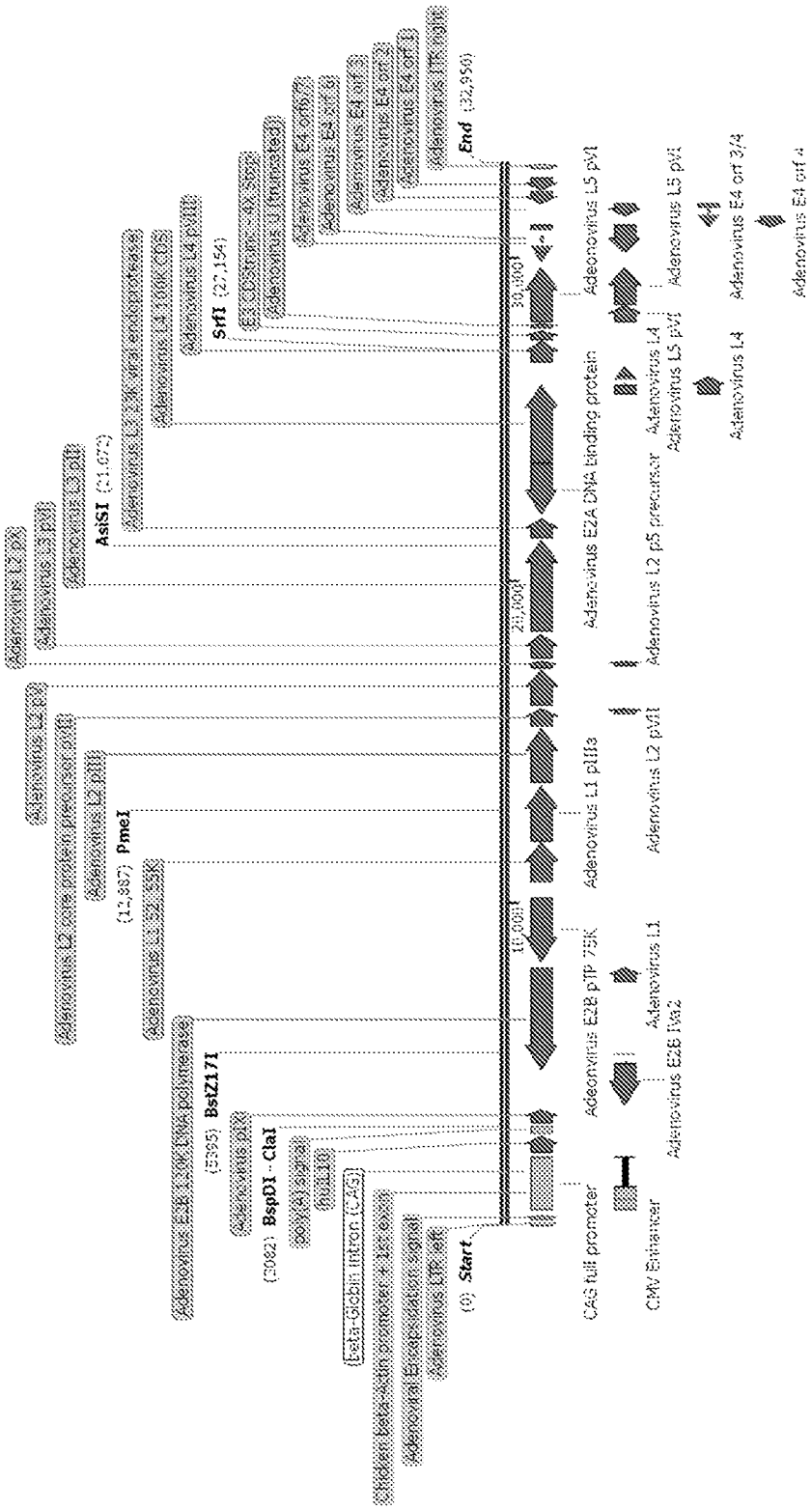

Adenovirus L2 pX
Adenovirus L3 pVII
Adenovirus L3 pVI
AsiSI (21,672)
Adenovirus L3 hexon
Adenovirus L3 protease
Adenovirus L4 pVIII
SrfI (27,154)
E3 CR3 hexon 6K 55kD
Adenovirus L5 (truncated)
Adenovirus E4 orf6/7
Adenovirus E4 orf6
Adenovirus E4 orf 3
Adenovirus E4 orf 2
Adenovirus E4 orf 1
Adenovirus ITR right
End (32,956)
Adenovirus L2 pVI
Adenovirus L2 core protein precursor pVII
Adenovirus L2 pVII
PmeI (17,387)
Adenovirus L1 52,55K
Adenovirus E3B 12.5K DNA polymerase
(5395) BstZ17I
Adenovirus pIX
(3062) BspDI - ClaI
SV40 early polyA
pUC19
Beta-Globin intron (CAG)
Chicken beta-actin promoter + 1st exon
Adenoviral Encapsulation signal
Adenovirus ITR left
(0) Start
30,000
20,000
10,000
Adenovirus L5 pVI
Adenovirus E2A DNA binding protein
Adenovirus L4
Adenovirus L2 p5 precursor
Adenovirus L5 pVI
Adenovirus L5 pVI
Adenovirus L4
Adenovirus E4 orf 3/4
Adenovirus E4 orf 4
Adenovirus L1 pIIIa
Adenovirus L2 pVII
Adenovirus E2B pTP 75K
Adenovirus L1
Adenovirus E2B IVa2
CAG full promoter
CMV Enhancer

