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(54) Title: COMPOSITIONS AND METHODS TO TREAT VIRAL INFECTIONS

(57) Abstract: The invention provides methods and compositions for treating viral infections. A nuclease is used to cleave viral nucleic acid in an infected cell. The nuclease cleaves viral nucleic acid in a sequence-specific manner and thus does not cleave genes or other important genomic features from a genome of the infected host. In a preferred embodiment, the nuclease is a CRISPR-associated protein such as Cas9 and is delivered to the infected cells as a ribonucleoprotein that includes the Cas9 and a guide RNA designed to target HSV or CMV nucleic acid. Additionally or alternatively, the nuclease can be delivered encoded on a plasmid or as mRNA to be expressed within the target cells. Methods and compositions may be used to treat a patient or may be used to treat tissues, cells, or organs ex vivo.

COMPOSITIONS AND METHODS TO TREAT VIRAL INFECTIONS

Cross-Reference to Related Application

This application claims the benefit and priority of both U.S. Provisional Application No. 62/168,259, filed May 29, 2015, and U.S. Provisional Application No. 62/168,262, filed May 29, 2015, the contents of which are incorporated by reference.

Technical Field

The invention generally relates to treatment of viral infections and cleaving foreign nucleic acids in cells.

Background

Viral infections present significant medical problems. For example, herpes simplex virus (HSV) may cause oral herpes, which manifest as blisters around face or mouth, or genital herpes that manifest as blisters that can break open and result in small ulcers. Tingling or shooting pains may occur before the blisters appear. Additionally, HSV has the ability to lie dormant within a cell indefinitely in a latent infection and not be fully eradicated even after treatment. Because latent infections can evade immune surveillance and reactivate the lytic cycle at any time, there is a persistent risk to an infected individual of outbreak and the pain and suffering associated with it.

Worldwide, about 90% of people are infected with one or both of HSV-1 and HSV-2 with HSV-1 infection much more prevalent than HSV-2 infection. About 65% of persons in the United States have antibodies to HSV-1 and about 16% of Americans between the ages of 14 and 49 are infected with HSV-2.

Cytomegalovirus (CMV) is an example of another problematic virus. Some people get infected when they receive an organ transplant. The CMV infection disseminates to the lungs, liver, pancreas, kidneys, stomach, intestine, brain, and parathyroid glands, causing organ failure and even death. When disseminated to the eye, the virus can cause retinal detachment and blindness. Additionally, a fetus may be infected with cytomegalovirus (CMV) in utero, ultimately causing hearing loss, vision impairment, mental retardation, coordination problems, and death. Even when born healthy, a baby infected with CMV must be monitored throughout the developmental years for hearing or vision loss, or signs of mental impairment.

Summary

The invention provides methods and compositions for treating viral infections. A nuclease is used to cleave viral nucleic acid in an infected cell. The nuclease cleaves viral nucleic acid in a sequence-specific manner and thus does not cleave genes or other important genomic features from a genome of the infected host. In a preferred embodiment, the nuclease is a CRISPR-associated protein such as Cas9 and is delivered to the infected cells as a ribonucleoprotein that includes the Cas9 and a guide RNA designed to target HSV or CMV nucleic acid. Additionally or alternatively, the nuclease can be delivered encoded on a plasmid or as mRNA to be expressed within the target cells. Methods and compositions may be used to treat a patient or may be used to treat tissues, cells, or organs *ex vivo*.

Embodiments of the invention relate to HSV.

The invention provides compositions and methods for selectively treating HSV infection using a guided nuclease system that targets a specific region of the HSV genome such as oriS, UL9, RL2, or LAT. Methods of the invention may be used to remove even latent HSV genetic material from a host organism, without interfering with the integrity of the host's genetic material. A nuclease may be targeted to HSV nucleic acid where it can then disrupt the nucleic acid, thereby interfering with viral replication or transcription or even excising the viral genetic material from the host genome. Through use of a sequence-specific targeting moiety directed to HSV, the nuclease may be specifically targeted to remove only the HSV nucleic acid without acting on host material whether the viral nucleic acid exists as a particle within the cell or is integrated into the host genome. A sequence-specific moiety can include a guide RNA that targets viral genomic material for destruction by the nuclease and does not target the host cell genome. In some embodiments, a CRISPR/Cas9 nuclease and guide RNA (gRNA) that together target and selectively edit or destroy viral genomic material is used. CRISPR (clustered regularly interspaced short palindromic repeats) is a naturally-occurring element of the bacterial immune system that protects bacteria from phage infection. The guide RNA localizes the CRISPR/Cas9 complex to a HSV target sequence, such as oriS, UL9, RL2, or LAT. Binding of the complex

localizes the Cas9 endonuclease to the HSV genomic target sequence causing breaks in the viral genome.

The guided nuclease system may be introduced into an infected host transdermally through application of, for instance, a topical solution. Topical solutions may be applied directly to infected tissue.

The sequence specific moiety can target other nuclease systems to HSV nucleic acid including, for example, zinc finger nucleases, transcription activator-like effector nucleases (TALENs), meganucleases, or any other system that can be used to degrade or interfere with HSV nucleic acid without interfering with the regular function of the host's genetic material.

Aspects of the invention include a composition for treatment of a herpes simplex virus (HSV) infection. The composition comprises a vector encoding a nuclease and a sequence-specific targeting moiety complementary to HSV nucleic acid and capable of directing the nuclease to the HSV nucleic acid.

In certain embodiments, the HSV nucleic acid may comprise one or more of the following regions: origin of replication S (oriS), long unique region 9 (UL9), or long repeat region 2 (RL2). The composition may be configured to be administered transdermally, for example, through a topical solution. The nuclease may be a zinc-finger nuclease, a transcription activator-like effector nuclease, and a meganuclease.

In various embodiments, the nuclease may a Cas9 endonuclease and the sequence-specific binding module may comprise a guide RNA that specifically targets a portion of a viral genome. Compositions of the invention may be packaged for delivery to a human patient.

In certain embodiments, the targeting sequence may be a guide RNA that has no match > 60% within a human genome.

The vector may include a retrovirus, a lentivirus, an adenovirus, a herpesvirus, a poxvirus, an alphavirus, a vaccinia virus, an adeno-associated viruses, a plasmid, a nanoparticle, a cationic lipid, a cationic polymer, metallic nanoparticle, a nanorod, a liposome, microbubbles, a cell-penetrating peptide, or a liposphere.

In certain aspects, the invention includes a method for treating an HSV infection including the step of introducing into a cell of a host, a vector encoding a nuclease and a sequence-specific targeting moiety complementary to HSV nucleic acid. Additional steps include causing the sequence-specific targeting moiety to target the nuclease to the HSV nucleic acid and to cleave the HSV nucleic acid.

The HSV nucleic acid can include a portion of or all of one or more genomic regions including an origin of replication S (oriS), long unique region 9 (UL9) or long repeat region 2 (RL2). Methods of the invention may include transdermally administering the vector to the host and transdermal administration may include applying a topical solution comprising the vector.

The nuclease may be a zinc-finger nuclease, a transcription activator-like effector nuclease, or a meganuclease. In certain embodiments, the nuclease may include a Cas9 endonuclease and the sequence-specific binding module may include a guide RNA that specifically targets a portion of a viral genome.

In various methods of the invention, the host may be a living human subject and the steps may be performed in vivo. The targeting sequence may be a guide RNA and have no match > 60% within a human genome.

In certain methods, the vector may include a retrovirus, a lentivirus, an adenovirus, a herpesvirus, a poxvirus, an alphavirus, a vaccinia virus, an adeno-associated viruses, a plasmid, a nanoparticle, a cationic lipid, a cationic polymer, metallic nanoparticle, a nanorod, a liposome, microbubbles, a cell-penetrating peptide, or a liposphere.

The presented methods allow for HSV genome editing or destruction, which results in the inability of the HSV virus to proliferate without adversely affecting a host's uninfected cells. Compositions and methods of the invention can accordingly be used to treat an HSV infection through targeted disruption of HSV genomic function or by digestion of viral nucleic acid via one or multiple breaks caused by targeting sites such as oriS, UL9, RL2, or LAT for endonuclease action in the HSV genome.

In preferred aspects, the invention provides a composition for treatment of a herpes simplex virus (HSV) infection. The composition includes a ribonucleoprotein that includes: a nuclease; and a sequence-specific targeting moiety complementary to HSV nucleic acid and capable of directing the nuclease to the HSV nucleic acid. The HSV nucleic acid may include an origin of replication S (oriS) region, a long unique region 9 (UL9), a long repeat region 2 (RL2), or a combination thereof. Preferably, the nuclease comprises a Cas9 endonuclease and the sequence-specific binding module comprises a guide RNA that specifically targets a portion of a viral genome. The targeting sequence is a guide RNA and has no match > 60% within a human genome.

The composition may be configured to be administered transdermally. In some embodiments, the composition includes a pharmaceutically acceptable carrier for topical application to infected tissue.

Embodiments of the invention relate to CMV.

The invention provides methods for treating cytomegalovirus (CMV) infections in tissues, so that infected individuals will not suffer the effects of CMV infections, such as hearing/vision loss, mental impairment, multi-organ failure, and death. CMV infection is treated by using a nuclease that targets CMV nucleic acid. The nuclease cleaves and thus interferes with the function of the CMV nucleic acid, which prevents CMV from infecting the tissue or patient. The nuclease may be introduced into a patient with a CMV infection, or into an organ prior to transplant. For example, a heart needed for a life-saving transplant may be exposed to the CMV targeted nuclease to incapacitate the CMV genome prior to transplantation. Thus, the heart transplant recipient does not need to choose between accepting the infected heart and passing, only to wait and hope that the next heart will be CMV-free.

In certain aspects, the invention provides compositions for treatment of a cytomegalovirus (CMV) infection The composition comprise a nuclease; and a sequence-specific targeting moiety complementary to CMV nucleic acid and capable of directing the nuclease to the CMV nucleic acid.

In certain aspects, the invention provides methods for treating a cytomegalovirus (CMV) infection in an organ. The methods comprise introducing a nuclease that cleaves the CMV nucleic acid into an organ and then causing the nuclease to target and cleave CMV nucleic acid. The organ can be from a transplant donor, or the organ can be grown or created from cells. In some aspects, the organ is a heart, liver, kidney, eye, lung, pancreas, intestine, thymus, or any other biological tissue to be transplanted into a transplant recipient. It should be appreciated that preparing the nuclease for delivery can involve associating the nuclease with a viral or non-viral vector. For example, the nuclease could be prepared for delivery into an organ by encasing or binding the nuclease within a liposome.

In some embodiments of the invention, the tissue (or organ, used interchangeably herein) may be a tissue that is to be used in transplantation. For example, the tissue may be an organ supplied by a donor, to be transplanted into a recipient. In some embodiments, the tissues of the organ are treated with the nuclease to cleave and incapacitate the CMV nucleic acid.

In some embodiments, the transplant donor is treated with or exposed to the nuclease. According to methods of the invention, the donor receives the nuclease as a therapeutic agent to eliminate CMV from tissues within the patient. It should be appreciated that any means of delivery into the patient may be used. For example, the nuclease may be delivered by oral, parenteral, inhalation, topical, rectal, or vaginal means.

In some embodiments, the transplant recipient is treated with or exposed to the nuclease following transplantation. According to methods of the invention, the transplant recipient is treated with the nuclease to prevent CMV from spreading within and throughout the patient. It should be appreciated that the nuclease may be delivered by any known means in the art.

In some embodiments, the tissue is located within a patient. According to methods of the invention, a patient may be treated with the CMV targeting nuclease to prevent infection. For example, the patient may be a pregnant woman. The nuclease targets the CMV genome and destroys or disrupts the function of the virus. Thus, the pregnant woman does not pass the virus to the fetus in utero.

In some aspects, the invention provides methods of treating a cytomegalovirus (CMV) infection in a fetus. The methods comprise providing a nuclease that cleaves CMV nucleic acid and preparing the nuclease for delivery into a pregnant woman. It should be appreciated that the nuclease may be delivered into the pregnant woman, the uterus of the pregnant women, or the fetus in utero, by techniques known in the art.

It should also be appreciated that any type of nuclease may be used to cleave CMV nucleic acid. The nuclease may be a zinc finger nuclease, a transcription activator-like effector nuclease, or a meganuclease. The nuclease may be a structure specific nuclease or a sequence specific nuclease. In some embodiments, the nuclease is a Cas9 endonuclease. The methods of the invention may further comprise cleaving the CMV nucleic acid using the nuclease.

In some embodiments, the methods comprise delivering to the cell or tissue a guide RNA that targets the nuclease to a portion of the CMV nucleic acid. In some embodiments, for example, a guide RNA targets the Cas9 endonuclease to a portion of the CMV nucleic acid. In certain embodiments, the guide RNA is designed to have no perfect match in a human genome. The guide RNAs may target the nuclease to a regulatory element in the genome of CMV.

The nuclease may be delivered to the tissue by a viral vector. The viral vector may be retrovirus, lentivirus, adenovirus, herpesvirus, poxvirus, alphavirus, vaccinia virus or adeno-associated viruses. In some embodiments, the nuclease may be delivered by a plasmid, a nanoparticle, a cationic lipid, a cationic polymer, metallic nanoparticle, a nanorod, a liposome, a cell-penetrating peptide, a liposphere, and polyethyleneglycol (PEG).

In some embodiments, the nuclease is a CRISPR/Cas9 endonuclease. A guide RNA that specifically targets one or more portions of a genome of CMV within a cell or tissue of the transplant may be used. The CRISPR/Cas9 complex binds to and alters the CMV genome. In other embodiments, the invention may make use of a CRISPR/Cas9 nuclease and guide RNA (gRNA) that together target and selectively edit or destroy CMV genomic material. The CRISPR (clustered regularly interspaced short palindromic repeats) is an element of the bacterial immune system that protects bacteria from phage infection. The guide RNA localizes the CRISPR/Cas9 complex to a CMV target sequence. Binding of the complex localizes the Cas9 endonuclease to the CMV genomic target sequence causing breaks in the CMV genome. In a preferred embodiment, the guide RNA is designed to target multiple sites on the CMV genome in order to disrupt the CMV nucleic acid and reduce the chance that it will functionally recombine.

The presented methods allow for CMV genome destruction, which results in the inability of the virus to proliferate, with no observed cytotoxicity to the cells. Aspects of the invention provide for designing a CRISPR/gRNA/Cas9 complex to selectively target CMV genomic material (DNA or RNA), delivering the CRISPR/gRNA/Cas9 complex to a cell or tissue containing the CMV genome, and cutting the CMV genome in order to incapacitate the virus. The presented methods allows for targeted disruption of CMV genomic function or, in a preferred embodiment, digestion of CMV nucleic acid via multiple breaks caused by targeting multiple sites for nuclease action in the CMV genome. Aspects of the invention provide for transfection of a CRISPR/gRNA/Cas9 complex cocktail to completely suppressed CMV proliferation. Additional aspects and advantages of the invention will be apparent upon consideration of the following detailed description thereof.

Brief Description of the Drawings

FIG. 1 diagrams a method of targeting an HSV infection.

FIG. 2 is a map of an HSV genome.

FIG. 3 shows amplicons of HSV genomic regions before and after digestion with cas9.

FIG. 3 is a gel with lanes showing genomic DNA size bands for cells treated with the RL2, LATi, LATp, UL9, OriS, and US12 guide sequences with and without Cas9.

FIG. 4 shows the results of quantitative PCR assays showing different levels of decreasing of HSV DNA in the CRISPR treated samples.

FIG. 5 shows the EGFP marker fused after the Cas9 protein, allowing selection of Cas9-positive cells.

FIG. 6 shows that including an ori-P in the plasmid promoted active plasmid replication inside the cells, which increased the transfection efficiency to >60%.

FIG. 7 is a diagram of an EBV genome, with structure-, transformation-, and latency-related targets called out.

FIG. 8 shows the genome context around guide RNA sgEBV2 and PCR primer locations.

FIG. 9 shows the large deletion induced by sgEBV2 (lanes 1-3 are before, 5 days after, and 7 days after sgEBV2 treatment, respectively).

FIG. 10 shows the genome context around guide RNA sgEBV3/4/5 and PCR primer locations.

FIG. 11 shows the large deletions induced by sgEBV3/5 and sgEBV4/5. Lane 1 and 2 are 3F/5R PCR amplicons before and 8 days after sgEBV3/5 treatment. Lane 3 and 4 are 4F/5R PCR amplicons before and 8 days after sgEBV4/5 treatment.

FIG. 12 shows that Sanger sequencing confirmed genome cleavage and repair ligation 8 days after sgEBV3/5.

FIG. 13 shows that Sanger sequencing confirmed genome cleavage and repair ligation 8 days after sgEBV4/5.

FIG. 14 shows relative cell proliferation after targeting various combinations of regions in an EBV genome with guide RNAs.

FIG. 15 gives flow cytometry scattering signals from before sgEBV1-7 treatments.

FIG. 16 gives flow cytometry scattering signals from 5 days after sgEBV1-7 treatments

FIG. 17 gives flow cytometry scattering signals from 8 days after sgEBV1-7 treatments.

FIG. 18 shows Annexin V Alexa647 and DAPI staining results before sgEBV1-7 treatments.

FIG. 19 shows Annexin V Alexa647 and DAPI staining results 5 days after sgEBV1-7 treatments.

FIG. 20 shows Annexin V Alexa647 and DAPI staining results 8 days after sgEBV1-7 treatments.

FIGS. 21 and 22 show microscopy revealed apoptotic cell morphology after sgEBV1-7 treatment.

FIG. 23 shows nuclear morphology before sgEBV1-7 treatment.

FIGS. 24-26 show nuclear morphology after sgEBV1-7 treatment.

FIG. 27 shows EBV load after different CRISPR treatments by digital PCR. Cas9 and Cas9-oriP had two replicates, and sgEBV1-7 had 5 replicates.

FIGS. 28 shows a single Raji cell as captured on a microfluidic chip.

FIG. 29 shows a single sgEBV1-7 treated cell as captured on the chip.

FIG. 30 is a histogram of EBV quantitative PCR Ct values from single cells before treatment.

FIG. 31 is a histogram of EBV quantitative PCR Ct values from single live cells 7 days after sgEBV1-7 treatment.

FIG. 32 represents SURVEYOR assay of EBV CRISPR (lanes numbered from left to right: Lane 1: NEB 100bp ladder; Lane 2: sgEBV1 control; Lane 3: sgEBV1; Lane 4: sgEBV5 control; Lane 5: sgEBV5; Lane 6: sgEBV7 control; Lane 7: sgEBV7; Lane 8: sgEBV4).

FIG. 33 shows that the CRISPR treatments were not cytotoxic to the EBV-negative Burkitt's lymphoma cell line DG-75

FIG. 34 shows that the CRISPR treatments were not cytotoxic to primary human lung fibroblasts IMR90.

FIG. 35 shows ZFN being used to cut viral nucleic acid.

FIG. 36 shows a composition for treating a viral infection.

FIG. 37 depicts a flow chart of embodiments of the invention for targeting CMV.

FIG. 38 is a map of a CMV genome.

FIG. 39 depicts results from an in vitro CRISPR endonuclease assay, showing PCR amplicons from CMV genome and the corresponding products from in vitro CRISPR digestion (shown in pairs).

FIG. 40 depicts results from an in vitro CRISPR endonuclease assay, showing PCR amplicons from CMV genome and the corresponding products from in vitro CRISPR digestion (shown in pairs).

FIG. 41 depicts results from an in vitro CRISPR endonuclease assay, showing PCR amplicons from CMV genome and the corresponding products from in vitro CRISPR digestion (shown in pairs).

FIG. 42 shows a method 3201 for treating a cell 3237 to remove foreign nucleic acid.

Detailed Description

Embodiments of the invention relate to compositions and methods for treating HSV infections including HSV-1 and HSV-2 infections using guided nuclease systems targeted to specific regions of the HSV genome such as RL2, LAT, UL9, or OriS. Compositions of the invention disrupt HSV viral nucleic acid in host cells through nuclease activity without affecting the host's genome. Through disruption of the HSV genome in infected host cells, methods and compositions of the invention may eradicate even latent HSV infections.

Additionally or alternatively, embodiments of the invention related to methods for treating cytomegalovirus (CMV). Methods of the invention are used to incapacitate or disrupt CMV within a cell, a tissue, or a patient by systematically causing large or repeated insertions or deletions in the CMV genome, reducing the probability of reconstructing the full genome. The insertions or deletions in the genome incapacitates or destroys the virus, thus treating CMV. In some embodiments, the nuclease may be a CRISPR/Cas9 complex. In some embodiments, the nuclease is guided by a sequence, such as a guided RNA. In some embodiments, tissues, such as organs are treated with the nuclease prior to transplantation. In some embodiments, a transplant donor or recipient are treated before and after a transplantation surgery. In some embodiments, a patient infected with CMV is treated with the nuclease.

FIG. 1 diagrams a method of targeting an HSV infection. The method includes obtaining a targetable nuclease (e.g., as a protein or a gene for a nuclease). Any suitable nuclease can be used such as ZFN, TALENs, or meganucleases. In a preferred embodiment, the nuclease is Cas9. A sequence is provided that targets the nuclease to specific targets on the HSV genome such as oriS, UL9, RL2, or LAT. The nuclease gene and encoded gRNAs may be provided in a DNA vector, such as a plasmid or an adenovirus based vector, and the vector may further optionally include a promoter. That composition is then introduced into the HSV-infected cells. Any suitable transfection or delivery method may be used. Once in the cell, the genes are expressed and the Cas9 enzyme uses the gRNA to target, and cleave, the HSV genome. Since the gRNA is specific to the HSV genome with no match to the human genome according to methods and criteria described herein, the method leaves the host genome intact and does not interfere with normal human genetic function.