FIG. 1

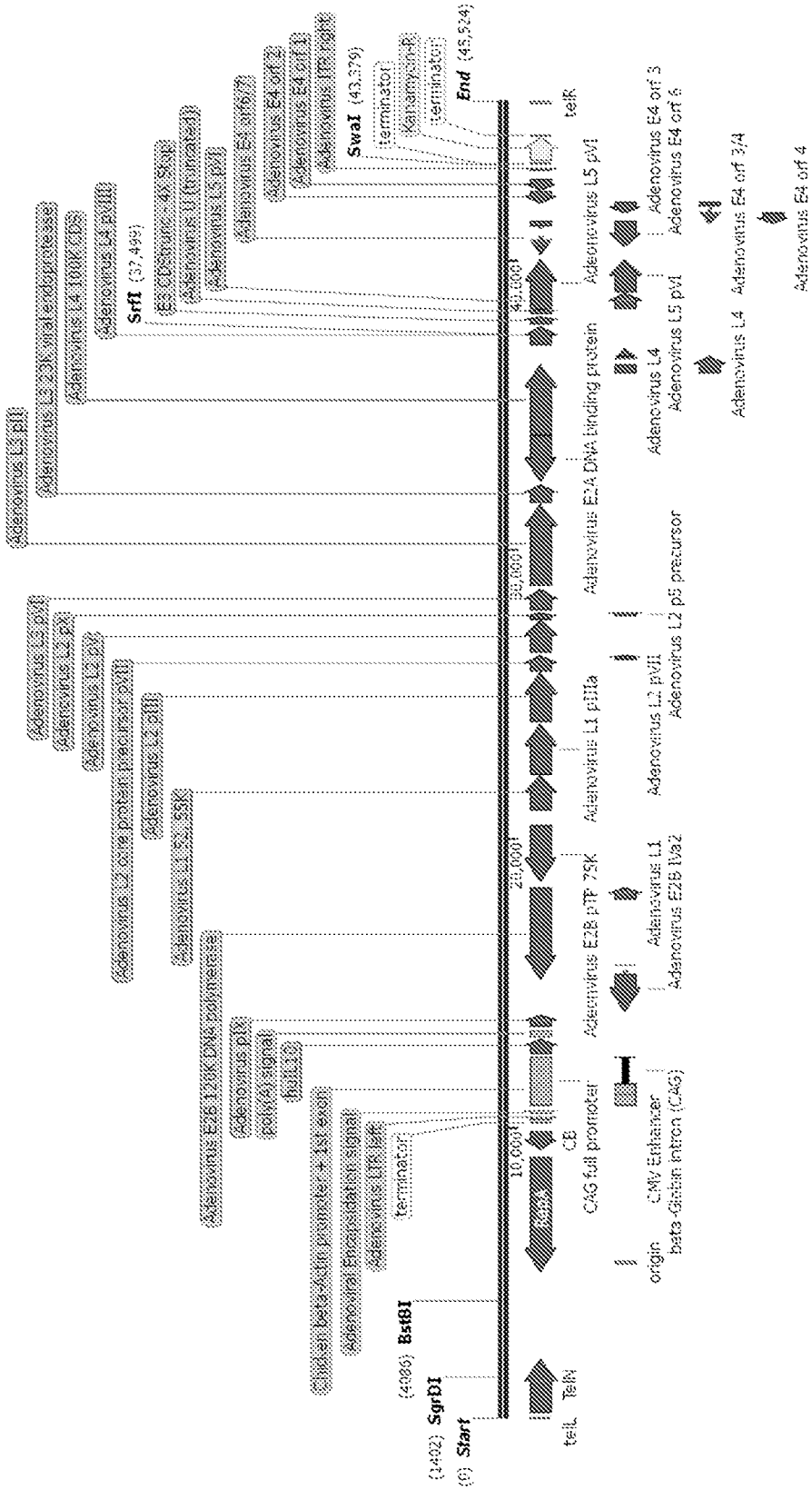

Adenovirus L1 pIII
Adenovirus L2 100K maleic anhydrase
Adenovirus L4 100K CDS
Adenovirus L4 pVIII
SrfI (32,499)
E3 CDS (nt 29,792 - 30,793)
Adenovirus L1 (truncated)
Adenovirus L3 pVI
Adenovirus E3 orf 6/7
Adenovirus E4 orf 2
Adenovirus E4 orf 1
Adenovirus IVa2 rc/nt
SwaI (43,379)
terminator
Kanamycin-R
terminator
End (45,524)
Adenovirus L1 pIX
Adenovirus L2 pX
Adenovirus L2 pV
Adenovirus L2 protease precursor pVII
Adenovirus L2 pVII
Adenovirus L1 52,55K
Adenovirus E2B 120K DNA polymerase
Adenovirus pIX
polyA signal
fbLUC
Chicken beta-Actin promoter + 1st exon
Adenoviral Encapsidation signal
Adenovirus L1 IIIa
terminator
(4086) BstBI
(1492) SgrDI
(6) Start
10,000
20,000
30,000
40,000
tetR
tetL
CE
CAG full promoter
Adenovirus E2B pTP 75K
Adenovirus L1 pIIIa
Adenovirus E2A DNA binding protein
Adenovirus L5 pVI
tetR
origin
CMV Enhancer
beta-Globin intron (CAG)
Adenovirus L1
Adenovirus E2B IVa2
Adenovirus L2 pVII
Adenovirus L2 p5 precursor
Adenovirus L4
Adenovirus L5 pVI
Adenovirus E4 orf 3
Adenovirus E4 orf 6
Adenovirus L4
Adenovirus E4 orf 3/4
Adenovirus E4 orf 4

FIG. 2

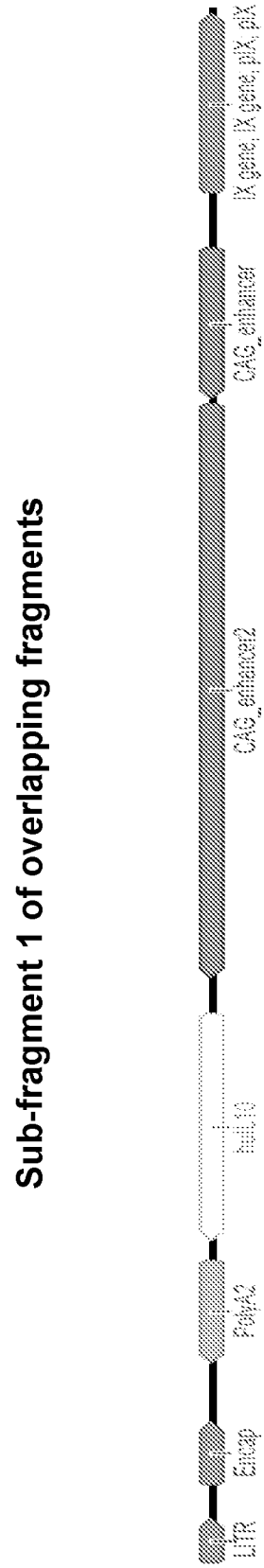

Sub-fragment 1 of overlapping fragments
LTR
Ecoop
PolyA2
hsiL10
CAG_enhancer2
CAG_enhancer
IX gene, IX gene, pIX, pIX