Discussion of HSV as well as regions for targeting can be found in Summers, et al., 2002, Herpes Simplex Virus Type 1 Origins of DNA Replication Play No Role in the Regulation of Flanking Promoters, J Virol., 76(14); Eom, et al., 2003, Replication-initiator protein (UL9) of the herpes simplex virus 1 binds NFB42 and is degraded via the ubiquitin–proteasome pathway, Proc Natl Acad Sci U S A, 100(17): 9803–9807; McGeoch, et al., 1991, Comparative sequence analysis of the long repeat regions and adjoining parts of the long unique regions in the genomes of herpes simplex viruses types 1 and 2, J Gen Virol., 72 (Pt 12):3057-75; the contents of each of which are incorporated by reference in their entirety for all purposes.

FIG. 2 shows the HSV genome and the HSV oriS palindrome dimer (oriS), RL2-ICP0 (RL2), LAT Intron (LATi), LAT promoter (LATp), UL9, and US12 genes which may be targeted by CRISPR guide RNAs.

FIG. 3 shows amplicons of HSV genomic regions before and after digestion with cas9 and corresponding guide RNAs. T7 in vitro transcription was used to produce the complete guide RNA with scaffold for RL2, LATi, LATp, UL9, oriS, and US12. Flanking regions of the genome targets were PCR amplified from HSV2 strain G genomic DNA (from ATCC). Cas9 protein (from PNA Bio), guide RNA and target DNA were mixed and incubated for in vitro endonuclease assay. High endonuclease activities were revealed by DNA gel electrophoresis of the digested DNA as shown in FIG. 3 which shows PCR amplicons of RL2, LATi, LATp, UL9, oriS, and US12 before and after digestion with cas9 and the guide RNA targeting each respective genomic region.

FIG. 37 depicts a flow chart of embodiments of the invention for targeting CMV. In general, the method 100 comprises introducing a nuclease 105 that targets the CMV genome. The nuclease can be introduced into an organ or a pregnant woman. The organ or the fetus can be suspected of having a CMV infection. The method further comprises causing the nuclease to target and cleave 110 a CMV nucleic acid. The nuclease is able to bind to and alter the CMV genome.

i. Treating infected cell

Cells may be treated with a nuclease encoded in a nucleic acid (e.g., delivered as mRNA or a plasmid) or delivered in active form. Where the nuclease is a CRISPR-associated protein such as Cas9, an active ribonucleoprotein (RNP) may be delivered to the cells.

FIG. 42 shows a method 3201 for treating a cell 3237 to remove foreign nucleic acid such as a viral nucleic acid 3251 from a herpes simplex virus. The method 3201 may be used to treat an infection, or the method 3201 may be used in vitro in research and development to remove foreign nucleic acid from subject cells such as cells from a human.

The method 3201 includes the steps of: forming 3225 a ribonucleoprotein (RNP) 3231 that includes a nuclease 3205 and an RNA 3213; obtaining a cell 3237 from a donor; delivering 3245 (preferably *in vitro*) the RNP 3231 to the cell 3237; and cleaving viral nucleic acid 3251 within the cell 3237 with the RNP 3231. The method 3201 may include providing the cell 3237 for transplantation into a patient.

The delivering 3245 may include electroporation, or the RNP may be packaged in a liposome for the delivering 3245. In some embodiments, the viral nucleic acid 3251 will exist as an episomal viral genome, i.e., an episome or episomal vector, of a virus. The RNA 3213 has a portion that is substantially complementary to a target within a viral nucleic acid 3251 and preferably not substantially complementary to any location on a human genome. In the preferred embodiments, the virus is a herpes family virus such as one selected from the group consisting of HSV-1, HSV-2, Varicella zoster virus, Epstein-Barr virus, and Cytomegalovirus. The virus may be in a latent stage in the cell.

In a preferred embodiment, the nuclease 3205 is a Crisper-associated protein such as, preferably, Cas9. The RNA 3213 may be a single guide RNA (sgRNA) (providing the functionality of crRNA and tracrRNA). In the preferred embodiment, the nuclease 3205 and the RNA 3213 are delivered to the cell as the RNP 3231.

In preferred embodiments, the cell 3237 has the viral nucleic acid 3251 therein, and the method further comprises cleaving the viral nucleic acid using the nuclease.

In some embodiments, it may be found that RNP is preferable (e.g., to plasmid DNA) for clinical applications, particularly for parenteral delivery. RNP is the active pre-formed drug which offers advantages to DNA (AAV) or mRNA. No need to transcribe, translate, or assemble drug components within cell. Delivery of RNP 3231 may offer improved drug properties, e.g. earlier onset activity and controlled clearance (toxicity).

FIG. 36 shows a composition for treating a viral infection according to certain embodiments. The composition preferably includes a vector (which may be a plasmid, linear DNA, or a viral vector) that codes for a nuclease and a targeting moiety (e.g., a gRNA) that targets the nuclease to HSV and may be complimentary to a genomic region of HSV such as RL2, LAT, UL9, or OriS. The composition may optionally include one or more of a promoter, replication origin, other elements, or combinations thereof as described further herein.

ii. Nuclease

Methods of the invention include using a programmable or targetable nuclease to specifically target viral nucleic acid for destruction. Any suitable targeting nuclease can be used including, for example, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeat (CRISPR) nucleases, meganucleases, other endo- or exo-nucleases, or combinations thereof. See Schiffer, 2012, Targeted DNA mutagenesis for the cure of chronic viral infections, J Virol 88(17):8920-8936, incorporated by reference.

CRISPR methodologies employ a nuclease, CRISPR-associated (Cas9), that complexes with small RNAs as guides (gRNAs) to cleave DNA in a sequence-specific manner upstream of the protospacer adjacent motif (PAM) in any genomic location. CRISPR may use separate guide RNAs known as the crRNA and tracrRNA. These two separate RNAs have been combined into a single RNA to enable site-specific mammalian genome cutting through the design of a short guide RNA. Cas9 and guide RNA (gRNA) may be synthesized by known methods. Cas9/guide-RNA (gRNA) uses a non-specific DNA cleavage protein Cas9, and an RNA oligo to hybridize to target and recruit the Cas9/gRNA complex. See Chang et al., 2013, Genome editing with RNA-guided Cas9 nuclease in zebrafish embryos, Cell Res 23:465-472; Hwang et al., 2013, Efficient genome editing in zebrafish using a CRISPR-Cas system, Nat. Biotechnol 31:227-229; Xiao et al., 2013, Chromosomal deletions and inversions mediated by TALENS and CRISPR/Cas in zebrafish, Nucl Acids Res 1-11.

CRISPR(Clustered Regularly Interspaced Short Palindromic Repeats) is found in bacteria and is believed to protect the bacteria from phage infection. It has recently been used as a means to alter gene expression in eukaryotic DNA, but has not been proposed as an anti-viral therapy or more broadly as a way to disrupt genomic material. Rather, it has been used to introduce insertions or deletions as a way of increasing or decreasing transcription in the DNA of a targeted cell or population of cells. See for example, Horvath et al., Science (2010) 327:167–170; Terns et al., Current Opinion in Microbiology (2011) 14:321–327; Bhaya et al. Annu Rev Genet (2011) 45:273–297; Wiedenheft et al. Nature (2012) 482:331–338); Jinek M et al. Science (2012) 337:816–821; Cong L et al. Science (2013) 339:819–823; Jinek M et al. (2013) eLife 2:e00471; Mali P et al. (2013) Science 339:823–826; Qi LS et al. (2013) Cell 152:1173–1183; Gilbert LA et al. (2013) Cell 154:442–451; Yang H et al. (2013) Cell 154:1370–1379; and Wang H et al. (2013) Cell 153:910–918); the contents of each of which are incorporated by reference in their entirety for all purposes.

In an aspect of the invention, the Cas9 endonuclease causes a double strand break in at least two locations in the genome. These two double strand breaks cause a fragment of the genome to be deleted. Even if viral repair pathways anneal the two ends, there will still be a deletion in the genome. One or more deletions using the mechanism will incapacitate the viral genome. The result is that the host cell will be free of viral infection.

In embodiments of the invention, nucleases cleave the genome of the target virus. A nuclease is an enzyme capable of cleaving the phosphodiester bonds between the nucleotide subunits of nucleic acids. Endonucleases are enzymes that cleave the phosphodiester bond within a polynucleotide chain. Some, such as Deoxyribonuclease I, cut DNA relatively nonspecifically (without regard to sequence), while many, typically called restriction endonucleases or restriction enzymes, cleave only at very specific nucleotide sequences. In a preferred embodiment of the invention, the Cas9 nuclease is incorporated into the compositions and methods of the invention, however, it should be appreciated that any nuclease may be utilized.

In preferred embodiments of the invention, the Cas9 nuclease is used to cleave the genome. The Cas9 nuclease is capable of creating a double strand break in the genome. The Cas9 nuclease has two functional domains: RuvC and HNH, each cutting a different strand. When both of these domains are active, the Cas9 causes double strand breaks in the genome.

In some embodiments of the invention, insertions into the genome can be designed to cause incapacitation, or altered genomic expression. Additionally, insertions/deletions are also used to introduce a premature stop codon either by creating one at the double strand break or by shifting the reading frame to create one downstream of the double strand break. Any of these outcomes of the NHEJ repair pathway can be leveraged to disrupt the target gene. The changes introduced by the use of the CRISPR/gRNA/Cas9 system are permanent to the genome.

In some embodiments of the invention, at least one insertion is caused by the CRISPR/gRNA/Cas9 complex. In a preferred embodiment, numerous insertions are caused in the genome, thereby incapacitating the virus. In an aspect of the invention, the number of insertions lowers the probability that the genome may be repaired.

In some embodiments of the invention, at least one deletion is caused by the CRISPR/gRNA/Cas9 complex. In a preferred embodiment, numerous deletions are caused in the genome, thereby incapacitating the virus. In an aspect of the invention, the number of deletions lowers the probability that the genome may be repaired. In a highly-preferred embodiment, the CRISPR/Cas9/gRNA system of the invention causes significant genomic disruption, resulting in effective destruction of the viral genome, while leaving the host genome intact.

TALENs uses a nonspecific DNA-cleaving nuclease fused to a DNA-binding domain that can be to target essentially any sequence. For TALEN technology, target sites are identified and expression vectors are made. Linearized expression vectors (e.g., by Notl) may be used as template for mRNA synthesis. A commercially available kit may be use such as the mMESSAGE mMACHINE SP6 transcription kit from Life Technologies (Carlsbad, CA). See Joung & Sander, 2013, TALENs: a widely applicable technology for targeted genome editing, Nat Rev Mol Cell Bio 14:49-55.

TALENs and CRISPR methods provide one-to-one relationship to the target sites, i.e. one unit of the tandem repeat in the TALE domain recognizes one nucleotide in the target site, and the crRNA, gRNA, or sgRNA of CRISPR/Cas system hybridizes to the complementary sequence in the DNA target. Methods can include using a pair of TALENs or a Cas9 protein with one gRNA to generate double-strand breaks in the target. The breaks are then repaired via non-homologous end-joining or homologous recombination (HR).

FIG. 35 shows ZFN being used to cut viral nucleic acid. Briefly, the ZFN method includes introducing into the infected host cell at least one vector (e.g., RNA molecule) encoding a targeted ZFN 305 and, optionally, at least one accessory polynucleotide. See, e.g., U.S. Pub. 2011/0023144 to Weinstein, incorporated by reference The cell includes target sequence 311. The cell is incubated to allow expression of the ZFN 305, wherein a double-stranded break 317 is introduced into the targeted chromosomal sequence 311 by the ZFN 305. In some embodiments, a donor polynucleotide or exchange polynucleotide 321 is introduced. Swapping a portion of the viral nucleic acid with irrelevant sequence can fully interfere transcription or replication of the viral nucleic acid. Target DNA 311 along with exchange polynucleotide 321 may be repaired by an error-prone non-homologous end-joining DNA repair process or a homology-directed DNA repair process.

Typically, a ZFN comprises a DNA binding domain (i.e., zinc finger) and a cleavage domain (i.e., nuclease) and this gene may be introduced as mRNA (e.g., 5′ capped, polyadenylated, or both). Zinc finger binding domains may be engineered to recognize and bind to any nucleic acid sequence of choice. See, e.g., Qu et al., 2013, Zinc-finger-nucleases mediate specific and efficient excision of HIV-1 proviral DAN from infected and latently infected human T cells, Nucl Ac Res 41(16):7771-7782, incorporated by reference. An engineered zinc finger binding domain may have a novel binding specificity compared to a naturally-occurring zinc finger protein. Engineering methods include, but are not limited to, rational design and various types of selection. A zinc finger binding domain may be designed to recognize a target DNA sequence via zinc finger recognition regions (i.e., zinc fingers). See for example, U.S. Pat. Nos. 6,607,882; 6,534,261 and 6,453,242, incorporated by reference. Exemplary methods of selecting a zinc finger recognition region may include phage display and two-hybrid systems, and are disclosed in U.S. Pat. 5,789,538; U.S. Pat. 5,925,523; U.S. Pat. 6,007,988; U.S. Pat. 6,013,453; U.S. Pat. 6,410,248; U.S. Pat. 6,140,466; U.S. Pat. 6,200,759; and U.S. Pat. 6,242,568, each of which is incorporated by reference.

A ZFN also includes a cleavage domain. The cleavage domain portion of the ZFNs may be obtained from any suitable endonuclease or exonuclease such as restriction endonucleases and homing endonucleases. See, for example, Belfort & Roberts, 1997, Homing endonucleases: keeping the house in order, Nucleic Acids Res 25(17):3379-3388. A cleavage domain may be derived from an enzyme that requires dimerization for cleavage activity. Two ZFNs may be required for cleavage, as each nuclease comprises a monomer of the active enzyme dimer. Alternatively, a single ZFN may comprise both monomers to create an active enzyme dimer. Restriction endonucleases present may be capable of sequence-specific binding and cleavage of DNA at or near the site of binding. Certain restriction enzymes (e.g., Type IIS) cleave DNA at sites removed from the recognition site and have separable binding and cleavage domains. For example, the Type IIS enzyme FokI, active as a dimer, catalyzes double-stranded cleavage of DNA, at 9 nucleotides from its recognition site on one strand and 13 nucleotides from its recognition site on the other. The FokI enzyme used in a ZFN may be considered a cleavage monomer. Thus, for targeted double-stranded cleavage using a FokI cleavage domain, two ZFNs, each comprising a FokI cleavage monomer, may be used to reconstitute an active enzyme dimer. See Wah, et al., 1998, Structure of FokI has implications for DNA cleavage, PNAS 95:10564-10569; U.S. Pat. 5,356,802; U.S. Pat. 5,436,150; U.S. Pat. 5,487,994; U.S. Pub. 2005/0064474; U.S. Pub. 2006/0188987; and U.S. Pub. 2008/0131962, each incorporated by reference.

Virus targeting using ZFN may include introducing at least one donor polynucleotide comprising a sequence into the cell. A donor polynucleotide preferably includes the sequence to be introduced flanked by an upstream and downstream sequence that share sequence similarity with either side of the site of integration in the chromosome. The upstream and downstream sequences in the donor polynucleotide are selected to promote recombination between the chromosomal sequence of interest and the donor polynucleotide. Typically, the donor polynucleotide will be DNA. The donor polynucleotide may be a DNA plasmid, a bacterial artificial chromosome (BAC), a yeast artificial chromosome (YAC), a viral vector, a linear piece of DNA, a PCR fragment, a naked nucleic acid, and may employ a delivery vehicle such as a liposome. The sequence of the donor polynucleotide may include exons, introns, regulatory sequences, or combinations thereof. The double stranded break is repaired via homologous recombination with the donor polynucleotide such that the desired sequence is integrated into the chromosome. In the ZFN-mediated process, a double stranded break introduced into the target sequence by the ZFN is repaired, via homologous recombination with the exchange polynucleotide, such that the sequence in the exchange polynucleotide may be exchanged with a portion of the target sequence. The presence of the double stranded break facilitates homologous recombination and repair of the break. The exchange polynucleotide may be physically integrated or, alternatively, the exchange polynucleotide may be used as a template for repair of the break, resulting in the exchange of the sequence information in the exchange polynucleotide with the sequence information in that portion of the target sequence. Thus, a portion of the viral nucleic acid may be converted to the sequence of the exchange polynucleotide. ZFN methods can include using a vector to deliver a nucleic acid molecule encoding a ZFN and, optionally, at least one exchange polynucleotide or at least one donor polynucleotide to the infected cell.

Meganucleases are endodeoxyribonucleases characterized by a large recognition site (double-stranded DNA sequences of 12 to 40 base pairs); as a result this site generally occurs only once in any given genome. For example, the 18-base pair sequence recognized by the I-SceI meganuclease would on average require a genome twenty times the size of the human genome to be found once by chance (although sequences with a single mismatch occur about three times per human-sized genome). Meganucleases are therefore considered to be the most specific naturally occurring restriction enzymes. Meganucleases can be divided into five families based on sequence and structure motifs: LAGLIDADG, GIY-YIG, HNH, His-Cys box and PD-(D/E)XK. The most well studied family is that of the LAGLIDADG proteins, which have been found in all kingdoms of life, generally encoded within introns or inteins although freestanding members also exist. The sequence motif, LAGLIDADG, represents an essential element for enzymatic activity. Some proteins contained only one such motif, while others contained two; in both cases the motifs were followed by ~75-200 amino acid residues having little to no sequence similarity with other family members. Crystal structures illustrates mode of sequence specificity and cleavage mechanism for the LAGLIDADG family: (i) specificity contacts arise from the burial of extended β-strands into the major groove of the DNA, with the DNA binding saddle having a pitch and contour mimicking the helical twist of the DNA; (ii) full hydrogen bonding potential between the protein and DNA is never fully realized; (iii) cleavage to generate the characteristic 4-nt 3'-OH overhangs occurs across the minor groove, wherein the scissile phosphate bonds are brought closer to the protein catalytic core by a distortion of the DNA in the central "4-base" region; (iv) cleavage occurs via a proposed two-metal mechanism, sometimes involving a unique "metal sharing" paradigm; (v) and finally, additional affinity and/or specificity contacts can arise from "adapted" scaffolds, in regions outside the core α/β fold. See Silva et al., 2011, Meganucleases and other tools for targeted genome engineering, Curr Gene Ther 11(1):11-27, incorporated by reference.

In some embodiments of the invention, a template sequence is inserted into the genome. In order to introduce nucleotide modifications to genomic DNA, a DNA repair template containing the desired sequence must be present during homology directed repair (HDR). The DNA template is normally transfected into the cell along with the gRNA/Cas9. The length and binding position of each homology arm is dependent on the size of the change being introduced. In the presence of a suitable template, HDR can introduce significant changes at the Cas9 induced double strand break.

Some embodiments of the invention may utilize modified version of a nuclease. Modified versions of the Cas9 enzyme containing a single inactive catalytic domain, either RuvC- or HNH-, are called ‘nickases’. With only one active nuclease domain, the Cas9 nickase cuts only one strand of the target DNA, creating a single-strand break or 'nick'. Similar to the inactive dCas9 (RuvC- and HNH-), a Cas9 nickase is still able to bind DNA based on gRNA specificity, though nickases will only cut one of the DNA strands. The majority of CRISPR plasmids are derived from S. pyogenes and the RuvC domain can be inactivated by a D10A mutation and the HNH domain can be inactivated by an H840A mutation.

A single-strand break, or nick, is normally quickly repaired through the HDR pathway, using the intact complementary DNA strand as the template. However, two proximal, opposite strand nicks introduced by a Cas9 nickase are treated as a double strand break, in what is often referred to as a 'double nick' or 'dual nickase' CRISPR system. A double-nick induced double strain break can be repaired by either NHEJ or HDR depending on the desired effect on the gene target. At these double strain breaks, insertions and deletions are caused by the CRISPR/Cas9 complex. In an aspect of the invention, a deletion is caused by positioning two double strand breaks proximate to one another, thereby causing a fragment of the genome to be deleted.

iii. Targeting moiety

A nuclease may use the targeting specificity of a guide RNA (gRNA). As discussed below, guide RNAs or single guide RNAs are specifically designed to target a virus genome.

A CRISPR/Cas9 gene editing complex of the invention works optimally with a guide RNA that targets the viral genome. Guide RNA (gRNA) (which includes single guide RNA (sgRNA), crisprRNA (crRNA), transactivating RNA (tracrRNA), any other targeting oligo, or any combination thereof) leads the CRISPR/Cas9 complex to the viral genome in order to cause viral genomic disruption. In an aspect of the invention, CRISPR/Cas9/gRNA complexes are designed to target specific viruses within a cell. It should be appreciated that any virus can be targeted using the composition of the invention. Identification of specific regions of the virus genome aids in development and designing of CRISPR/Cas9/gRNA complexes.

In an aspect of the invention, the CRISPR/Cas9/gRNA complexes are designed to target latent viruses within a cell. Once transfected within a cell, the CRISPR/Cas9/gRNA complexes cause repeated insertions or deletions to render the genome incapacitated, or due to number of insertions or deletions, the probability of repair is significantly reduced.

As an example, the Epstein–Barr virus (EBV), also called human herpesvirus 4 (HHV-4) is inactivated in cells by a CRISPR/Cas9/gRNA complex of the invention. EBV is a virus of the herpes family, and is one of the most common viruses in humans. The virus is approximately 122 nm to 180 nm in diameter and is composed of a double helix of DNA wrapped in a protein capsid. In this example, the Raji cell line serves as an appropriate in vitro model. The Raji cell line is the first continuous human cell line from hematopoietic origin and cell lines produce an unusual strain of Epstein-Barr virus while being one of the most extensively studied EBV models. To target the EBV genomes in the Raji cells, a CRISPR/Cas9 complex with specificity for EBV is needed. The design of EBV-targeting CRISPR/Cas9 plasmids consisting of a U6 promoter driven chimeric guide RNA (sgRNA) and a ubiquitous promoter driven Cas9 that were obtained from Addgene, Inc. Commercially available guide RNAs and Cas9 nucleases may be used with the present invention. An EGFP marker fused after the Cas9 protein allowed selection of Cas9-positive cells.