FIG. 3

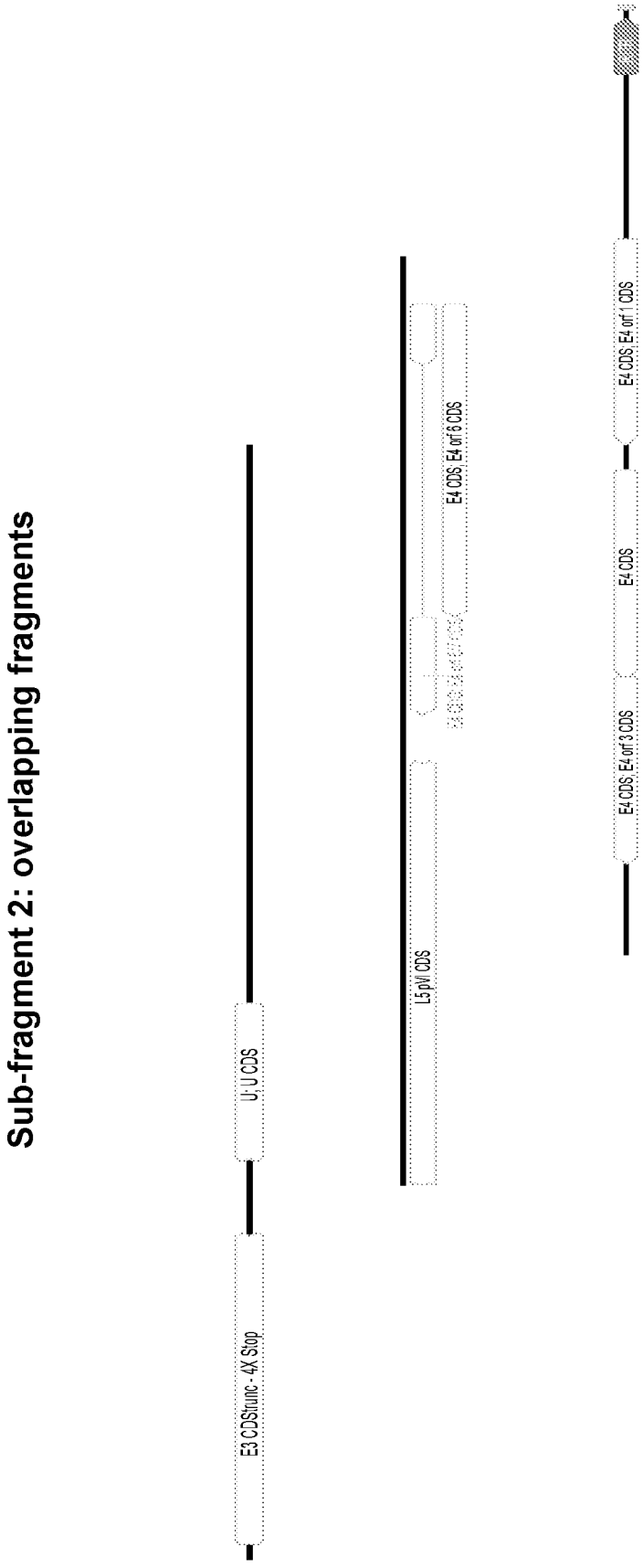

Sub-fragment 2: overlapping fragments
E3 CDStrunc - 4X Stop
U1 U CDS
L5 pVI CDS
E4 CDS; E4 orf 6 CDS
E4 CDS; E4 orf 3 CDS
E4 CDS
E4 CDS; E4 orf 1 CDS

FIG. 4

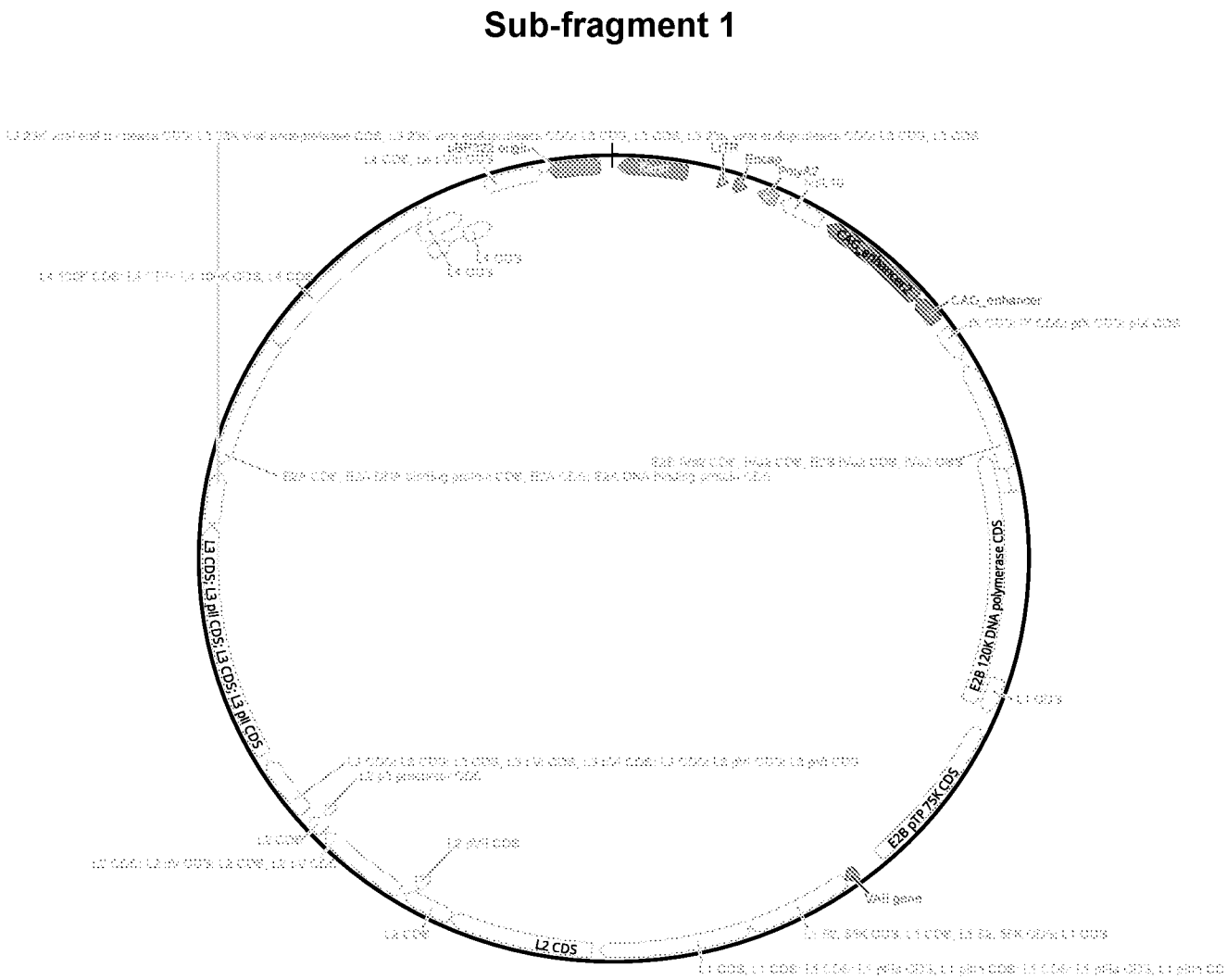

Sub-fragment 1
CAG enhancer
CAG_enhancer
E2B 120K DNA polymerase CDS
E2B pTP 75K CDS
L2 CDS
L3 CDS; L3 pIII CDS; L3 CDS; L3 pIII CDS