In an aspect of the invention, guide RNAs are designed, whether or not commercially purchased, to target a specific viral genome. The viral genome is identified and guide RNA to target selected portions of the viral genome are developed and incorporated into the composition of the invention. In an aspect of the invention, a reference genome of a particular strain of the virus is selected for guide RNA design.

For example, guide RNAs that target the EBV genome are a component of the system in the present example. In relation to EBV, for example, the reference genome from strain B95-8 was used as a design guide. Within a genome of interest, such as EBV, selected regions, or genes are targeted. For example, six regions can be targeted with seven guide RNA designs for different genome editing purposes.

FIG. 7 is a diagram of an EBV genome, with structure-, transformation-, and latency-related targets called out. FIG. 7 additionally shows where sgEBV1, sgEBV2, sgEBV3, sgEBV4/5, sgEBV6, and sgEBV7 target the EBV genome.

In relation to EBV, EBNA1 is the only nuclear Epstein-Barr virus (EBV) protein expressed in both latent and lytic modes of infection. While EBNA1 is known to play several important roles in latent infection, EBNA1 is crucial for many EBV functions including gene regulation and latent genome replication. Therefore, guide RNAs sgEBV4 and sgEBV5 were selected to target both ends of the EBNA1 coding region in order to excise this whole region of the genome. These “structural” targets enable systematic digestion of the EBV genome into smaller pieces. EBNA3C and LMP1 are essential for host cell transformation, and guide RNAs sgEBV3 and sgEBV7 were designed to target the 5’ exons of these two proteins respectively.

iv. Introduce to cell

Methods of the invention include introducing into a cell a nuclease and a sequence-specific targeting moiety. The nuclease is targeted to viral nucleic acid by means of the sequence-specific targeting moiety where it then cleaves the viral nucleic acid without interfering with a host genome. Any suitable method can be used to deliver the nuclease to the infected cell or tissue. For example, the nuclease or the gene encoding the nuclease may be delivered by injection, orally, or by hydrodynamic delivery. The nuclease or the gene encoding the nuclease may be delivered to systematic circulation or may be delivered or otherwise localized to a specific tissue type. The nuclease or gene encoding the nuclease may be modified or programmed to be active under only certain conditions such as by using a tissue-specific promoter so that the encoded nuclease is preferentially or only transcribed in certain tissue types.

In some embodiments, specific CRISPR/Cas9/gRNA complexes are introduced into a cell. A guide RNA is designed to target at least one category of sequences of the viral genome. In addition to latent infections this invention can also be used to control actively replicating viruses by targeting the viral genome before it is packaged or after it is ejected.

In some embodiments, a cocktail of guide RNAs may be introduced into a cell. The guide RNAs are designed to target numerous categories of sequences of the viral genome. By targeting several areas along the genome, the double strand break at multiple locations fragments the genome, lowering the possibility of repair. Even with repair mechanisms, the large deletions render the virus incapacitated.

In some embodiments, several guide RNAs are added to create a cocktail to target different categories of sequences. For example, two, five, seven or eleven guide RNAs may be present in a CRISPR cocktail targeting three different categories of sequences. However, any number of gRNAs may be introduced into a cocktail to target categories of sequences. In preferred embodiments, the categories of sequences are important for genome structure, host cell transformation, and infection latency, respectively.

In some aspects of the invention, in vitro experiments allow for the determination of the most essential targets within a viral genome. For example, to understand the most essential targets for effective incapacitation of a genome, subsets of guide RNAs are transfected into model cells. Assays can determine which guide RNAs or which cocktail is the most effective at targeting essential categories of sequences.

For example, in the case of the EBV genome targeting, seven guide RNAs in the CRISPR cocktail targeted three different categories of sequences which are identified as being important for EBV genome structure, host cell transformation, and infection latency, respectively. To understand the most essential targets for effective EBV treatment, Raji cells were transfected with subsets of guide RNAs.

FIG. 14 shows relative cell proliferation after targeting various combinations of regions in an EBV genome with guide RNAs. Although sgEBV4/5 reduced the EBV genome by 85%, they could not suppress cell proliferation as effectively as the full cocktail (FIG. 14). Guide RNAs targeting the structural sequences (sgEBV1/2/6) could stop cell proliferation completely, despite not eliminating the full EBV load (26% decrease). Given the high efficiency of genome editing and the proliferation arrest, it was suspect that the residual EBV genome signature in sgEBV1/2/6 was not due to intact genomes but to free-floating DNA that has been digested out of the EBV genome, i.e. as a false positive.

Once CRISPR/Cas9/gRNA complexes are constructed, the complexes are introduced into a cell. It should be appreciated that complexes can be introduced into cells in an in vitro model or an in vivo model. In an aspect of the invention, CRISPR/Cas9/gRNA complexes are designed to not leave intact genomes of a virus after transfection and complexes are designed for efficient transfection.

Aspects of the invention allow for CRISPR/Cas9/gRNA to be transfected into cells by various methods, including viral vectors and non-viral vectors. Viral vectors may include retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses. It should be appreciated that any viral vector may be incorporated into the present invention to effectuate delivery of the CRISPR/Cas9/gRNA complex into a cell. Some viral vectors may be more effective than others, depending on the CRISPR/Cas9/gRNA complex designed for digestion or incapacitation. In an aspect of the invention, the vectors contain essential components such as origin of replication, which is necessary for the replication and maintenance of the vector in the host cell.

In an aspect of the invention, viral vectors are used as delivery vectors to deliver the complexes into a cell. Use of viral vectors as delivery vectors are known in the art. See for example U.S. Pub. 2009/0017543 to Wilkes et al., the contents of which are incorporated by reference.

A retrovirus is a single-stranded RNA virus that stores its nucleic acid in the form of an mRNA genome (including the 5' cap and 3' PolyA tail) and targets a host cell as an obligate parasite. In some methods in the art, retroviruses have been used to introduce nucleic acids into a cell. Once inside the host cell cytoplasm the virus uses its own reverse transcriptase enzyme to produce DNA from its RNA genome, the reverse of the usual pattern, thus retro (backwards). This new DNA is then incorporated into the host cell genome by an integrase enzyme, at which point the retroviral DNA is referred to as a provirus. For example, the recombinant retroviruses such as the Moloney murine leukemia virus have the ability to integrate into the host genome in a stable fashion. They contain a reverse transcriptase that allows integration into the host genome. Retroviral vectors can either be replication-competent or replication-defective. In some embodiments of the invention, retroviruses are incorporated to effectuate transfection into a cell, however the CRISPR/Cas9/gRNA complexes are designed to target the viral genome.

In some embodiments of the invention, lentiviruses, which are a subclass of retroviruses, are used as viral vectors. Lentiviruses can be adapted as delivery vehicles (vectors) given their ability to integrate into the genome of non-dividing cells, which is the unique feature of lentiviruses as other retroviruses can infect only dividing cells. The viral genome in the form of RNA is reverse-transcribed when the virus enters the cell to produce DNA, which is then inserted into the genome at a random position by the viral integrase enzyme. The vector, now called a provirus, remains in the genome and is passed on to the progeny of the cell when it divides.

As opposed to lentiviruses, adenoviral DNA does not integrate into the genome and is not replicated during cell division. Adenovirus and the related AAV would be potential approaches as delivery vectors since they do not integrate into the host's genome. In some aspects of the invention, only the viral genome to be targeted is effected by the CRISPR/Cas9/gRNA complexes, and not the host's cells. Adeno-associated virus (AAV) is a small virus that infects humans and some other primate species. AAV can infect both dividing and non-dividing cells and may incorporate its genome into that of the host cell. For example, because of its potential use as a gene therapy vector, researchers have created an altered AAV called self-complementary adeno-associated virus (scAAV). Whereas AAV packages a single strand of DNA and requires the process of second-strand synthesis, scAAV packages both strands which anneal together to form double stranded DNA. By skipping second strand synthesis scAAV allows for rapid expression in the cell. Otherwise, scAAV carries many characteristics of its AAV counterpart. Methods of the invention may incorporate herpesvirus, poxvirus, alphavirus, or vaccinia virus as a means of delivery vectors.

In certain embodiments of the invention, non-viral vectors may be used to effectuate transfection. Methods of non-viral delivery of nucleic acids include lipofection, nucleofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, artificial virions, and agent-enhanced uptake of DNA. Lipofection is described in e.g., U.S. Pat. Nos. 5,049,386, 4,946,787; and 4,897,355, the contents of each of which are incorporated by reference in their entirety for all purposes.) and lipofection reagents are sold commercially (e.g., Transfectam and Lipofectin). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides include those described in U.S. Pat. 7,166,298 to Jessee or U.S. Pat. 6,890,554 to Jesse, the contents of each of which are incorporated by reference. Delivery can be to cells (e.g. in vitro or ex vivo administration) or target tissues (e.g. in vivo administration).

Synthetic vectors are typically based on cationic lipids or polymers which can complex with negatively charged nucleic acids to form particles with a diameter in the order of 100 nm. The complex protects nucleic acid from degradation by nuclease. Moreover, cellular and local delivery strategies have to deal with the need for internalization, release, and distribution in the proper subcellular compartment. Systemic delivery strategies encounter additional hurdles, for example, strong interaction of cationic delivery vehicles with blood components, uptake by the reticuloendothelial system, kidney filtration, toxicity and targeting ability of the carriers to the cells of interest. Modifying the surfaces of the cationic non-virals can minimize their interaction with blood components, reduce reticuloendothelial system uptake, decrease their toxicity and increase their binding affinity with the target cells. Binding of plasma proteins (also termed opsonization) is the primary mechanism for RES to recognize the circulating nanoparticles. For example, macrophages, such as the Kupffer cells in the liver, recognize the opsonized nanoparticles via the scavenger receptor.

In some embodiments of the invention, non-viral vectors are modified to effectuate targeted delivery and transfection. PEGylation (i.e. modifying the surface with polyethyleneglycol) is the predominant method used to reduce the opsonization and aggregation of non-viral vectors and minimize the clearance by reticuloendothelial system, leading to a prolonged circulation lifetime after intravenous (i.v.) administration. PEGylated nanoparticles are therefore often referred as “stealth” nanoparticles. The nanoparticles that are not rapidly cleared from the circulation will have a chance to encounter infected cells.

However, PEG on the surface can decrease the uptake by target cells and reduce the biological activity. Therefore, to attach targeting ligand to the distal end of the PEGylated component is necessary; the ligand is projected beyond the PEG “shield” to allow binding to receptors on the target cell surface. When cationic liposome is used as gene carrier, the application of neutral helper lipid is helpful for the release of nucleic acid, besides promoting hexagonal phase formation to enable endosomal escape. In some embodiments of the invention, neutral or anionic liposomes are developed for systemic delivery of nucleic acids and obtaining therapeutic effect in experimental animal model. Designing and synthesizing novel cationic lipids and polymers, and covalently or noncovalently binding gene with peptides, targeting ligands, polymers, or environmentally sensitive moieties also attract many attentions for resolving the problems encountered by non-viral vectors. The application of inorganic nanoparticles (for example, metallic nanoparticles, iron oxide, calcium phosphate, magnesium phosphate, manganese phosphate, double hydroxides, carbon nanotubes, and quantum dots) in delivery vectors can be prepared and surface-functionalized in many different ways.

Guided nuclease systems of the invention may be administered alone or as an active ingredient in combination with pharmaceutically acceptable carriers, diluents, adjuvants and vehicles. Vectors may be incorporated into topical or intravenous formulations which may comprise a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject agents from one organ, or portion of the body, to another organ, or portion of the body. Each carrier should be compatible with the other ingredients of the formulation.

In certain embodiments, compositions of the invention may be encapsulated in hydrogels. In another embodiment, composition as disclosed herein can comprise lipid-based formulations. Any of the known lipid-based drug delivery systems can be used in the practice of the invention. For instance, multivesicular liposomes, multilamellar liposomes and unilamellar liposomes can all be used so long as a sustained release rate of the encapsulated active compound can be established. Such formulations may be used to modify the release profile of the guided nuclease compositions. Methods of making controlled release multivesicular liposome drug delivery systems are described in PCT Application Publication Nos: WO 9703652, WO 9513796, and WO 9423697, the contents of which are incorporated herein by reference.

Synthetic membrane vesicles may comprise a combination of phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. Examples of lipids useful in synthetic membrane vesicle production include phosphatidylglycerols, phosphatidylcholines, phosphatidylserines, phosphatidylethanolamines, sphingolipids, cerebrosides, and gangliosides, with preferable embodiments including egg phosphatidylcholine, dipalmitoylphosphatidylcholine, distearoylphosphatidyleholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylglycerol, and dioleoylphosphatidylglycerol.

In preparing lipid-based vesicles containing compositions of the invention, such variables as the efficiency of compound encapsulation, labiality of the compound, homogeneity and size of the resulting population of vesicles, active compound-to-lipid ratio, permeability, instability of the preparation, and pharmaceutical acceptability of the formulation should be considered.

In another embodiment, guided nuclease systems of the invention can be delivered in a vesicle, in particular a liposome (see Langer (1990) Science 249:1527-1533), the contents of which are incorporated by reference in their entirety for all purposes). In yet another embodiment, guided nuclease systems can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer (1990) supra). In another embodiment, polymeric materials can be used (see Howard et al. (1989) J. Neurosurg. 71 : 105, the contents of which are incorporated by reference in their entirety for all purposes). In another embodiment a vector of the invention can be administered so that it becomes intracellular, e.g., by use of a retroviral vector (see, for example, U.S. Pat. No. 4,980,286), or by direct injection, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see e.g., Joliot et al., 1991, Proc. Natl. Acad. Sci. USA 88: 1864-1868), the contents of which are incorporated by reference in their entirety for all purposes. Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

In some embodiments of the invention, targeted controlled-release systems responding to the unique environments of tissues and external stimuli are utilized. Gold nanorods have strong absorption bands in the near-infrared region, and the absorbed light energy is then converted into heat by gold nanorods, the so-called ‘photothermal effect’. Because the near-infrared light can penetrate deeply into tissues, the surface of gold nanorod could be modified with nucleic acids for controlled release. When the modified gold nanorods are irradiated by near-infrared light, nucleic acids are released due to thermo-denaturation induced by the photothermal effect. The amount of nucleic acids released is dependent upon the power and exposure time of light irradiation.

In some embodiments of the invention, liposomes are used to effectuate transfection into a cell or tissue. The pharmacology of a liposomal formulation of nucleic acid is largely determined by the extent to which the nucleic acid is encapsulated inside the liposome bilayer. Encapsulated nucleic acid is protected from nuclease degradation, while those merely associated with the surface of the liposome is not protected. Encapsulated nucleic acid shares the extended circulation lifetime and biodistribution of the intact liposome, while those that are surface associated adopt the pharmacology of naked nucleic acid once they disassociate from the liposome.

In some embodiments, the complexes of the invention are encapsulated in a liposome. Unlike small molecule drugs, nucleic acids cannot cross intact lipid bilayers, predominantly due to the large size and hydrophilic nature of the nucleic acid. Therefore, nucleic acids may be entrapped within liposomes with conventional passive loading technologies, such as ethanol drop method (as in SALP), reverse-phase evaporation method, and ethanol dilution method (as in SNALP).

In some embodiments, linear polyethylenimine (L-PEI) is used as a non-viral vector due to its versatility and comparatively high transfection efficiency. L-PEI has been used to efficiently deliver genes in vivo into a wide range of organs such as lung, brain, pancreas, retina, bladder as well as tumor. L-PEI is able to efficiently condense, stabilize and deliver nucleic acids in vitro and in vivo.

Low-intensity ultrasound in combination with microbubbles has recently acquired much attention as a safe method of gene delivery. Ultrasound shows tissue-permeabilizing effect. It is non-invasive and site-specific, and could make it possible to destroy tumor cells after systemic delivery, while leave nontargeted organs unaffected. Ultrasound-mediated microbubbles destruction has been proposed as an innovative method for noninvasive delivering of drugs and nucleic acids to different tissues. Microbubbles are used to carry a drug or gene until a specific area of interest is reached, and then ultrasound is used to burst the microbubbles, causing site-specific delivery of the bioactive materials. Furthermore, the ability of albumin-coated microbubbles to adhere to vascular regions with glycocalix damage or endothelial dysfunction is another possible mechanism to deliver drugs even in the absence of ultrasound. See Tsutsui et al., 2004, The use of microbubbles to target drug delivery, Cardiovasc Ultrasound 2:23, the contents of which are incorporated by reference. In ultrasound-triggered drug delivery, tissue-permeabilizing effect can be potentiated using ultrasound contrast agents, gas-filled microbubbles. The use of microbubbles for delivery of nucleic acids is based on the hypothesis that destruction of DNA-loaded microbubbles by a focused ultrasound beam during their microvascular transit through the target area will result in localized transduction upon disruption of the microbubble shell while sparing non-targeted areas.

Besides ultrasound-mediated delivery, magnetic targeting delivery could be used for delivery. Magnetic nanoparticles are usually entrapped in gene vectors for imaging the delivery of nucleic acid. Nucleic acid carriers can be responsive to both ultrasound and magnetic fields, i.e., magnetic and acoustically active lipospheres (MAALs). The basic premise is that therapeutic agents are attached to, or encapsulated within, a magnetic micro- or nanoparticle. These particles may have magnetic cores with a polymer or metal coating which can be functionalized, or may consist of porous polymers that contain magnetic nanoparticles precipitated within the pores. By functionalizing the polymer or metal coating it is possible to attach, for example, cytotoxic drugs for targeted chemotherapy or therapeutic DNA to correct a genetic defect. Once attached, the particle/therapeutic agent complex is injected into the bloodstream, often using a catheter to position the injection site near the target. Magnetic fields, generally from high-field, high-gradient, rare earth magnets are focused over the target site and the forces on the particles as they enter the field allow them to be captured and extravasated at the target.

Synthetic cationic polymer-based nanoparticles (~100 nm diameter) have been developed that offer enhanced transfection efficiency combined with reduced cytotoxicity, as compared to traditional liposomes. The incorporation of distinct layers composed of lipid molecules with varying physical and chemical characteristics into the polymer nanoparticle formulation resulted in improved efficiency through better fusion with cell membrane and entry into the cell, enhanced release of molecules inside the cell, and reduced intracellular degradation of nanoparticle complexes.

In some embodiments, the complexes are conjugated to nano-systems for systemic therapy, such as liposomes, albumin-based particles, PEGylated proteins, biodegradable polymer-drug composites, polymeric micelles, dendrimers, among others. See Davis et al., 2008, Nanotherapeutic particles: an emerging treatment modality for cancer, Nat Rev Drug Discov. 7(9):771–782, incorporated by reference. Long circulating macromolecular carriers such as liposomes, can exploit the enhanced permeability and retention effect for preferential extravasation from tumor vessels. In certain embodiments, the complexes of the invention are conjugated to or encapsulated into a liposome or polymerosome for delivery to a cell. For example, liposomal anthracyclines have achieved highly efficient encapsulation, and include versions with greatly prolonged circulation such as liposomal daunorubicin and pegylated liposomal doxorubicin. See Krishna et al., Carboxymethylcellulose-sodium based transdermal drug delivery system for propranolol, J Pharm Pharmacol. 1996 Apr; 48(4):367-70.

Liposomal delivery systems provide stable formulation, provide improved pharmacokinetics, and a degree of ‘passive’ or ‘physiological’ targeting to tissues. Encapsulation of hydrophilic and hydrophobic materials, such as potential chemotherapy agents, are known. See for example U.S. Pat. No. 5,466,468 to Schneider, which discloses parenterally administrable liposome formulation comprising synthetic lipids; U.S. Pat. No. 5,580,571, to Hostetler et al. which discloses nucleoside analogues conjugated to phospholipids; U.S. Pat. No. 5,626,869 to Nyqvist, which discloses pharmaceutical compositions wherein the pharmaceutically active compound is heparin or a fragment thereof contained in a defined lipid system comprising at least one amphiphatic and polar lipid component and at least one nonpolar lipid component.

Liposomes and polymerosomes can contain a plurality of solutions and compounds. In certain embodiments, the complexes of the invention are coupled to or encapsulated in polymersomes. As a class of artificial vesicles, polymersomes are tiny hollow spheres that enclose a solution, made using amphiphilic synthetic block copolymers to form the vesicle membrane. Common polymersomes contain an aqueous solution in their core and are useful for encapsulating and protecting sensitive molecules, such as drugs, enzymes, other proteins and peptides, and DNA and RNA fragments. The polymersome membrane provides a physical barrier that isolates the encapsulated material from external materials, such as those found in biological systems. Polymerosomes can be generated from double emulsions by known techniques, see Lorenceau et al., 2005, Generation of Polymerosomes from Double-Emulsions, Langmuir 21(20):9183-6, incorporated by reference.

Some embodiments of the invention provide for a gene gun or a biolistic particle delivery system. A gene gun is a device for injecting cells with genetic information, where the payload may be an elemental particle of a heavy metal coated with plasmid DNA. This technique may also be referred to as bioballistics or biolistics. Gene guns have also been used to deliver DNA vaccines. The gene gun is able to transfect cells with a wide variety of organic and non-organic species, such as DNA plasmids, fluorescent proteins, dyes, etc.