FIG. 5

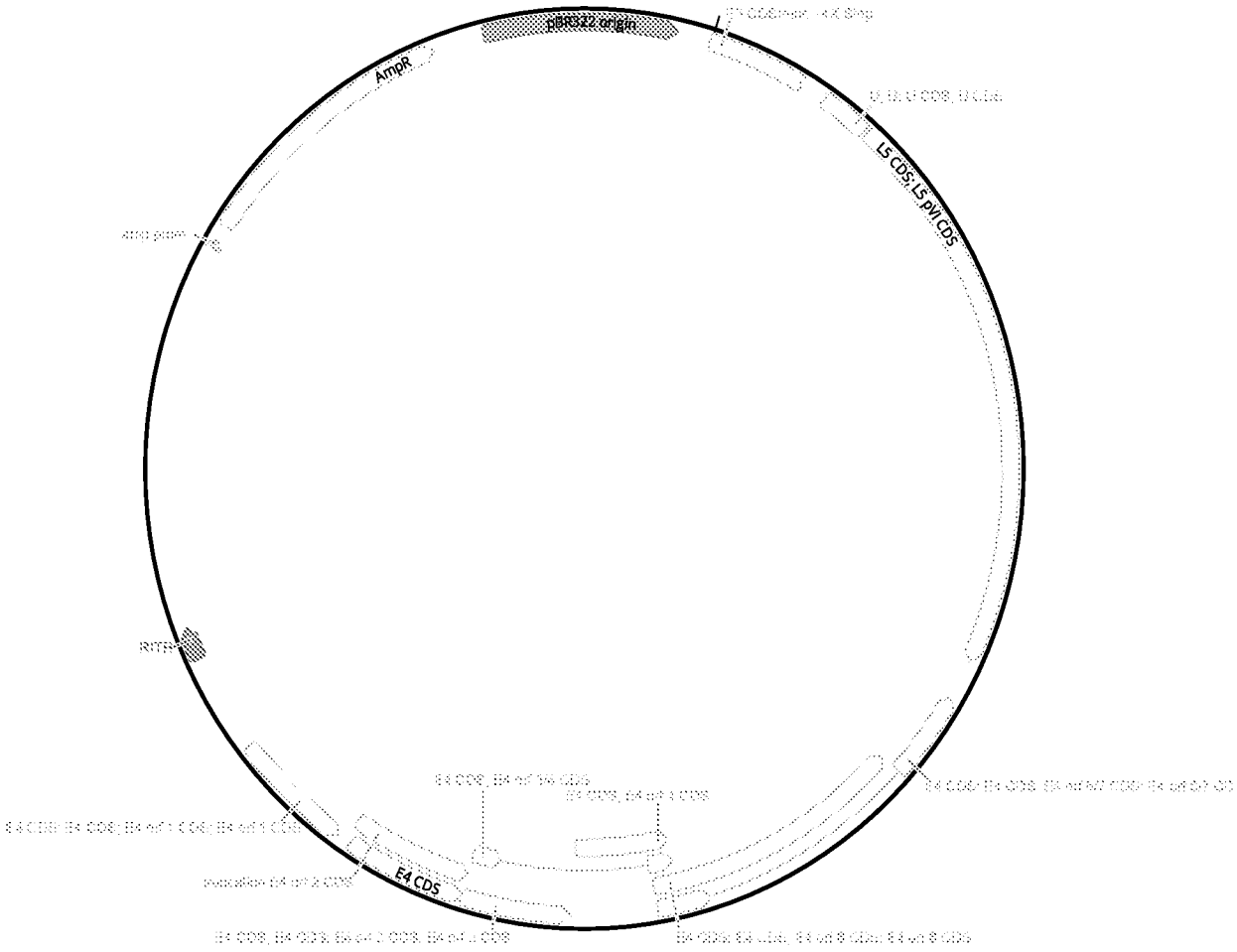


FIG. 6

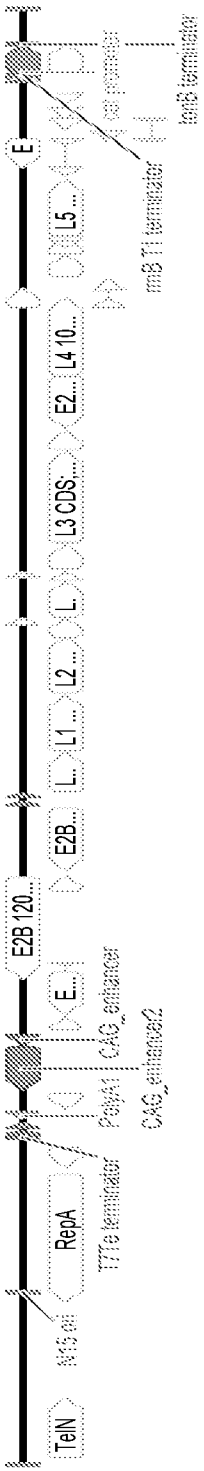

TelN
f115 ori
RepA
T7Te terminator
P4tpA1
CAG_enhancer
CAG_enhancer2
E...
E2B 120...
E2B...
L...
L1...
L2...
L...
L3 CDS:...
E2...
L4 10...
L5...
E
T7 promoter
mB T1 terminator
fd terminator