Aspects of the invention provide for numerous uses of delivery vectors. Selection of the delivery vector is based upon the cell or tissue targeted and the specific makeup of the CRISPR/Cas9/gRNA. For example, in the EBV example discussed above, since lymphocytes are known for being resistant to lipofection, nucleofection (a combination of electrical parameters generated by a device called Nucleofector, with cell-type specific reagents to transfer a substrate directly into the cell nucleus and the cytoplasm) was necessitated for DNA delivery into the Raji cells. The Lonza pmax promoter drives Cas9 expression as it offered strong expression within

Raji cells. At 24 hours after nucleofection, obvious EGFP signals were observed from a small proportion of cells through fluorescent microscopy.

FIG. 6 shows that including an ori-P in the plasmid promoted active plasmid replication inside the cells, which increased the transfection efficiency to >60%. The left panel shows that untreated cells and cells treated with a Cas9 exhibited similarly low number of cells with fluorescent markers. The right panels shows that the oriP promoted higher transfection.

The EGFP-positive cell population decreased dramatically, however, <10% transfection efficiency 48 hours after nucleofection was measured (FIG. 6). A CRISPR plasmid that included the EBV origin of replication sequence, oriP yielded a transfection efficiency >60% (FIG. 6).

Aspects of the invention utilize the CRISPR/Cas9/gRNA complexes for the targeted delivery. Common known pathways include transdermal, transmucal, nasal, ocular and pulmonary routes. Drug delivery systems may include liposomes, proliposomes, microspheres, gels, prodrugs, cyclodextrins, etc. Aspects of the invention utilize nanoparticles composed of biodegradable polymers to be transferred into an aerosol for targeting of specific sites or cell populations in the lung, providing for the release of the drug in a predetermined manner and degradation within an acceptable period of time. Controlled-release technology (CRT), such as transdermal and transmucosal controlled-release delivery systems, nasal and buccal aerosol sprays, drug-impregnated lozenges, encapsulated cells, oral soft gels, iontophoretic devices to administer drugs through skin, and a variety of programmable, implanted drug-delivery devices are used in conjunction with the complexes of the invention of accomplishing targeted and controlled delivery.

It should be appreciated that the CMV targeting nuclease can be delivered into a cell, organ, patient, or fetus by the techniques described herein and by techniques known in the art. The CMV targeting nucleases of the invention may be prepared for delivery by association (binding, encapsulating, etc.) with vectors and/or guided RNAs.

Aspects of the invention provide for delivering the CMV targeting nuclease across the placenta, or trans placental. As the conduit to the fetus, the placenta is both a drug target and a drug barrier. Alternatively, the nuclease can be introduced into the amniotic sac or into the fetus. For example, the nuclease can be introduced into the amniotic sac or into the fetus by injection. Alternatively, the fetus may be treated for CMV infection using the methods of the invention after birth, using any known technique in the art for delivering a therapeutic to an infant.

v. Cut viral nucleic acid

Once inside the cell, the CRISPR/Cas9/gRNA complexes target the viral genome. In an aspect of the invention, the complexes are targeted to viral genomes. In addition to latent infections this invention can also be used to control actively replicating viruses by targeting the viral genome before it is packaged or after it is ejected. In some embodiments, methods and compositions of the invention use a nuclease such as Cas9 to target latent viral genomes, thereby reducing the chances of proliferation. The nuclease may form a complex with a gRNA (e.g., crRNA + tracrRNA or sgRNA). The complex cuts the viral nucleic acid in a targeted fashion to incapacitate the viral genome. As discussed above, the Cas9 endonuclease causes a double strand break in the viral genome. By targeted several locations along the viral genome and causing not a single strand break, but a double strand break, the genome is effectively cut a several locations along the genome. In a preferred embodiment, the double strand breaks are designed so that small deletions are caused, or small fragments are removed from the genome so that even if natural repair mechanisms join the genome together, the genome is render incapacitated.

After introduction into a cell, the CRISPR/Cas9/gRNA complexes act on the viral genome, genes, transcripts, or other viral nucleic acid. The double-strand DNA breaks generated by CRISPR are repaired with small deletions. These deletions will disrupt the protein coding and hence create knockout effects.

The nuclease, or a gene encoding the nuclease, may be delivered into an infected cell by transfection. For example, the infected cell can be transfected with DNA that encodes Cas9 and gRNA (on a single piece or separate pieces). The gRNAs are designed to localize the Cas9 endonuclease at one or several locations along the viral genome. The Cas9 endonuclease causes double strand breaks in the genome, causing small fragments to be deleted from the viral genome. Even with repair mechanisms, the deletions render the viral genome incapacitated.

Cells and tissues treated with a nuclease according to the methods of the invention are then provided for transplantation. In some embodiments, organs are treated with the nuclease to render the tissue CMV free, prior to transplantation.

In some embodiments of the invention, the nucleases are prepared for use in organs for transplant. Organ transplantation is the moving of an organ from one body to another or from a donor site to another location on the person's own body, to replace the recipient's damaged or absent organ. Organ can also be created or re-grown from the person's own cells (stem cells, or cells extracted from the failing organs) or from cells of another person. Organs can either be from a living or cadaveric source. Organs that can be transplanted are the heart, kidneys, liver, lungs, pancreas, intestine, and thymus. Tissues include bones, tendons (both referred to as musculoskeletal grafts), cornea, skin, heart valves, nerves and veins. Cornea and musculoskeletal grafts are commonly transplanted tissues, or organs.

vi. Host genome

It will be appreciated that method and compositions of the invention can be used to target viral nucleic acid without interfering with host genetic material. Methods and compositions of the invention employ a targeting moiety such as a guide RNA that has a sequence that hybridizes to a target within the viral sequence. Methods and compositions of the invention may further use a targeted nuclease such as the cas9 enzyme, or a vector encoding such a nuclease, which uses the gRNA to bind exclusively to the viral genome and make double stranded cuts, thereby removing the viral sequence from the host.

Where the targeting moiety includes a guide RNA, the sequence for the gRNA, or the guide sequence, can be determined by examination of the viral sequence to find regions of about 20 nucleotides that are adjacent to a protospacer adjacent motif (PAM) and that do not also appear in the host genome adjacent to the protospacer motif.

Preferably a guide sequence that satisfies certain similarity criteria (e.g., at least 60% identical with identity biased toward regions closer to the PAM) so that a gRNA/cas9 complex made according to the guide sequence will bind to and digest specified features or targets in the viral sequence without interfering with the host genome. Preferably, the guide RNA corresponds to a nucleotide string next to a protospacer adjacent motif (PAM) (e.g., NGG, where N is any nucleotide) in the viral sequence. Preferably, the host genome lacks any region that (1) matches the nucleotide string according to a predetermined similarity criteria and (2) is also adjacent to the PAM. The predetermined similarity criteria may include, for example, a requirement of at least 12 matching nucleotides within 20 nucleotides 5' to the PAM and may also include a requirement of at least 7 matching nucleotides within 10 nucleotides 5' to the PAM. An annotated viral genome (e.g., from GenBank) may be used to identify features of the viral sequence and finding the nucleotide string next to a protospacer adjacent motif (PAM) in the viral sequence within a selected feature (e.g., a viral replication origin, a terminal repeat, a replication factor binding site, a promoter, a coding sequence, or a repetitive region) of the viral sequence. The viral sequence and the annotations may be obtained from a genome database.

Where multiple candidate gRNA targets are found in the viral genome, selection of the sequence to be the template for the guide RNA may favor the candidate target closest to, or at the 5' most end of, a targeted feature as the guide sequence. The selection may preferentially favor sequences with neutral (e.g., 40% to 60%) GC content. Additional background regarding the RNA-directed targeting by endonuclease is discussed in U.S. Pub. 2015/0050699; U.S. Pub. 20140356958; U.S. Pub. 2014/0349400; U.S. Pub. 2014/0342457; U.S. Pub. 2014/0295556; and U.S. Pub. 2014/0273037, the contents of each of which are incorporated by reference for all purposes. Due to the existence of human genomes background in the infected cells, a set of steps are provided to ensure high efficiency against the viral genome and low off-target effect on the human genome. Those steps may include (1) target selection within viral genome, (2) avoiding PAM+target sequence in host genome, (3) methodologically selecting viral target that is conserved across strains, (4) selecting target with appropriate GC content, (5) control of nuclease expression in cells, (6) vector design, (7) validation assay, others and various combinations thereof. A targeting moiety (such as a guide RNA) preferably binds to targets within certain categories such as (i) latency related targets, (ii) infection and symptom related targets, and (iii) structure related targets.

A first category of targets for gRNA includes latency-related targets. The viral genome requires certain features in order to maintain the latency. These features include, but not limited to, master transcription regulators, latency-specific promoters, signaling proteins communicating with the host cells, etc. If the host cells are dividing during latency, the viral genome requires a replication system to maintain genome copy level. Viral replication origin, terminal repeats, and replication factors binding to the replication origin are great targets. Once the functions of these features are disrupted, the viruses may reactivate, which can be treated by conventional antiviral therapies.

A second category of targets for gRNA includes infection-related and symptom-related targets. Virus produces various molecules to facilitate infection. Once gained entrance to the host cells, the virus may start lytic cycle, which can cause cell death and tissue damage (HBV). In certain cases, such as HPV16, cell products (E6 and E7 proteins) can transform the host cells and cause cancers. Disrupting the key genome sequences (promoters, coding sequences, etc) producing these molecules can prevent further infection, and/or relieve symptoms, if not curing the disease.

A third category of targets for gRNA includes structure-related targets. Viral genome may contain repetitive regions to support genome integration, replication, or other functions. Targeting repetitive regions can break the viral genome into multiple pieces, which physically destroys the genome.

Where the nuclease is a cas protein, the targeting moiety is a guide RNA. Each cas protein requires a specific PAM next to the targeted sequence (not in the guide RNA). This is the same as for human genome editing. The current understanding the guide RNA/nuclease complex binds to PAM first, then searches for homology between guide RNA and target genome. Sternberg et al., 2014, DNA interrogation by the CRISPR RNA-guided endonuclease Cas9, Nature 507(7490):62-67. Once recognized, the DNA is digested 3-nt upstream of PAM. These results suggest that off-target digestion requires PAM in the host DNA, as well as high affinity between guide RNA and host genome right before PAM.

It may be preferable to use a targeting moiety that targets portions of the viral genome that are highly conserved. Viral genomes are much more variable than human genomes. In order to target different strains, the guide RNA will preferably target conserved regions. As PAM is important to initial sequence recognition, it is also essential to have PAM in the conserved region.

In a preferred embodiment, methods of the invention are used to deliver a nucleic acid to cells. The nucleic acid delivered to the cells may include a gRNA having the determined guide sequence or the nucleic acid may include a vector, such as a plasmid, that encodes an enzyme that will act against the target genetic material. Expression of that enzyme allows it to degrade or otherwise interfere with the target genetic material. The enzyme may be a nuclease such as the Cas9 endonuclease and the nucleic acid may also encode one or more gRNA having the determined guide sequence.

The gRNA targets the nuclease to the target genetic material. Where the target genetic material includes the genome of a virus, gRNAs complementary to parts of that genome can guide the degredation of that genome by the nuclease, thereby preventing any further replication or even removing any intact viral genome from the cells entirely. By these means, latent viral infections can be targeted for eradication.

The host cells may grow at different rate, based on the specific cell type. High nuclease expression is necessary for fast replicating cells, whereas low expression help avoiding off-target cutting in non-infected cells. Control of nuclease expression can be achieved through several aspects. If the nuclease is expressed from a vector, having the viral replication origin in the vector can increase the vector copy number dramatically, only in the infected cells. Each promoter has different activities in different tissues. Gene transcription can be tuned by choosing different promoters. Transcript and protein stability can also be tuned by incorporating stabilizing or destabilizing (ubiquitin targeting sequence, etc) motif into the sequence.

Specific promoters may be used for the gRNA sequence, the nuclease (e.g., cas9), other elements, or combinations thereof. For example, in some embodiments, the gRNA is driven by a U6 promoter. A vector may be designed that includes a promoter for protein expression (e.g., using a promoter as described in the vector sold under the trademark PMAXCLONING by Lonza Group Ltd (Basel, Switzerland). A vector may be a plasmid (e.g., created by synthesis instrument 255 and recombinant DNA lab equipment). In certain embodiments, the plasmid includes a U6 promoter driven gRNA or chimeric guide RNA (sgRNA) and a ubiquitous promoter-driven cas9. Optionally, the vector may include a marker such as EGFP fused after the cas9 protein to allow for later selection of cas9+ cells. It is recognized that cas9 can use a gRNA (similar to the CRISPR RNA (crRNA) of the original bacterial system) with a complementary trans-activating crRNA (tracrRNA) to target viral sequences complementary to the gRNA. It has also been shown that cas9 can be programmed with a single RNA molecule, a chimera of the gRNA and tracrRNA. The single guide RNA (sgRNA) can be encoded in a plasmid and transcription of the sgRNA can provide the programming of cas9 and the function of the tracrRNA. See Jinek, 2012, A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity, Science 337:816-821 and especially figure 5A therein for background.

Using the above principles, methods and compositions of the invention may be used to target viral nucleic acid in an infected host without adversely influencing the host genome.

For additional background see Hsu, 2013, DNA targeting specificity of RNA-guided Cas9 nucleases, Nature Biotechnology 31(9):827-832; and Jinek, 2012, A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity, Science 337:816-821, the contents of each of which are incorporated by reference. Since the targeted locations are selected to be within certain categories such as (i) latency related targets, (ii) infection and symptom related targets, or (iii) structure related targets, cleavage of those sequences inactivates the virus and removes it from the host. Since the targeting RNA (the gRNA or sgRNA) is designed to satisfy according to similarity criteria that matches the target in the viral genetic sequence without any off-target matching the host genome, the latent viral genetic material is removed from the host without any interference with the host genome.

ii. Apoptotic pathway

In cases where a small number of cells are infected and it would suffice to ablate the entire cell (as well as the CMV genome), an aspect of the invention contemplates administration of a vector containing a promoter which is active in the latent viral state, wherein the promoter drives a cell-killing gene. HSV is a particularly interesting target for this approach as it has been estimated that only thousands to tens of thousands neurons are latently infected. See Hoshino et al., 2008, The number of herpes simplex virus-infected neurons and the number of viral genome copies per neuron correlate with latent viral load in ganglia, Virology 372(1):56-63, incorporated by reference. Examples of cell-killing genes include both (1) targetable nucleases that are targeted to the cell genome; and (2) apoptosis effectors such as BAX and BAK and proteins that destroy the integrity of the cell or mitochondrial membrane, such as alpha hemolysin. (Bayles, "Bacterial programmed cell death: making sense of a paradox," Nature Reviews Microbiology 12 pp.63-69 (2014)). Having a promoter that is only activated in latently infected cells could be used not only in this context but also be used to increase selectivity of nuclease therapy by making activity specific to infected cells; an example of such a promoter is Latency-Associated Promoter 1, or "LAP1". (Preston and Efstathiou, "Molecular Basis of HSV Latency and Reactivation", in Human Herpesviruses: Biology, Therapy and Immunoprophylaxis 2007.) In some embodiments, the invention provides methods and therapeutics that can be used to cause the death of host cells but only those cells that are infected. For example, the treatment can include delivering a gene for a protein that causes cell death, where the gene is under control of a viral regulatory element such as a promoter from the genome of the infecting virus or the gene is encoded in a vector that includes a viral origin of replication. Where the virus is present, the gene will be expressed and the gene product will cause the death of the cell. The gene can code for a protein important in apoptosis, or the gene can code for a nuclease that digests the host genome.

The apoptotic embodiments may be used to remove infected cells from within a sample that contains a mix of infected and uninfected cells. Using a targetable nuclease, a composition may be provided that includes a viral-driven promoter, a targetable nuclease, and guide RNAs that target the cellular (e.g., human) genome. In the presence of the virus, the nuclease will kill the cells. The sample will be left containing only uninfected cells.

An apoptosis protein may be used as the therapeutic. The therapeutic may be provided encoded within a vector, in which the vector also encodes a sequence that causes the therapeutic to be expressed within a cell that is infected by a virus. The sequence may be a regulatory element (e.g., a promoter and an origin of replication) from the genome of the virus. The therapeutic may provide a mechanism that selectively causes death of virus-infected cells. For example, a protein may be used that restores a deficient apoptotic pathway in the cell. The gene may be, for example, BAX, BAK, BCL-2, or alpha-hemolysin. Preferably, the therapeutic induces apoptosis in the cell that is infected by the virus and does not induce apoptosis in an uninfected cell.

In some embodiments, the invention provides a composition that includes a viral vector, plasmid, or other coding nucleic acid that encodes at least one gene that promotes apoptosis and at least one promoter associated a viral genome. Apoptosis regulator Bcl-2 is a family of proteins that govern mitochondrial outer membrane permeabilization (MOMP) and include pro-apoptotic proteins such as Bax, BAD, Bak, Bok, Bcl-rambo, Bcl-xs and BOK/Mtd.

Apoptosis regulator BAX, also known as bcl-2-like protein 4, is a protein that in humans is encoded by the BAX gene. BAX is a member of the Bcl-2 gene family. This protein forms a heterodimer with BCL2, and functions as an apoptotic activator. This protein is reported to interact with, and increase the opening of, the mitochondrial voltage-dependent anion channel (VDAC), which leads to the loss in membrane potential and the release of cytochrome c.

Bcl-2 homologous antagonist/killer is a protein that in humans is encoded by the BAK1 gene on chromosome 6. This protein localizes to mitochondria, and functions to induce apoptosis. It interacts with and accelerates the opening of the mitochondrial voltage-dependent anion channel, which leads to a loss in membrane potential and the release of cytochrome c.

Human genes encoding proteins that belong to this family include: BAK1, BAX, BCL2, BCL2A1, BCL2L1, BCL2L2, BCL2L10, BCL2L13, BCL2L14, BOK, and MCL1.

Incorporation by Reference

References and citations to other documents, such as patents, patent applications, patent publications, journals, books, papers, web contents, have been made throughout this disclosure. All such documents are hereby incorporated herein by reference in their entirety for all purposes.

Equivalents

Various modifications of the invention and many further embodiments thereof, in addition to those shown and described herein, will become apparent to those skilled in the art from the full contents of this document, including references to the scientific and patent literature cited herein. The subject matter herein contains important information, exemplification and guidance that can be adapted to the practice of this invention in its various embodiments and equivalents thereof.

Examples

Example 1: Targeting HSV

T7 in vitro transcription produced the complete guide RNA with scaffold. Flanking regions of the genome targets were PCR amplified from HSV2 strain G genomic DNA (from ATCC). Cas9 protein (from PNA Bio), guide RNA and target DNA were mixed and incubated for in vitro endonuclease assay.

To further test the efficiency against HSV within cells, we subcloned each HSV2 amplicon mentioned above into an expression vector. The same guide RNA sequences (RL2, LATi, LATp, UL9, OriS, and US12) were also cloned into a CRISPR plasmid, containing CMV promoter driven cas9 and U6 promoter driven sgRNA scaffold. We transfected both HSV2 amplicon clones and anti-HSV CRISPR plasmid into 293T cells with Lipofectamine 2000. 72 hours after transfection, cells were harvested for genomic DNA isolation.

FIG. 3 is a gel with lanes showing genomic DNA size bands for cells treated with the RL2, LATi, LATp, UL9, OriS, and US12 guide sequences with and without Cas9.

FIG. 4 shows the results of quantitative PCR assays showing different levels of decreasing of HSV DNA in the CRISPR treated samples. In vivo anti-HSV treatment with a transient cell model. We used a CRISPR plasmid with a scrambled sgRNA sequence as control. DNA sample input was normalized with each control sample. OriS demonstrated the highest viral DNA elimination activity followed by RL2, UL9, and LATi.

Example 2: Targeting EBV

Burkitt's lymphoma cell lines Raji, Namalwa, and DG-75 were obtained from ATCC and cultured in RPMI 1640 supplemented with 10% FBS and PSA, following ATCC recommendation. Human primary lung fibroblast IMR-90 was obtained from Coriell and cultured in Advanced DMEM/F-12 supplemented with 10% FBS and PSA.

Plasmids consisting of a U6 promoter driven chimeric guide RNA (sgRNA) and a ubiquitous promoter driven Cas9 were obtained from addgene, as described by Cong L et al. (2013) Multiplex Genome Engineering Using CRISPR/Cas Systems. Science 339:819–823. An EGFP marker fused after the Cas9 protein allowed selection of Cas9-positive cells (FIG. 6). We adapted a modified chimeric guide RNA design for more efficient Pol-III transcription and more stable stem-loop structure (Chen B et al. (2013) Dynamic Imaging of Genomic Loci in Living Human Cells by an Optimized CRISPR/Cas System. Cell 155:1479–1491).

We obtained pX458 from Addgene, Inc. A modified CMV promoter with a synthetic intron (pmax) was PCR amplified from Lonza control plasmid pmax-GFP. A modified guide RNA sgRNA(F+E) was ordered from IDT. EBV replication origin oriP was PCR amplified from B95-8 transformed lymphoblastoid cell line GM12891. We used standard cloning protocols to clone pmax, sgRNA(F+E) and oriP to pX458, to replace the original CAG promoter, sgRNA and f1 origin. We designed EBV sgRNA based on the B95-8 reference, and ordered DNA oligos from IDT. The original sgRNA place holder in pX458 serves as the negative control.