FIG. 7

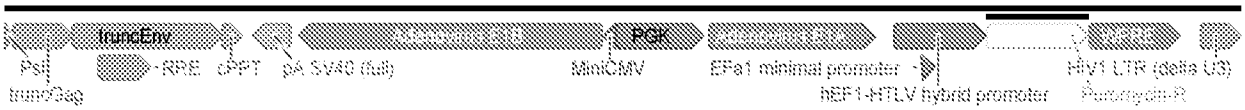

TruncEnv
Psi
truncGag
RRE
cPPT
pA SV40 (full)
MinCMV
PGK
EFa1 minimal promoter
hEF1-HTLV hybrid promoter
HIV1 LTR (delta U3)

FIG. 8

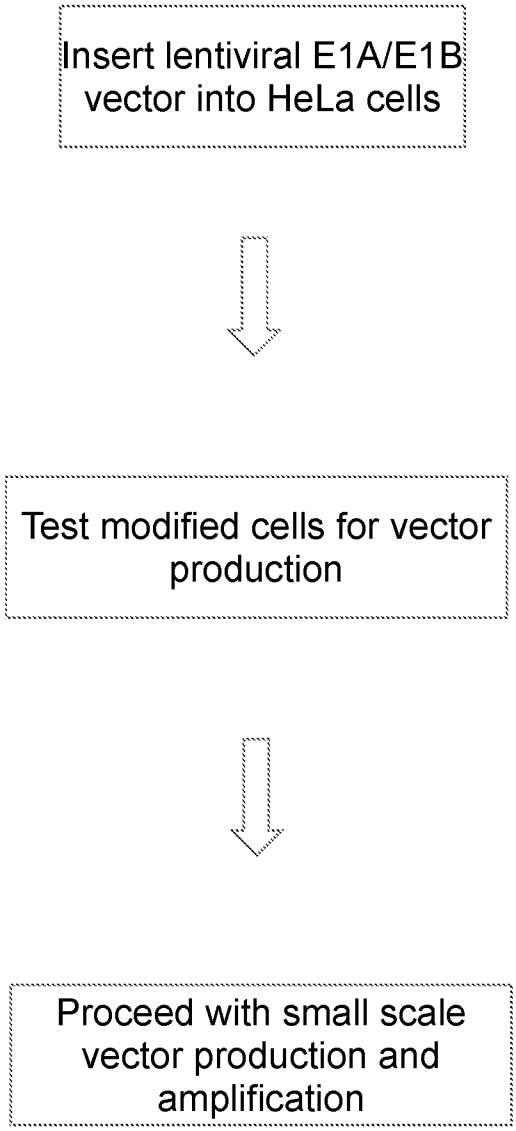

Insert lentiviral E1A/E1B vector into HeLa cells
Test modified cells for vector production
Proceed with small scale vector production and amplification