Lymphocytes are known for being resistant to lipofection, and therefore we used nucleofection for DNA delivery into Raji cells. We chose the Lonza pmax promoter to drive Cas9 expression as it offered strong expression within Raji cells. We used the Lonza Nucleofector II for DNA delivery. 5 million Raji or DG-75 cells were transfected with 5 ug plasmids in each 100-ul reaction. Cell line Kit V and program M-013 were used following Lonza recommendation. For IMR-90, 1 million cells were transfected with 5 ug plasmids in 100 ul

Solution V, with program T-030 or X-005. 24 hours after nucleofection, we observed obvious EGFP signals from a small proportion of cells through fluorescent microscopy. The EGFP-positive cell population decreased dramatically after that, however, and we measured <10% transfection efficiency 48 hours after nucleofection (FIG. 6). We attributed this transfection efficiency decrease to the plasmid dilution with cell division. To actively maintain the plasmid level within the host cells, we redesigned the CRISPR plasmid to include the EBV origin of replication sequence, oriP. With active plasmid replication inside the cells, the transfection efficiency rose to >60% (FIG. 6).

To design guide RNA targeting the EBV genome, we relied on the EBV reference genome from strain B95-8. We targeted six regions with seven guide RNA designs for different genome editing purposes.

Additional information such as primer design is shown in Wang and Quake, 2014, RNA-guided endonuclease provides a therapeutic strategy to cure latent herpesviridae infection, PNAS 111(36):13157-13162 and in the Supporting Information to that article published online at the PNAS website, and the contents of both of those documents are incorporated by reference for all purposes.

EBNA1 is crucial for many EBV functions including gene regulation and latent genome replication. Guide RNA sgEBV4 and sgEBV5 to both ends of the EBNA1 coding region in order to excise this whole region of the genome. Guide RNAs sgEBV1, 2 and 6 fall in repeat regions, so that the success rate of at least one CRISPR cut is multiplied. These "structural" targets enable systematic digestion of the EBV genome into smaller pieces. EBNA3C and LMP1 are essential for host cell transformation, and we designed guide RNAs sgEBV3 and sgEBV7 were designed to target the 5' exons of these two proteins respectively.

EBV Genome Editing. The double-strand DNA breaks generated by CRISPR are repaired with small deletions. FIGS. 8-13 represent CRISPR/Cas9 induced large deletions.

FIG. 8 shows the genome context around guide RNA sgEBV2 and PCR primer locations.

FIG. 9 shows the large deletion induced by sgEBV2. Lane 1-3 are before, 5 days after, and 7 days after sgEBV2 treatment, respectively.

FIG. 10 shows the genome context around guide RNA sgEBV3/4/5 and PCR primer locations.

FIG. 11 shows the large deletions induced by sgEBV3/5 and sgEBV4/5. Lane 1 and 2 are 3F/5R PCR amplicons before and 8 days after sgEBV3/5 treatment. Lane 3 and 4 are 4F/5R PCR amplicons before and 8 days after sgEBV4/5 treatment.

FIGS. 12 and 13 show that Sanger sequencing confirmed genome cleavage and repair ligation 8 days after sgEBV3/5 (FIG. 12) and sgEBV4/5 (FIG. 13) treatment. Areas 690 and 700 (FIG. 12) and areas 690 and 700 (FIG. 13) indicate the two ends before repair ligation.

These deletions will disrupt the protein coding and hence create knockout effects. SURVEYOR assays confirmed efficient editing of individual sites.

FIG. 32 represents SURVEYOR assay of EBV CRISPR (lanes numbered from left to right: Lane 1: NEB 100bp ladder; Lane 2: sgEBV1 control; Lane 3: sgEBV1; Lane 4: sgEBV5 control; Lane 5: sgEBV5; Lane 6: sgEBV7 control; Lane 7: sgEBV7; Lane 8: sgEBV4).

Beyond the independent small deletions induced by each guide RNA, large deletions between targeting sites can systematically destroy the EBV genome. Guide RNA sgEBV2 targets a region with twelve 125-bp repeat units (FIG. 8). PCR amplicon of the whole repeat region gave a ~1.8-kb band (FIG. 9). After 5 or 7 days of sgEBV2 transfection, we obtained ~0.4-kb bands from the same PCR amplification (FIG. 9). The ~1.4-kb deletion is the expected product of repair ligation between cuts in the first and the last repeat unit (FIG. 8).

DNA sequences flanking sgRNA targets were PCR amplified with Phusion DNA polymerase. SURVEYOR assays were performed following manufacturer's instruction. DNA amplicons with large deletions were TOPO cloned and single colonies were used for Sanger sequencing. EBV load was measured with Taqman digital PCR on Fluidigm BioMark. A Taqman assay targeting a conserved human locus was used for human DNA normalization. 1 ng of single-cell whole-genome amplification products from Fluidigm C1 were used for EBV quantitative PCR.

It is possible to delete regions between unique targets (FIG. 10). Six days after sgEBV4-5 transfection, PCR amplification of the whole flanking region (with primers EBV4F and 5R) returned a shorter amplicon, together with a much fainter band of the expected 2 kb (FIG. 11). Sanger sequencing of amplicon clones confirmed the direct connection of the two expected cutting sites (FIG. 13). A similar experiment with sgEBV3-5 also returned an even larger deletion, from EBNA3C to EBNA1 (FIG. 11).

Additional information such as primer design is shown in Wang and Quake, 2014, RNA-guided endonuclease provides a therapeutic strategy to cure latent herpesviridae infection, PNAS 111(36):13157-13162 and in the Supporting Information to that article published online at the PNAS website, and the contents of both of those documents are incorporated by reference for all purposes.

Cell Proliferation Arrest With EBV Genome Destruction. Two days after CRISPR transfection, EGFP-positive cells were flow sorted for further culture and counted the live cells daily. FIGS. 14-26 represent cell proliferation arrest with EBV genome destruction. FIG. 14 shows cell proliferation curves after different CRISPR treatments. Five independent sgEBV1-7 treatments are shown here. FIGS. 15-20 show flow cytometry scattering signals before (FIG. 15), 5 days after (FIG. 16) and 8 days after (FIG. 14) sgEBV1-7 treatments. FIG. 18-20 show Annexin V Alexa647 and DAPI staining results before (FIG. 18), 5 days after (FIG. 19) and 8 days after (FIG. 20) sgEBV1-7 treatments. Regions 300 and 200 correspond to subpopulation P3 and P4 in (FIGS. 15-17).

FIGS. 21 and 22 show microscopy revealed apoptotic cell morphology after sgEBV1-7 treatment.

FIG. 23 shows nuclear morphology before sgEBV1-7 treatment.

FIGS. 24-26 show nuclear morphology after sgEBV1-7 treatment.

As expected, cells treated with Cas9 plasmids which lacked oriP or sgEBV lost EGFP expression within a few days and proliferated with a rate similar rate to the untreated control group (FIG. 14). Plasmids with Cas9-oriP and a scrambled guide RNA maintained EGFP expression after 8 days, but did not reduce the cell proliferation rate. Treatment with the mixed cocktail sgEBV1-7 resulted in no measurable cell proliferation and the total cell count either remained constant or decreased (FIG. 14).

FIG. 15 shows that flow cytometry scattering signals clearly revealed alterations in the cell morphology after sgEBV1-7 treatment, as the majority of the cells shrank in size with increasing granulation (P4 to P3 shift).

FIG. 19 gives DAPI staining results showing that cells in population P3 also demonstrated compromised membrane permeability. To rule out the possibility of CRISPR cytotoxicity, especially with multiple guide RNAs, the same treatment was performed on two other samples: the EBV-negative Burkitt's lymphoma cell line DG-75 and primary human lung fibroblast IMR90.

FIG. 33 shows that the CRISPR treatments were not cytotoxic to the EBV-negative Burkitt's lymphoma cell line DG-75

FIG. 34 shows that the CRISPR treatments were not cytotoxic to primary human lung fibroblasts IMR90.

Eight and nine days after transfection the cell proliferation rates did not change from the untreated control groups, suggesting neglectable cytotoxicity.

Previous studies have attributed the EBV tumorigenic ability to its interruption of host cell apoptosis (Ruf IK et al. (1999) Epstein-Barr Virus Regulates c-MYC, Apoptosis, and Tumorigenicity in Burkitt Lymphoma. Molecular and Cellular Biology 19:1651–1660). Suppressing EBV activities may therefore restore the apoptosis process, which could explain the cell death observed in our experiment. Annexin V staining revealed a distinct subpopulation of cells with intact cell membrane but exposed phosphatidylserine, suggesting cell death through apoptosis (FIG. 18). Bright field microscopy showed obvious apoptotic cell morphology (FIG. 21) and fluorescent staining demonstrated drastic DNA fragmentation (FIG. 23). Altogether this evidence suggests restoration of the normal host cell apoptosis pathway after EBV genome destruction.

FIGS. 27-31 represent EBV load quantitation after CRISPR treatment.

FIG. 27 shows EBV load after different CRISPR treatments by digital PCR. Cas9 and Cas9-oriP had two replicates, and sgEBV1-7 had 5 replicates.

Complete Clearance Of EBV In A Subpopulation.

To study the potential connection between cell proliferation arrest and EBV genome editing, the EBV load was quantified in different samples with digital PCR targeting EBNA1. Another Taqman assay targeting a conserved human somatic locus served as the internal control for human DNA normalization. On average, each untreated Raji cell has 42 copies of EBV genome (FIG. 27). Cells treated with a Cas9 plasmid that lacked oriP or sgEBV did not have an obvious difference in EBV load difference from the untreated control. Cells treated with a Cas9-plasmid with oriP but no sgEBV had an EBV load that was reduced by ~50%. In conjunction with the prior observation that cells from this experiment did not show any difference in proliferation rate, we interpret this as likely due to competition for EBNA1 binding during plasmid replication. The addition of the guide RNA cocktail sgEBV1-7 to the transfection dramatically reduced the EBV load. Both the live and dead cells have >60% EBV decrease comparing to the untreated control.

Although seven guide RNAs were provided at the same molar ratio, the plasmid transfection and replication process is likely quite stochastic. Some cells will inevitably receive different subsets or mixtures of the guide RNA cocktail, which might affect the treatment efficiency. To control for such effects, the EBV load was measured at the single cell level by employing single-cell whole-genome amplification with an automated microfluidic system.

Freshly cultured Raji cells were loaded onto the microfluidic chip and captured 81 single cells.

For the sgEBV1-7 treated cells, the live cells were flow sorted eight days after transfection and captured 91 single cells.

FIGS. 28 shows a single Raji cell as captured on a microfluidic chip.

FIG. 29 shows a single sgEBV1-7 treated cell as captured on the chip.

Following manufacturer's instruction, ~150 ng amplified DNA was obtained from each single cell reaction chamber. For quality control purposes we performed 4-loci human somatic DNA quantitative PCR on each single cell amplification product (Wang J, Fan HC, Behr B, Quake SR (2012) Genome-wide single-cell analysis of recombination activity and de novo mutation rates in human sperm. Cell 150:402–412) and required positive amplification from at least one locus.

FIG. 30 is a histogram of EBV quantitative PCR Ct values from single cells before treatment. The dash line represents Ct values of one EBV genome per cell. A log-normal distribution of EBV load was displayed by the 69 untreated single-cell products that passed the quality control, with almost every cell displaying significant amounts of EBV genomic DNA.

We calibrated the quantitative PCR assay with a subclone of Namalwa Burkitt's lymphoma cells, which contain a single integrated EBV genome. The single-copy EBV measurements gave a Ct of 29.8, which enabled us to determine that the mean Ct of the 69 Raji single cell samples corresponded to 42 EBV copies per cells, in concordance with the bulk digital PCR measurement. For the sgEBV1-7 treated sample, 71 single-cell products passed the quality control and the EBV load distribution was dramatically wider.

FIG. 31 is a histogram of EBV quantitative PCR Ct values from single live cells 7 days after sgEBV1-7 treatment. The dash line represents Ct values of one EBV genome per cell.

While 22 cells had the same EBV load as the untreated cells, 19 cells had no detectable EBV and the remaining 30 cells displayed dramatic EBV load decrease from the untreated sample.

FIG. 32 represents SURVEYOR assay of EBV CRISPR. Lane 1 (lanes numbered from left to right): NEB 100bp ladder; Lane 2: sgEBV1 control; Lane 3: sgEBV1; Lane 4: sgEBV5 control; Lane 5: sgEBV5; Lane 6: sgEBV7 control; Lane 7: sgEBV7; Lane 8: sgEBV4. FIG. 33 represents CRISPR cytotoxicity test with EBV-negative Burkitt's lymphoma DG-75. FIG. 34 represents CRISPR cytotoxicity test with primary human lung fibroblast IMR-90.

Essential Targets For EBV Treatment. The seven guide RNAs in our CRISPR cocktail target three different categories of sequences which are important for EBV genome structure, host cell transformation, and infection latency, respectively. To understand the most essential targets for effective EBV treatment, we transfected Raji cells with subsets of guide RNAs. Although sgEBV4/5 reduced the EBV genome by 85%, they could not suppress cell proliferation as effectively as the full cocktail (FIG. 14). Guide RNAs targeting the structural sequences (sgEBV1/2/6) could stop cell proliferation completely, despite not eliminating the full EBV load (26% decrease). Given the high efficiency of genome editing and the proliferation arrest (FIG. 2), we suspect that the residual EBV genome signature in sgEBV1/2/6 was not due to intact genomes but to free-floating DNA that has been digested out of the EBV genome, i.e. as a false positive. We conclude that systematic destruction of EBV genome structure appears to be more effective than targeting specific key proteins for EBV treatment.

What is claimed is:

1. A composition for treatment of a herpes simplex virus (HSV) infection, the composition comprising a vector encoding:

a nuclease; and

a sequence-specific targeting moiety complementary to HSV nucleic acid and capable of directing the nuclease to the HSV nucleic acid.

2. The composition of claim 1, wherein the HSV nucleic acid is an origin of replication S (oriS) region.

3. The composition of claim 1, wherein the the HSV nucleic acid is a long unique region 9 (UL9) or long repeat region 2 (RL2).

4. The composition of claim 1, configured to be administered transdermally.

5. The composition of claim 1, wherein the composition comprises a topical solution.

6. The composition of claim 1, wherein the nuclease is one selected from the group consisting of a zinc-finger nuclease, a transcription activator-like effector nuclease, and a meganuclease.

7. The composition of claim 1, wherein the nuclease comprises a Cas9 endonuclease and the sequence-specific binding module comprises a guide RNA that specifically targets a portion of a viral genome.

8. The composition of claim 1, further being packaged for delivery to a human patient.

9. The composition of claim 1, wherein the targeting sequence is a guide RNA and has no match > 60% within a human genome.

10. The composition of claim 1, wherein the vector comprises one selected from the group consisting of: retrovirus, lentivirus, adenovirus, herpesvirus, poxvirus, alphavirus, vaccinia virus, adeno-associated viruses, a plasmid, a nanoparticle, a cationic lipid, a cationic polymer, metallic nanoparticle, a nanorod, a liposome, microbubbles, a cell-penetrating peptide, and a liposphere.

11. A method for treating a herpes simplex virus (HSV) infection, the method comprising:

introducing into a cell of a host, a vector encoding a nuclease and a sequence-specific targeting moiety complementary to HSV nucleic acid or a ribonucleoprotein that includes the nuclease and the sequence-specific targeting moiety;

causing the sequence-specific targeting moiety to direct the nuclease to the HSV nucleic acid; and

causing the nuclease to cleave the HSV nucleic acid.

12. The method of claim 11, wherein the HSV nucleic acid is an origin of replication S (oriS) region.

13. The method of claim 11, wherein the the HSV nucleic acid is a long unique region 9 (UL9) or long repeat region 2 (RL2).

14. The method of claim 11, further comprising transdermally administering the vector to the host.

15. The method of claim 14, further wherein the transdermal administration comprises applying a topical solution, the topical solution comprising the vector.

16. The method of claim 11, wherein the nuclease is one selected from the group consisting of a zinc-finger nuclease, a transcription activator-like effector nuclease, and a meganuclease.

17. The method of claim 11, wherein the nuclease comprises a Cas9 endonuclease and the sequence-specific binding module comprises a guide RNA that specifically targets a portion of a viral genome.

18. The method of claim 11, wherein the host is a living human subject and the steps are performed in vivo.

19. The method of claim 11, wherein the targeting sequence is a guide RNA and has no match > 60% within a human genome.

20. The method of claim 11, wherein the vector comprises one selected from the group consisting of: retrovirus, lentivirus, adenovirus, herpesvirus, poxvirus, alphavirus, vaccinia virus, adeno-associated viruses, a plasmid, a nanoparticle, a cationic lipid, a cationic polymer, metallic nanoparticle, a nanorod, a liposome, microbubbles, a cell-penetrating peptide, and a liposphere.

21. A composition for treatment of a herpes simplex virus (HSV) infection, the composition comprising a ribonucleoprotein that includes:

a nuclease; and

a sequence-specific targeting moiety complementary to HSV nucleic acid and capable of directing the nuclease to the HSV nucleic acid.

22. The composition of claim 21, wherein the HSV nucleic acid is an origin of replication S (oriS) region.

23. The composition of claim 21, wherein the the HSV nucleic acid is a long unique region 9 (UL9) or long repeat region 2 (RL2).

24. The composition of claim 21, configured to be administered transdermally.

25. The composition of claim 21, further comprising a pharmaceutically acceptable carrier for topical application to infected tissue.

27. The composition of claim 21, wherein the nuclease comprises a Cas9 endonuclease and the sequence-specific binding module comprises a guide RNA that specifically targets a portion of a viral genome.

29. The composition of claim 21, wherein the targeting sequence is a guide RNA and has no match > 60% within a human genome.

30. A composition for treatment of a cytomegalovirus (CMV) infection, the composition comprising:

a nuclease; and

a sequence-specific targeting moiety complementary to CMV nucleic acid and capable of directing the nuclease to the CMV nucleic acid.

31. The composition of claim 30, wherein the nuclease includes one selected from the group consisting of a zinc finger nuclease, a transcription activator-like effector nuclease, and a meganuclease.

32. The composition of claim 30, wherein the nuclease is a Cas9 endonuclease.

33. The composition of claim 32, wherein the nuclease further comprises a guide RNA that targets the Cas9 endonuclease to a portion of the CMV nucleic acid.

34. The composition of claim 30, wherein the nuclease causes at least one double strand breaks in the CMV nucleic acid.

35. The composition of claim 30, wherein the nuclease causes at least one insertion in the CMV nucleic acid.

36. A method of treating a cytomegalovirus (CMV) infection in an organ, the method comprising the steps of:

introducing into an organ a composition comprising a nuclease and a sequence-specific targeting moiety complementary to CMV nucleic acid and capable of directing the nuclease to the CMV nucleic acid; and

causing the nuclease to target and cleave CMV nucleic acid.

37. The method of claim 36, wherein the organ is from a transplant donor.

38. The method of claim 36, wherein the organ is selected from the group consisting of a heart, liver, kidney, eye, lung, pancreas, intestine, and thymus.

39. The method of claim 36, wherein the nuclease includes one selected from the group consisting of a zinc finger nuclease, a transcription activator-like effector nuclease, and a meganuclease.

40. The method of claim 36, wherein the nuclease is a Cas9 endonuclease.

41. The method of claim 40, wherein the nuclease further comprises a guide RNA that targets the Cas9 endonuclease to a portion of the CMV nucleic acid.

42. The method of claim 36, wherein the introducing step comprises delivering the nuclease in a viral vector for delivery.

43. The method of claim 42, wherein the viral vector is selected from the group consisting of retrovirus, lentivirus, adenovirus, herpesvirus, poxvirus, alphavirus, vaccinia virus and adeno-associated viruses.

44. The method of claim 36, wherein the introducing step comprises delivering the nuclease in a vector that includes one selected from the group consisting of a plasmid, a nanoparticle, a cationic lipid, a cationic polymer, metallic nanoparticle, a nanorod, a liposome, a cell-penetrating peptide, a liposphere, and polyethyleneglycol (PEG).

45. The method of claim 36, wherein the nuclease causes at least one double strand breaks in the CMV nucleic acid.

46. The method of claim 36, wherein the nuclease causes at least one insertion in the CMV nucleic acid.

47. A method of treating a cytomegalovirus (CMV) infection in a fetus, the method comprising the steps of:

introducing into a pregnant worman a composition comprising a nuclease and a sequence-specific targeting moiety complementary to CMV nucleic acid and capable of directing the nuclease to the CMV nucleic acid; and

causing the nuclease to target and cleave CMV nucleic acid.

48. The method of claim 47, wherein the nuclease includes one selected from the group consisting of a zinc finger nuclease, a transcription activator-like effector nuclease, and a meganuclease.

49. The method of claim 47, wherein the nuclease is a Cas9 endonuclease.

50. The method of claim 47, wherein the nuclease is associated with a guide RNA that targets the Cas9 endonuclease to a portion of the CMV nucleic acid.

51. The method of claim 47, wherein the introducing step comprises delivering the nuclease within a viral vector.

52. The method of claim 51, wherein the viral vector is selected from the group consisting of retrovirus, lentivirus, adenovirus, herpesvirus, poxvirus, alphavirus, vaccinia virus and adeno-associated viruses.

53. The method of claim 47, wherein the introducing step comprises delivering the nuclease with a vector that includes one selected from the group consisting of a plasmid, a nanoparticle, a

cationic lipid, a cationic polymer, metallic nanoparticle, a nanorod, a liposome, a cell-penetrating peptide, a liposphere, and polyethyleneglycol (PEG).

54. The method of claim 47, wherein the nuclease causes at least one double strand breaks in the CMV nucleic acid.

55. The method of claim 47, wherein the nuclease causes at least one insertion in the CMV nucleic acid.

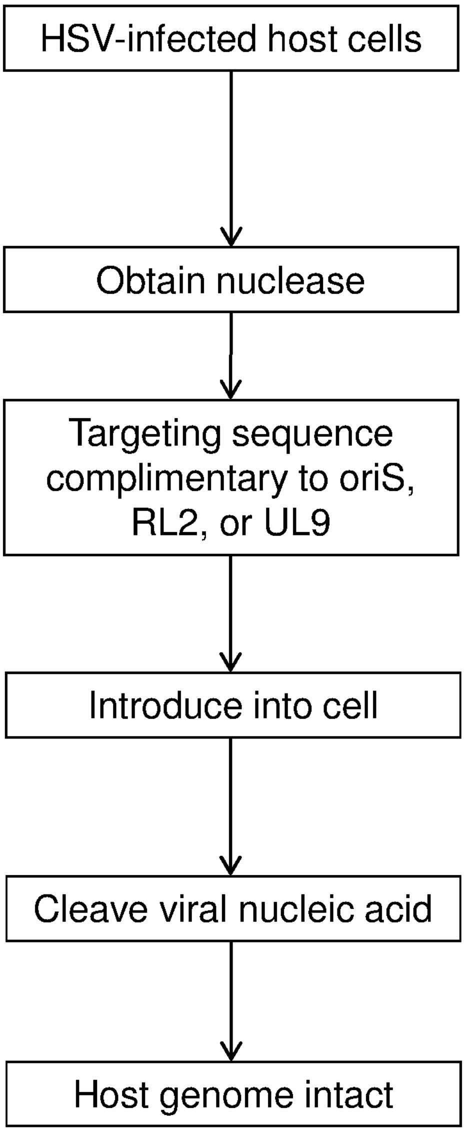
HSV-infected host cells
Obtain nuclease
Targeting sequence complimentary to oriS, RL2, or UL9
Introduce into cell
Cleave viral nucleic acid
Host genome intact

FIG. 1

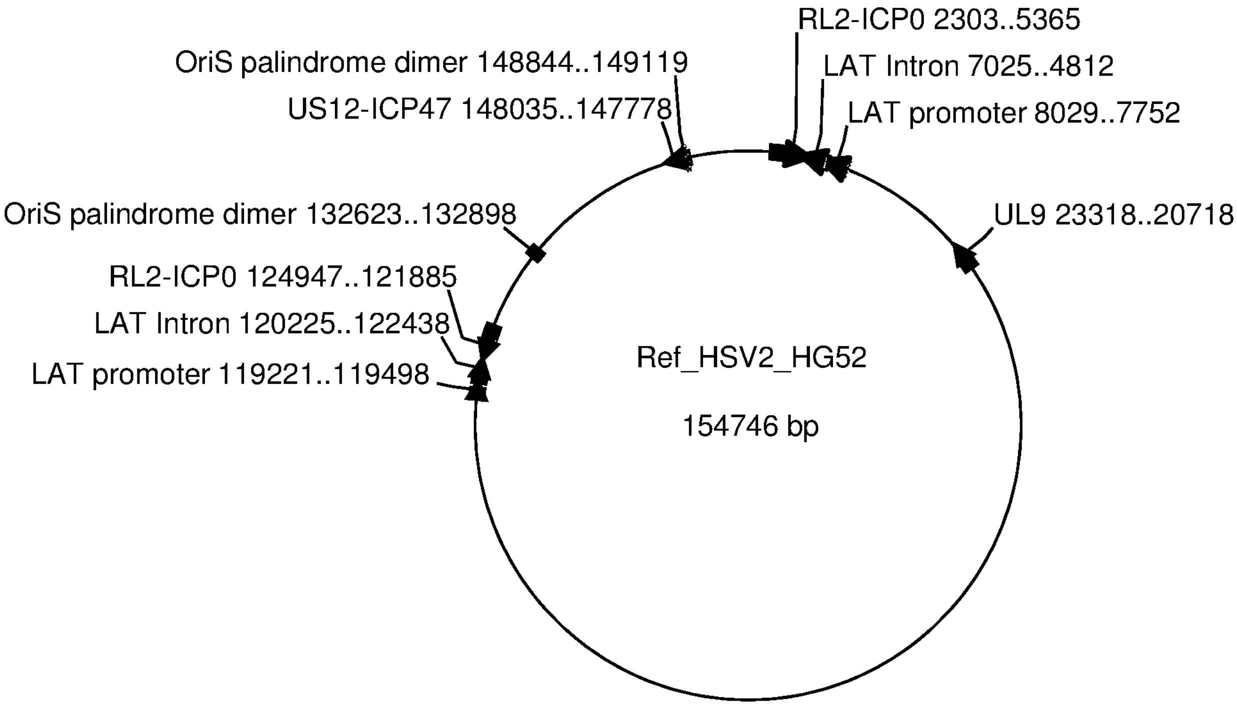
RL2-ICP0 2303..5365
OriS palindrome dimer 148844..149119
LAT Intron 7025..4812
US12-ICP47 148035..147778
LAT promoter 8029..7752
OriS palindrome dimer 132623..132898
UL9 23318..20718
RL2-ICP0 124947..121885
LAT Intron 120225..122438
LAT promoter 119221..119498
Ref_HSV2_HG52
154746 bp

FIG. 2

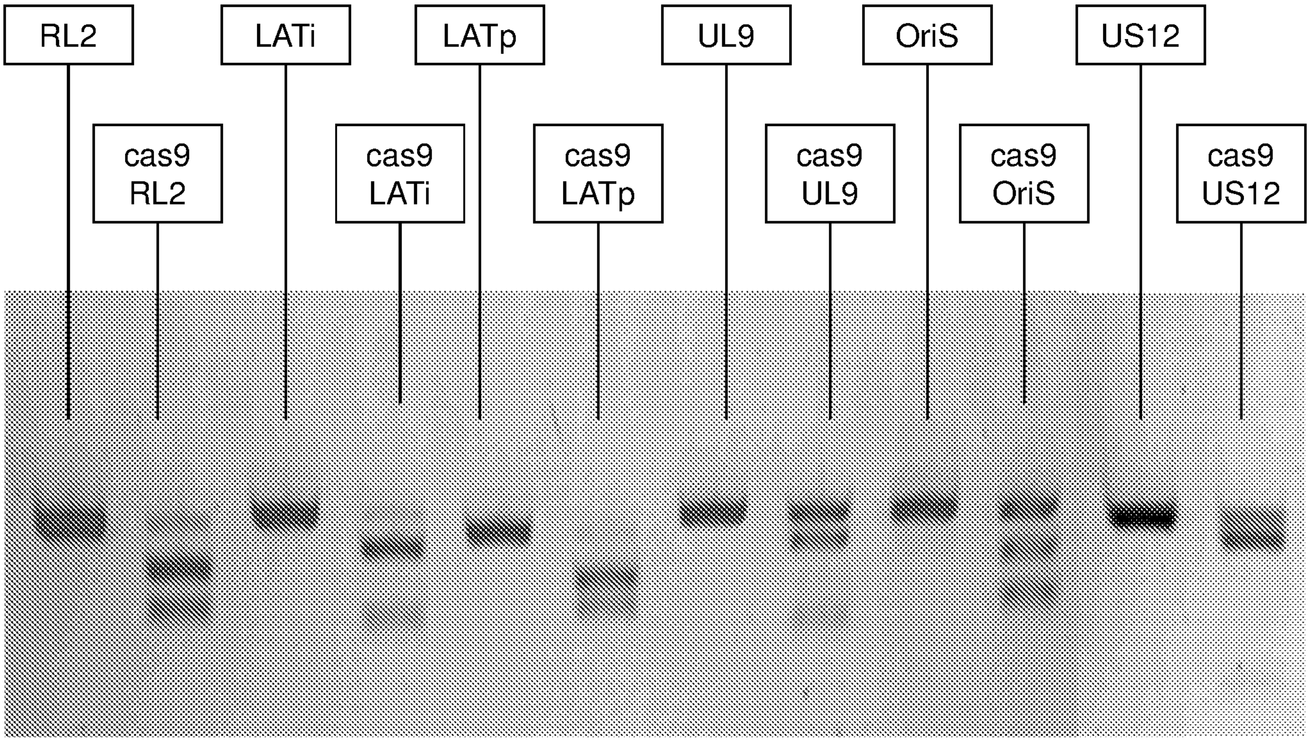
RL2
cas9 RL2
LATi
cas9 LATi
LATp
cas9 LATp
UL9
cas9 UL9
OriS
cas9 OriS
US12
cas9 US12

FIG. 3

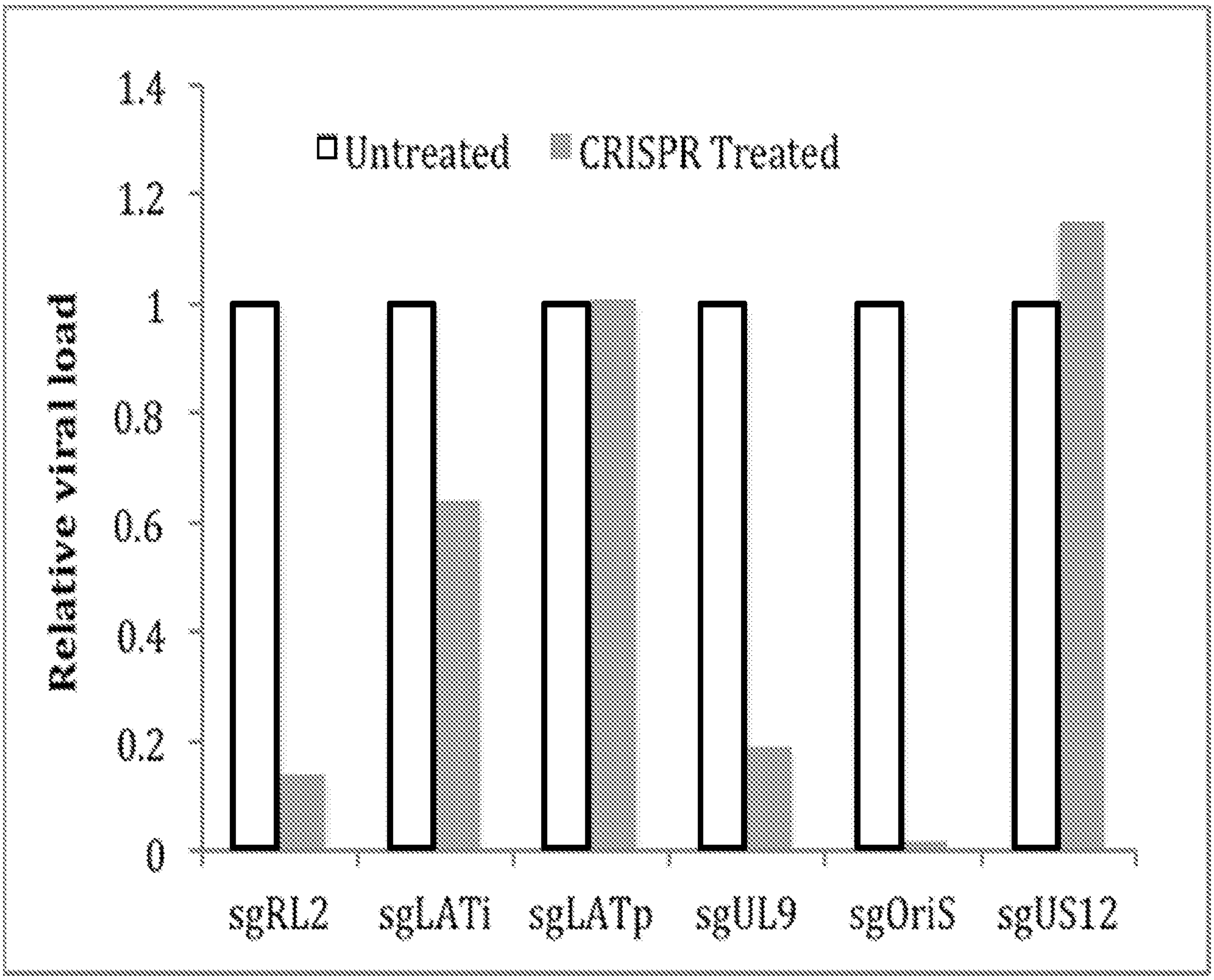
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative viral load
Untreated
CRISPR Treated
sgRL2
sgLATi
sgLATp
sgUL9
sgOriS
sgUS12

FIG. 4

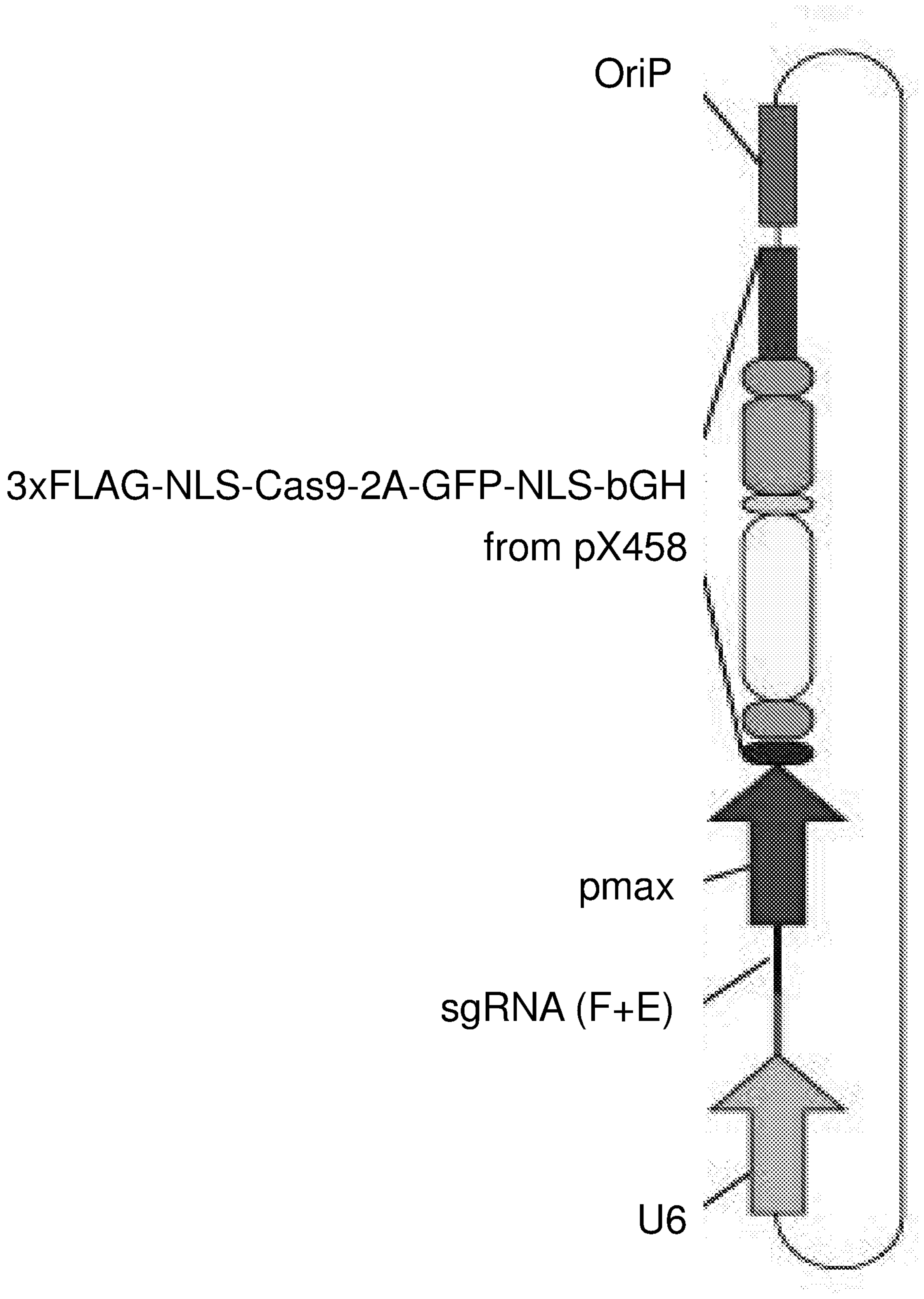
OriP
3xFLAG-NLS-Cas9-2A-GFP-NLS-bGH
from pX458
pmax
sgRNA (F+E)
U6

FIG. 5

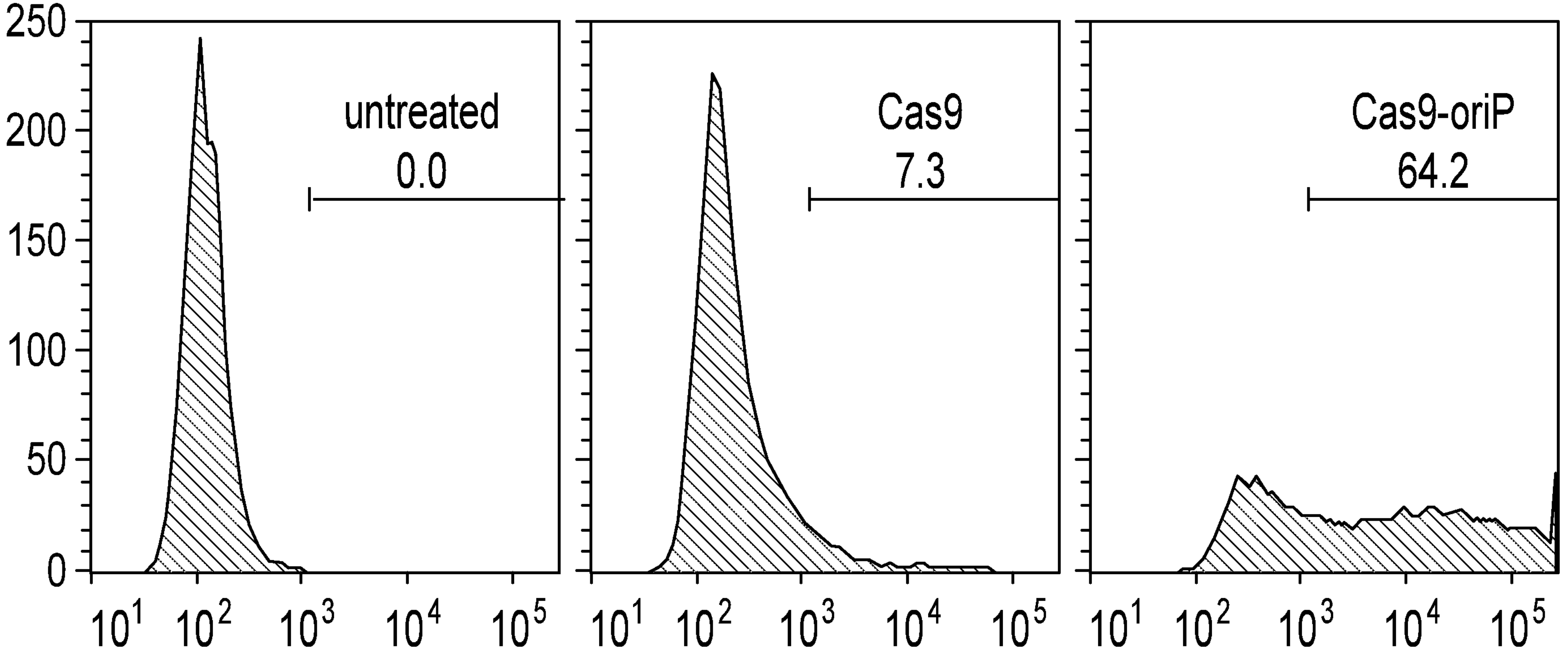
250
200
150
100
50
0
untreated
0.0
Cas9
7.3
Cas9-oriP
64.2
10^1
10^2
10^3
10^4
10^5

FIG. 6

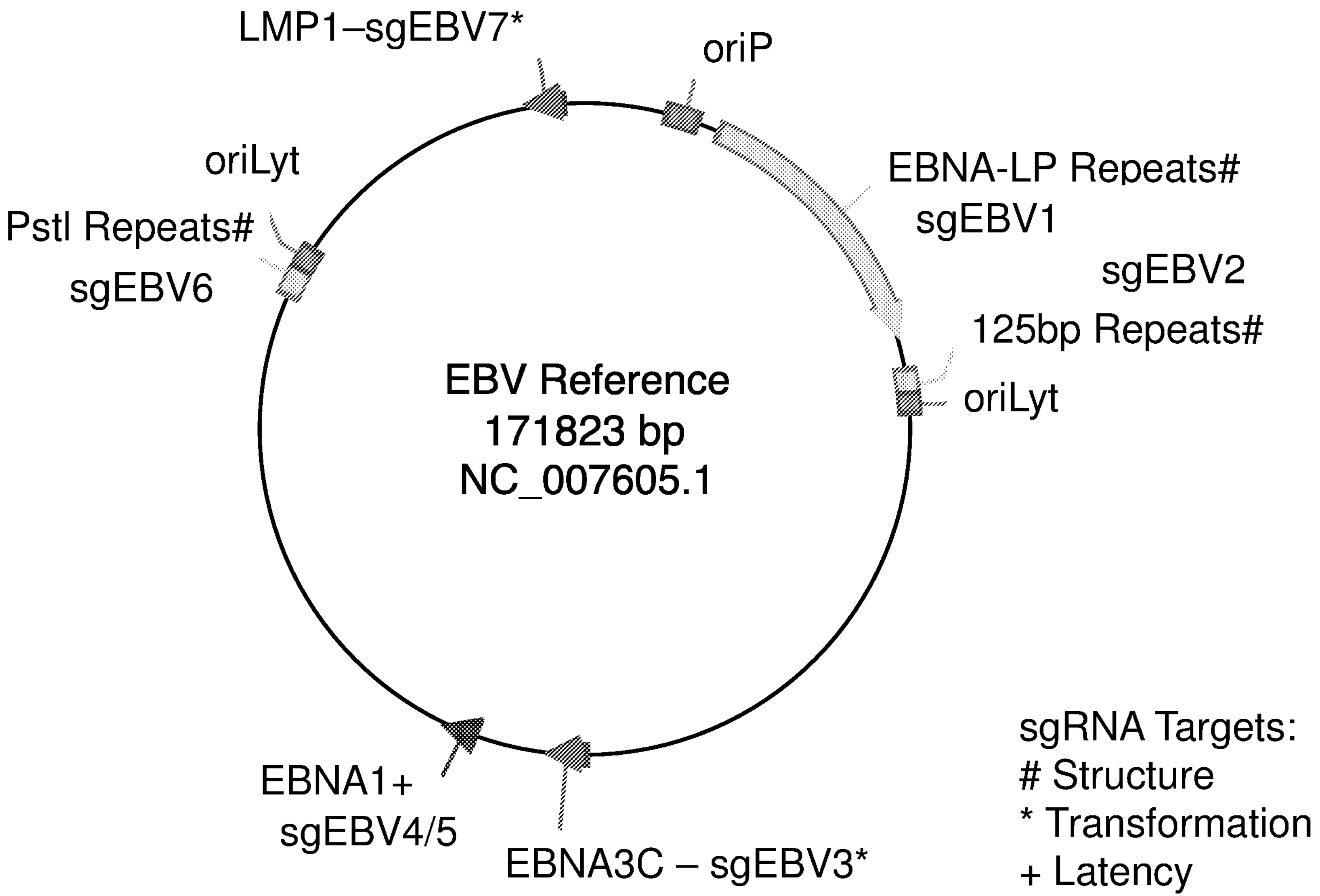
LMP1–sgEBV7*
oriP
EBNA-LP Repeats#
sgEBV1
sgEBV2
125bp Repeats#
oriLyt
oriLyt
PstI Repeats#
sgEBV6
EBV Reference
171823 bp
NC_007605.1
EBNA1+
sgEBV4/5
EBNA3C – sgEBV3*
sgRNA Targets:
Structure
* Transformation
+ Latency