FIG. 9

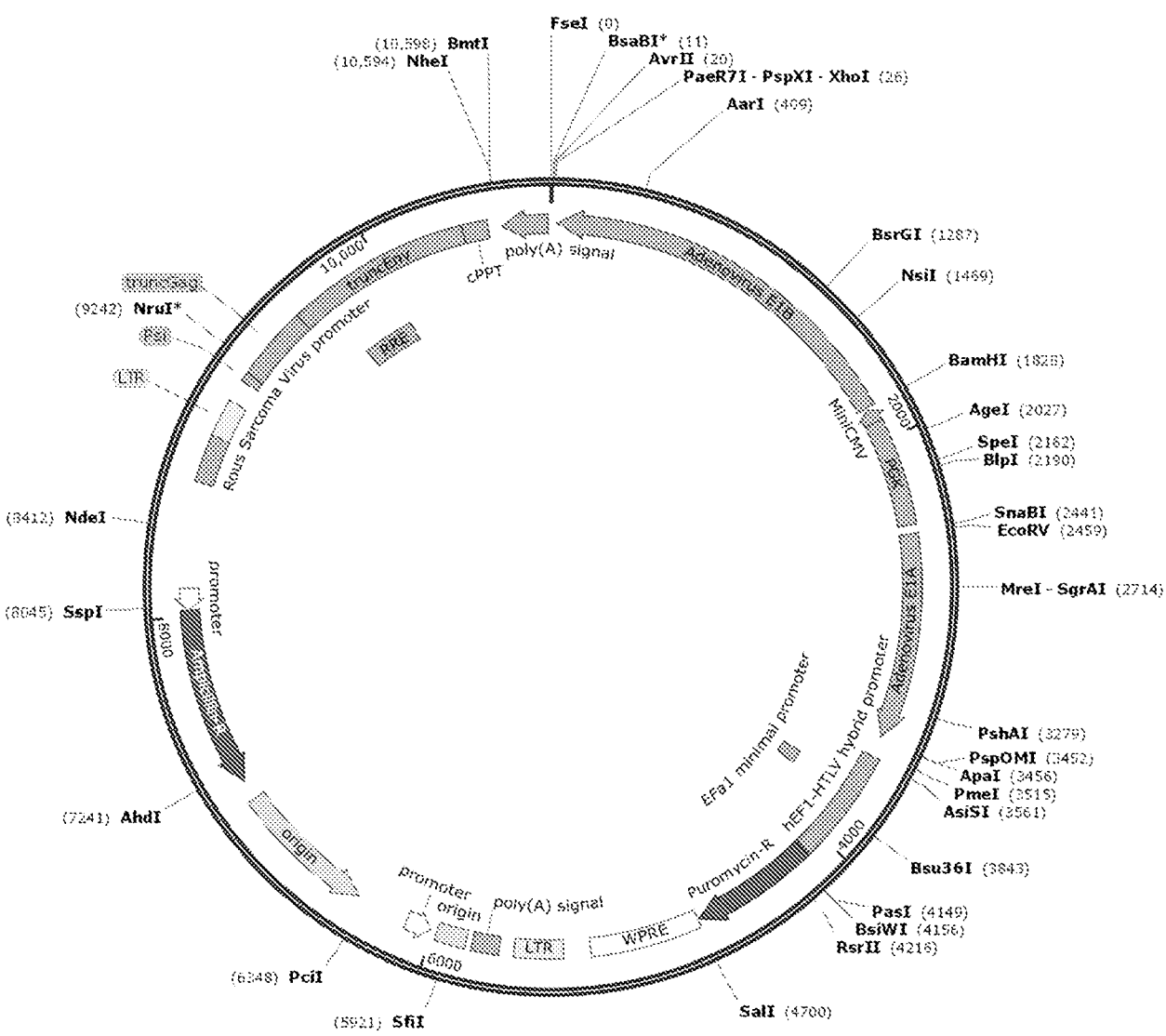

FseI (0)
BsaBI* (11)
AvrII (20)
PaeR7I - PspXI - XhoI (26)
AarI (409)
BsrGI (1287)
NsiI (1469)
BamHI (1828)
AgeI (2027)
SpeI (2162)
BlpI (2190)
SnaBI (2441)
EcoRV (2459)
MreI - SgrAI (2714)
PshAI (3279)
PspOMI (3452)
ApaI (3456)
PmeI (3515)
AsiSI (3561)
Bsu36I (3843)
PasI (4149)
BsiWI (4156)
RsrII (4216)
SalI (4700)
(5921) SfiI
(6348) PciI
(7241) AhdI
(8045) SspI
(8412) NdeI
(9242) NruI*
(10,594) NheI
(10,598) BmtI
poly(A) signal
cPPT
RRE
Rous Sarcoma Virus promoter
MiniCMV
EF1a minimal promoter
hEF1-HTLV hybrid promoter
PuromycinR
WPRE
LTR
origin
promoter
ampicillin
2000
4000
6000
8000
10,000

FIG. 10