FIG. 7

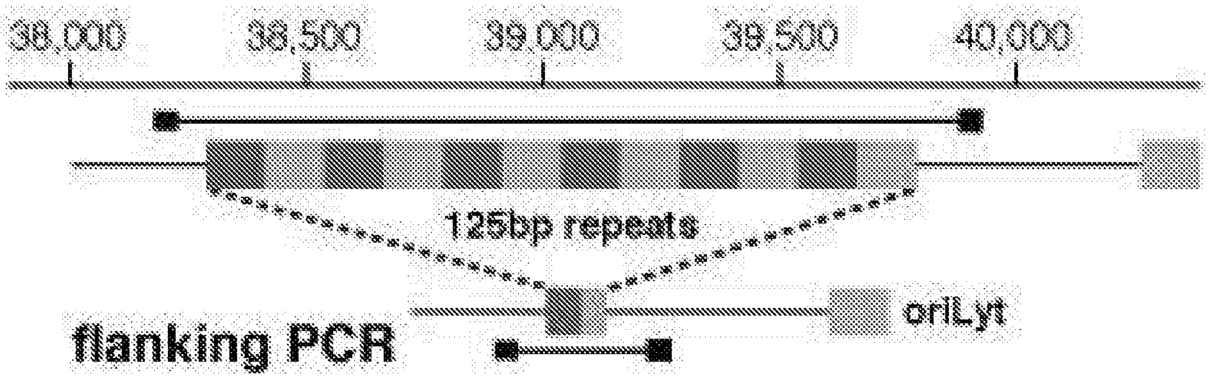
38,000
38,500
39,000
39,500
40,000
125bp repeats
oriLyt
flanking PCR

FIG. 8

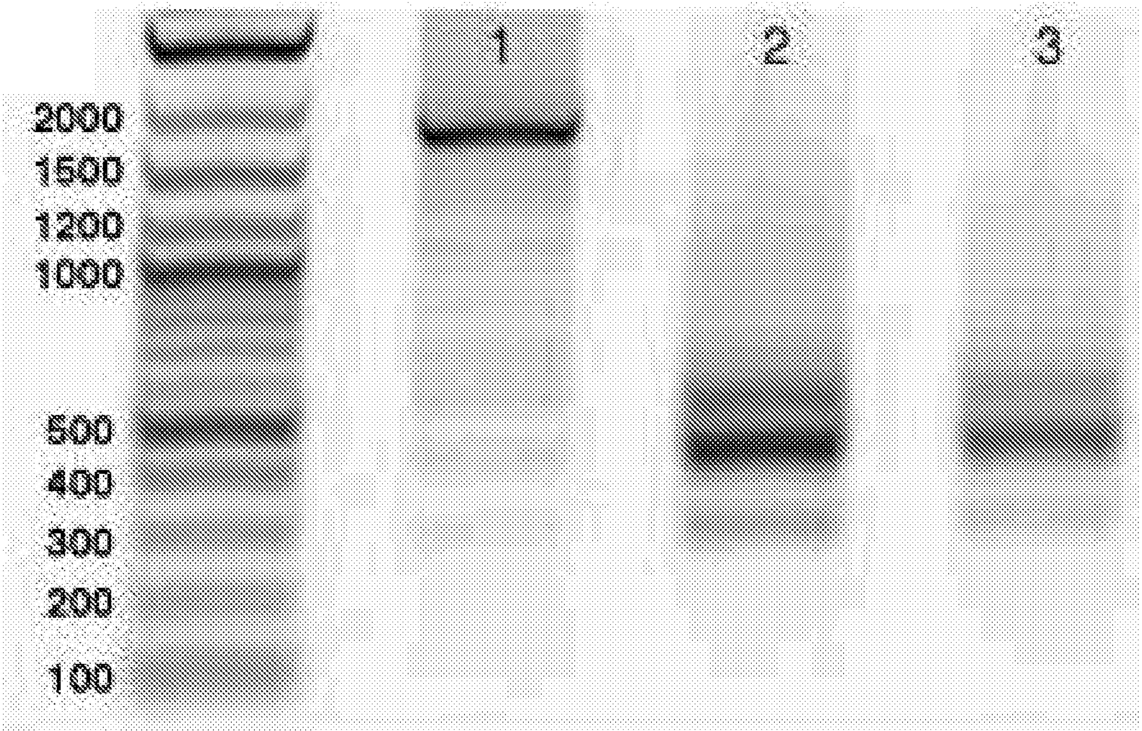
1
2
3
2000
1500
1200
1000
500
400
300
200
100

FIG. 9

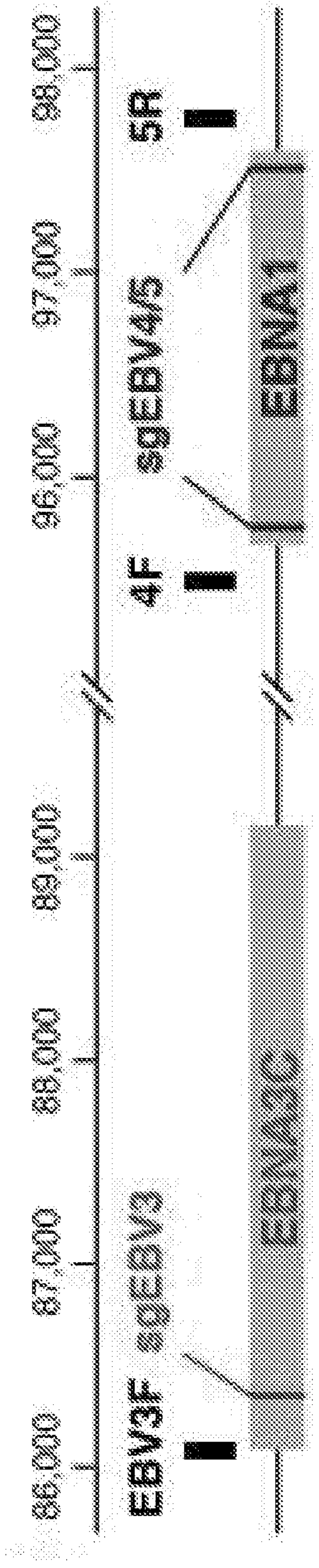
86,000
87,000
88,000
89,000
96,000
97,000
98,000
EBV3F
sgEBV3
EBNA3C
4F
sgEBV4/5
EBNA1
5R

FIG. 10

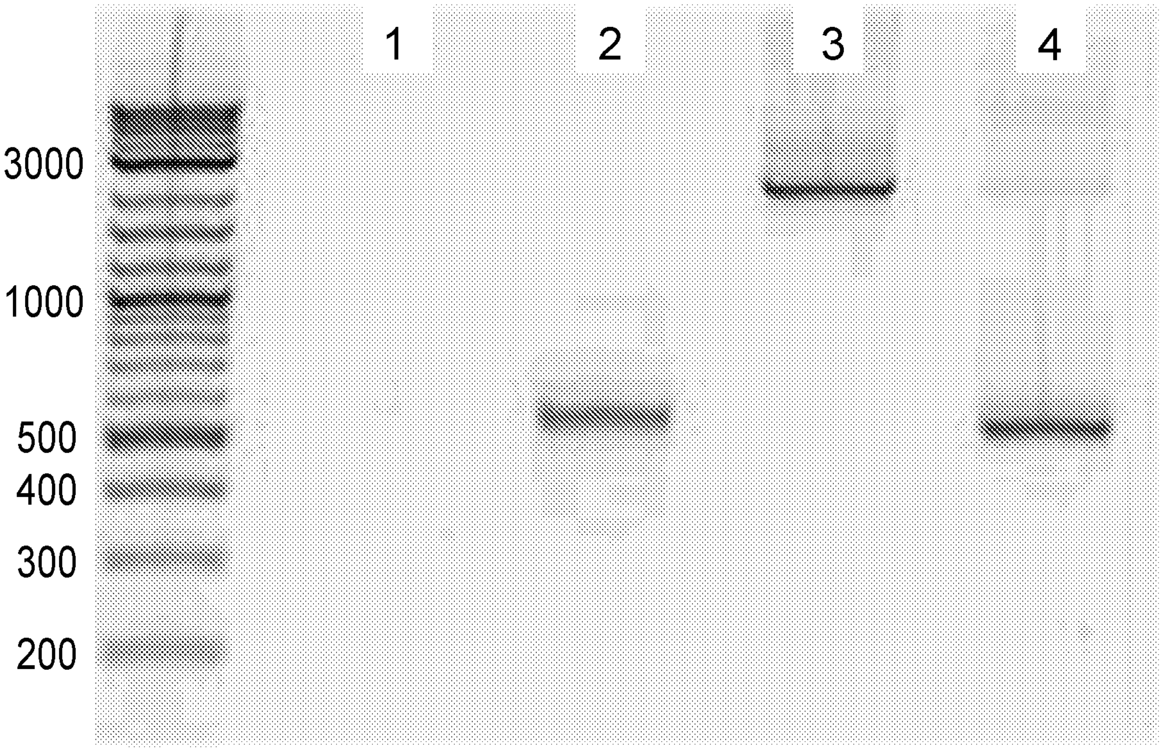

FIG. 11

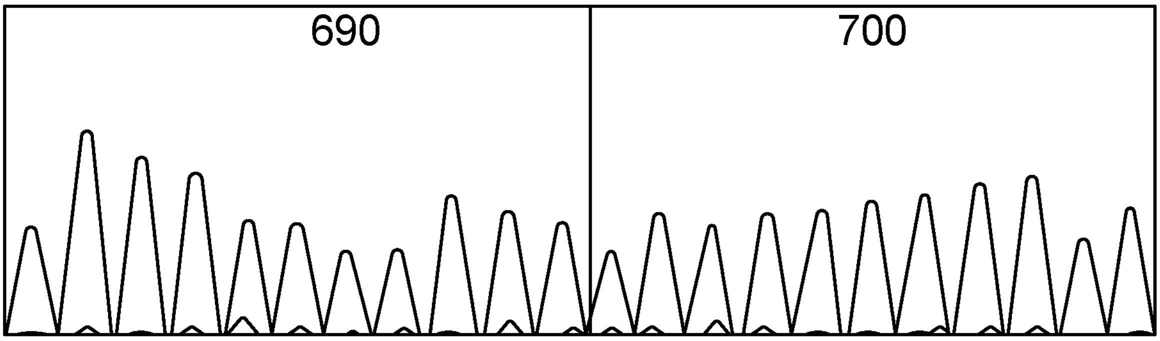

FIG. 12

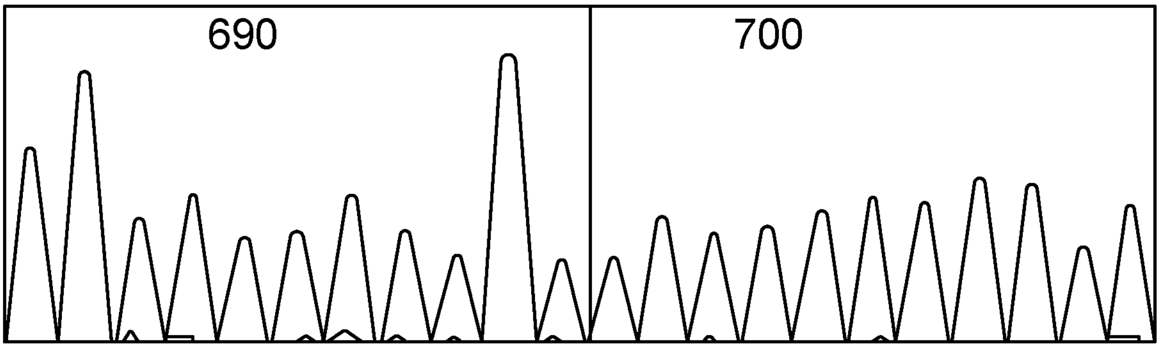

FIG. 13

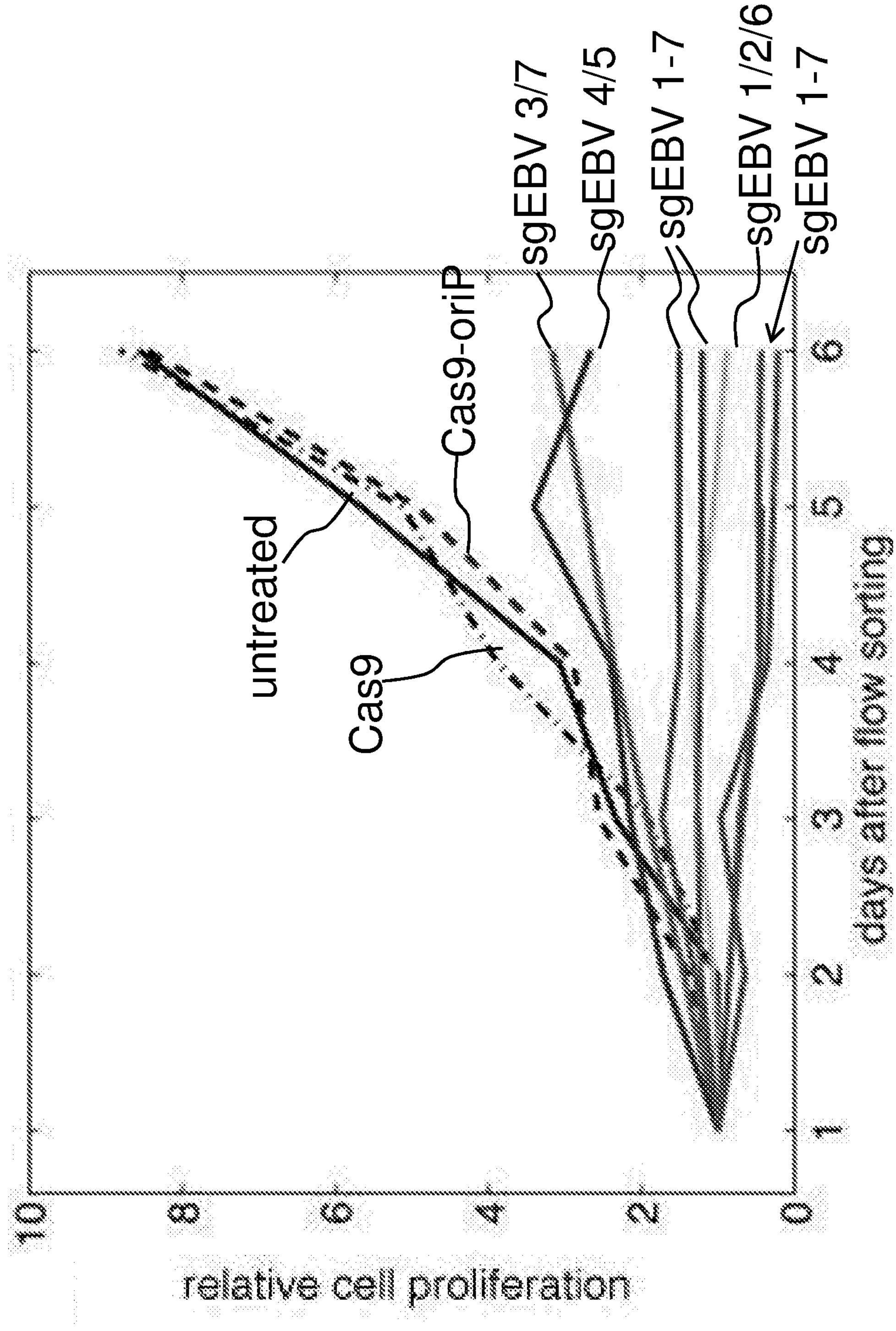
relative cell proliferation
0
2
4
6
8
10
days after flow sorting
1
2
3
4
5
6
untreated
Cas9
Cas9-oriP
sgEBV 3/7
sgEBV 4/5
sgEBV 1-7
sgEBV 1/2/6
sgEBV 1-7

FIG. 14

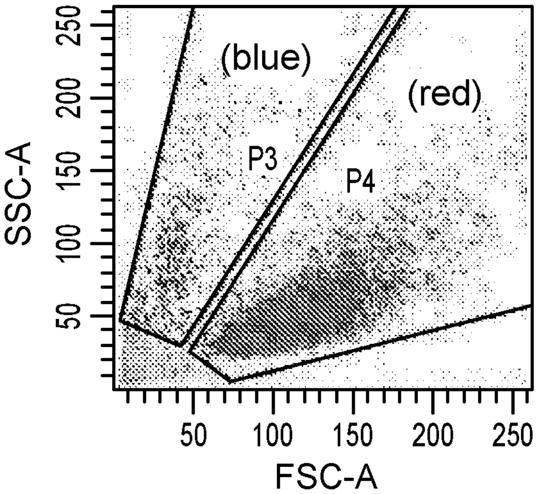

FIG. 15

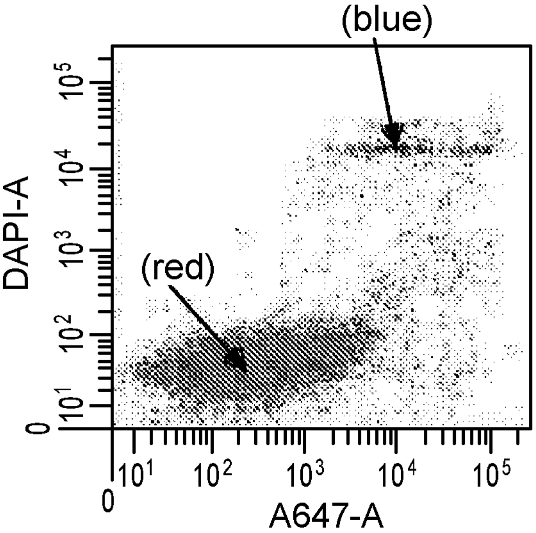

FIG. 18

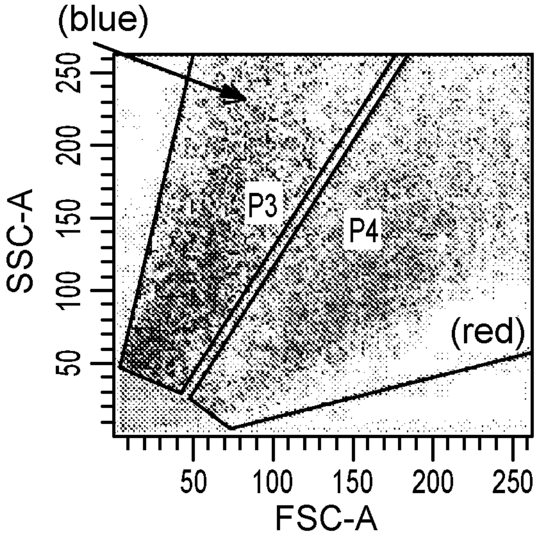

FIG. 16

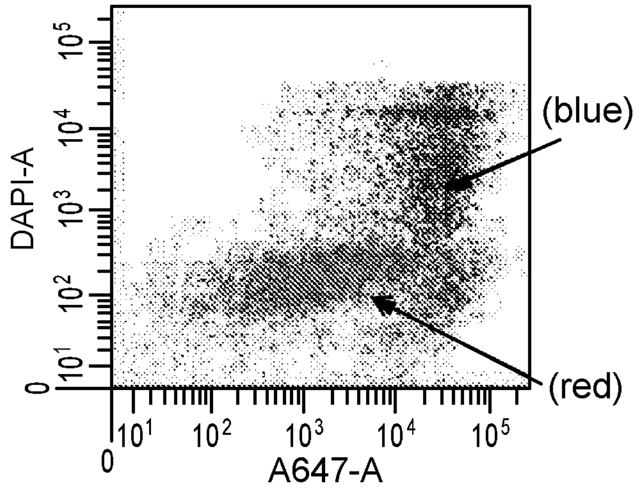

FIG. 19

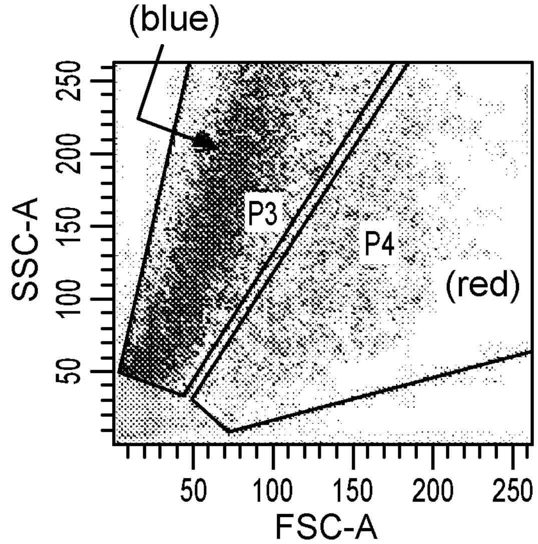

FIG. 17

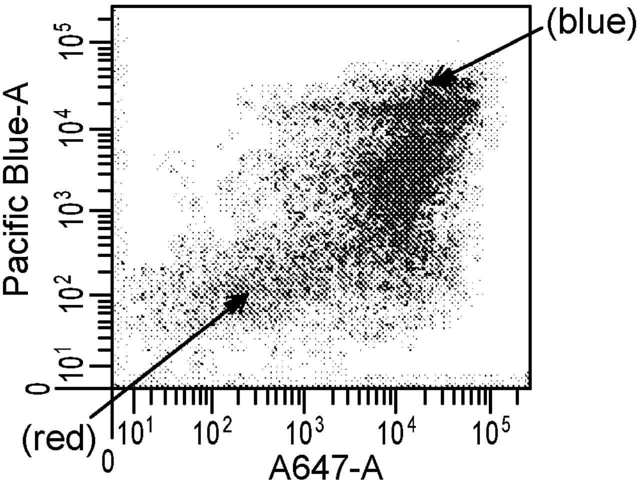

FIG. 20

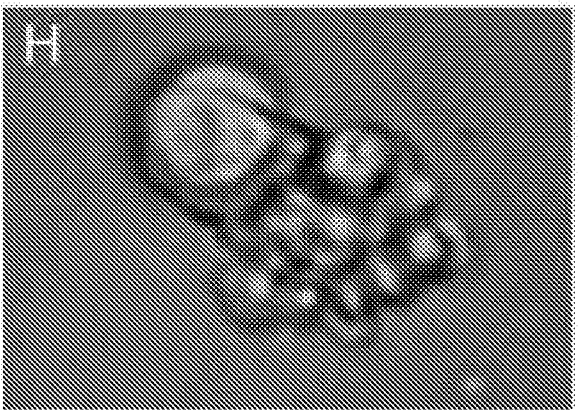

FIG. 21

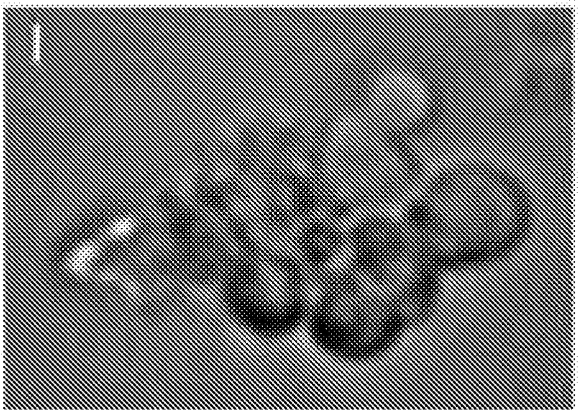

FIG. 22

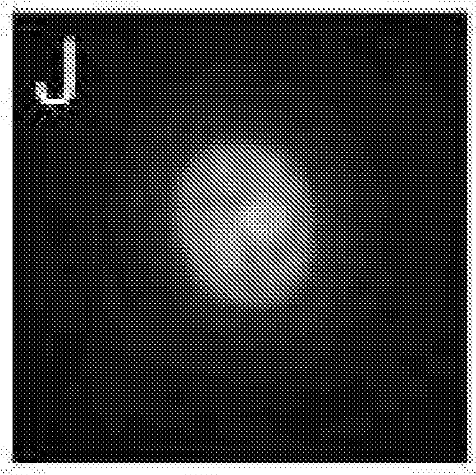

FIG. 23

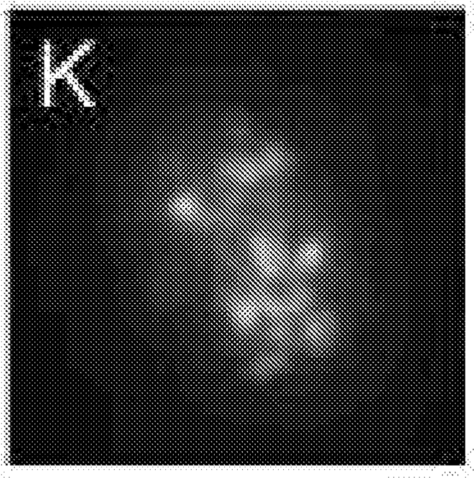

FIG. 24

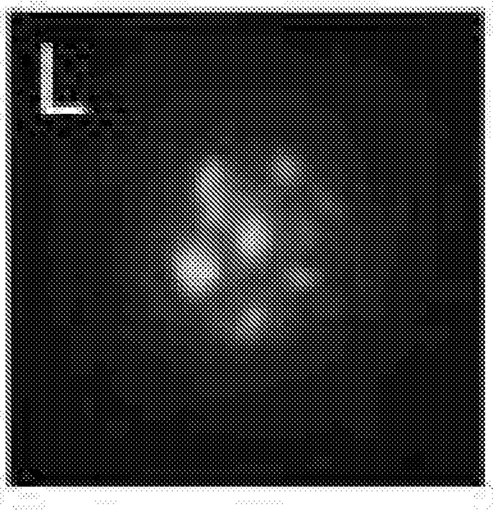

FIG. 25

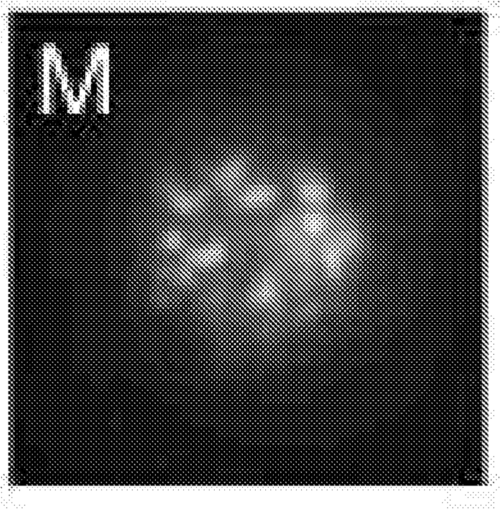

FIG. 26

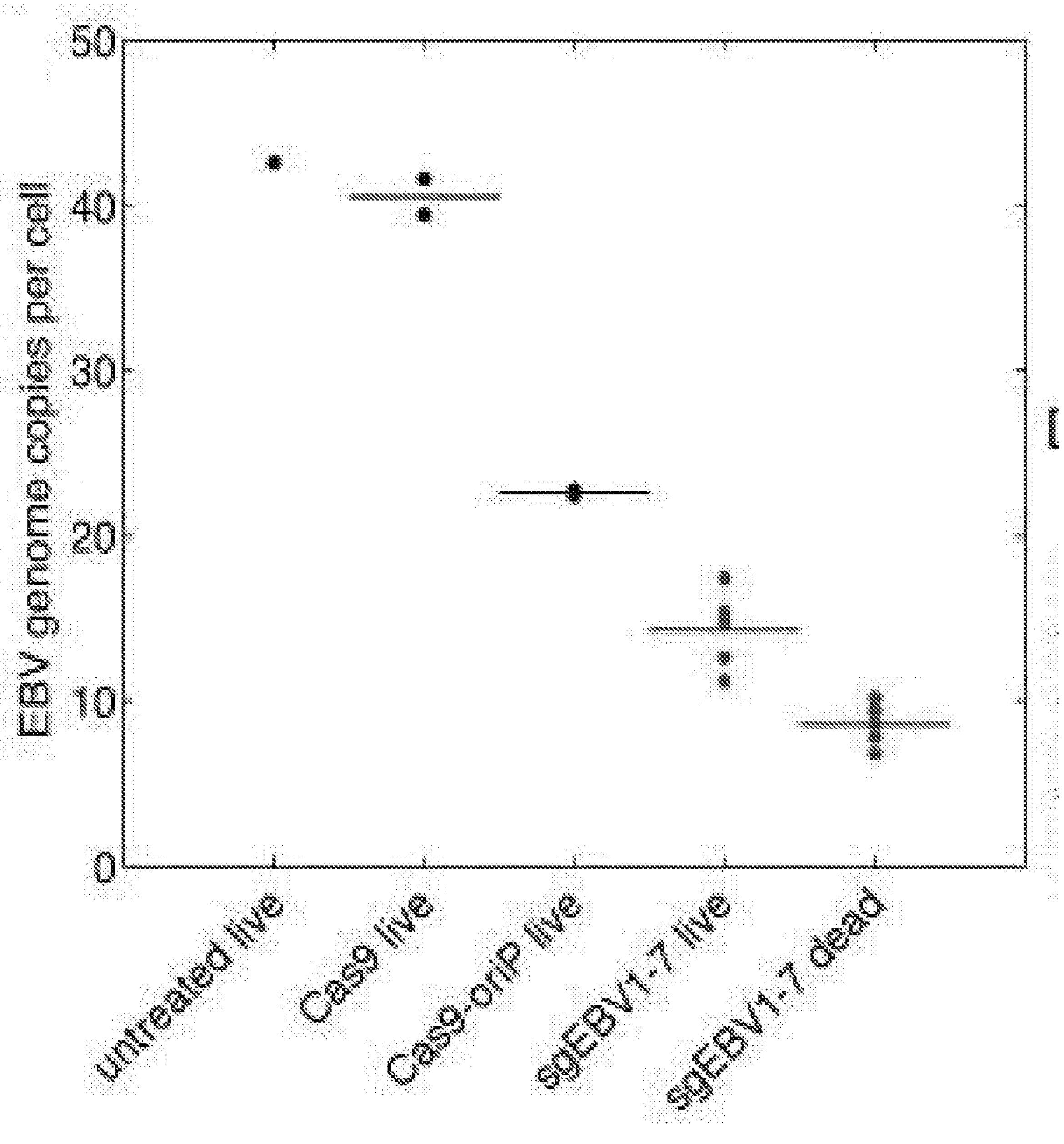
EBV genome copies per cell
50
40
30
20
10
0
untreated live
Cas9 live
Cas9-oriP live
sgEBV1-7 live
sgEBV1-7 dead

FIG. 27

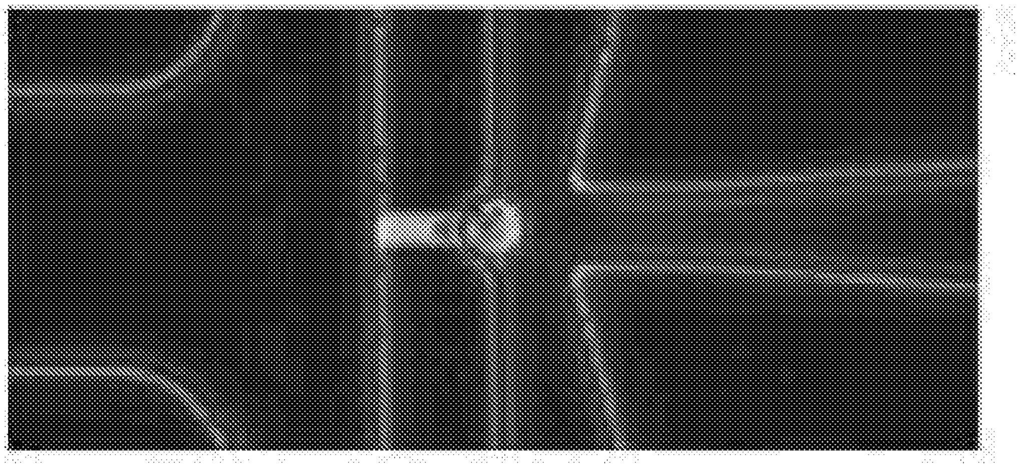

FIG. 28

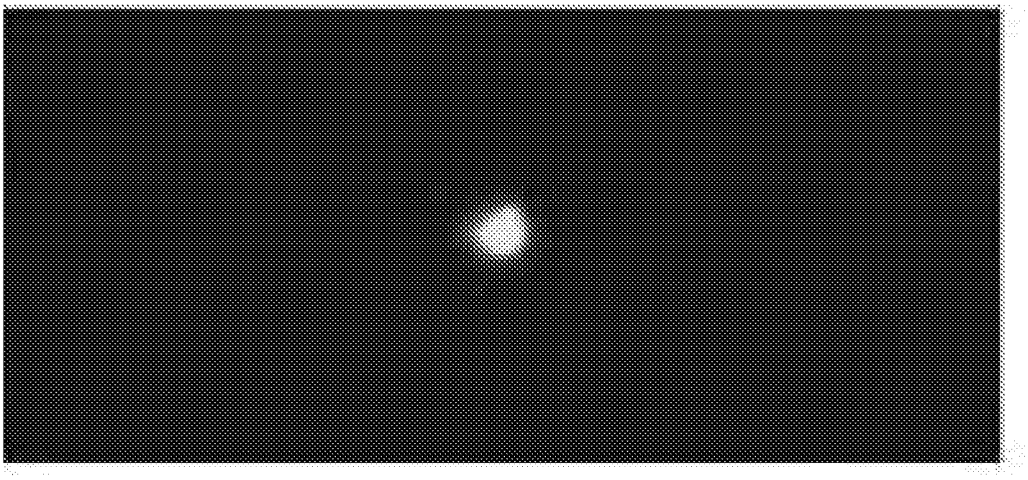

FIG. 29

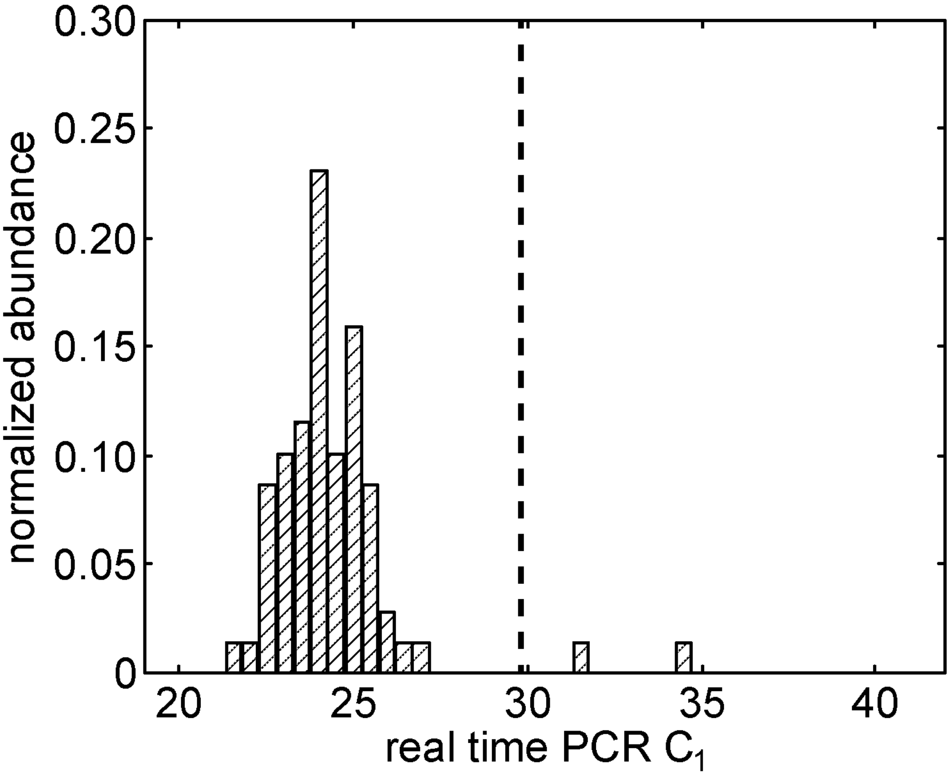
normalized abundance
0.30
0.25
0.20
0.15
0.10
0.05
0
20
25
30
35
40
real time PCR C_1

FIG. 30

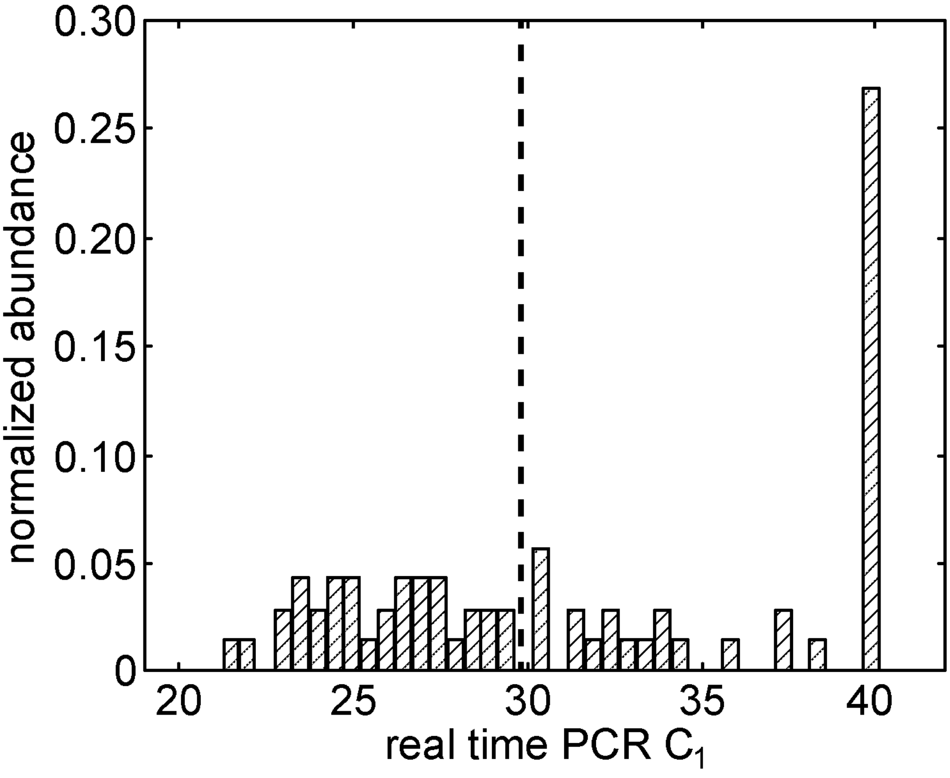
normalized abundance
0.30
0.25
0.20
0.15
0.10
0.05
0
20
25
30
35
40
real time PCR C_1

FIG. 31

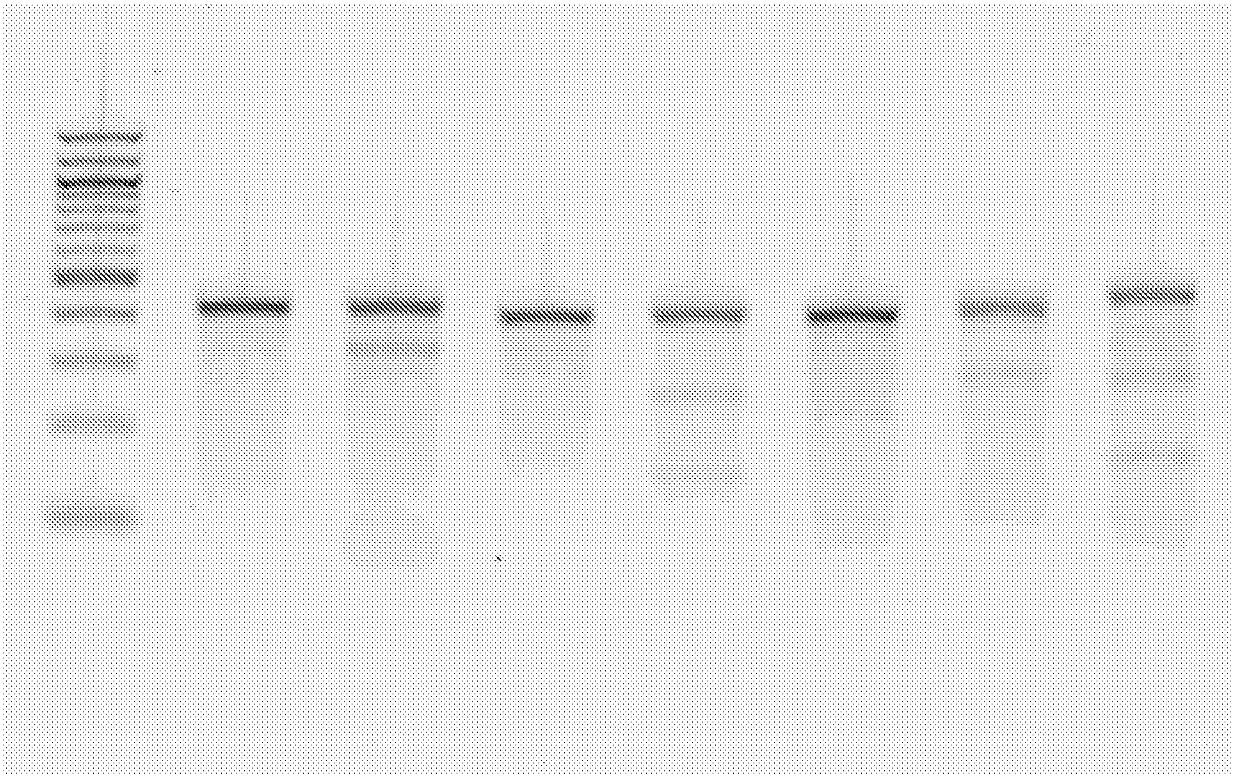

FIG. 32

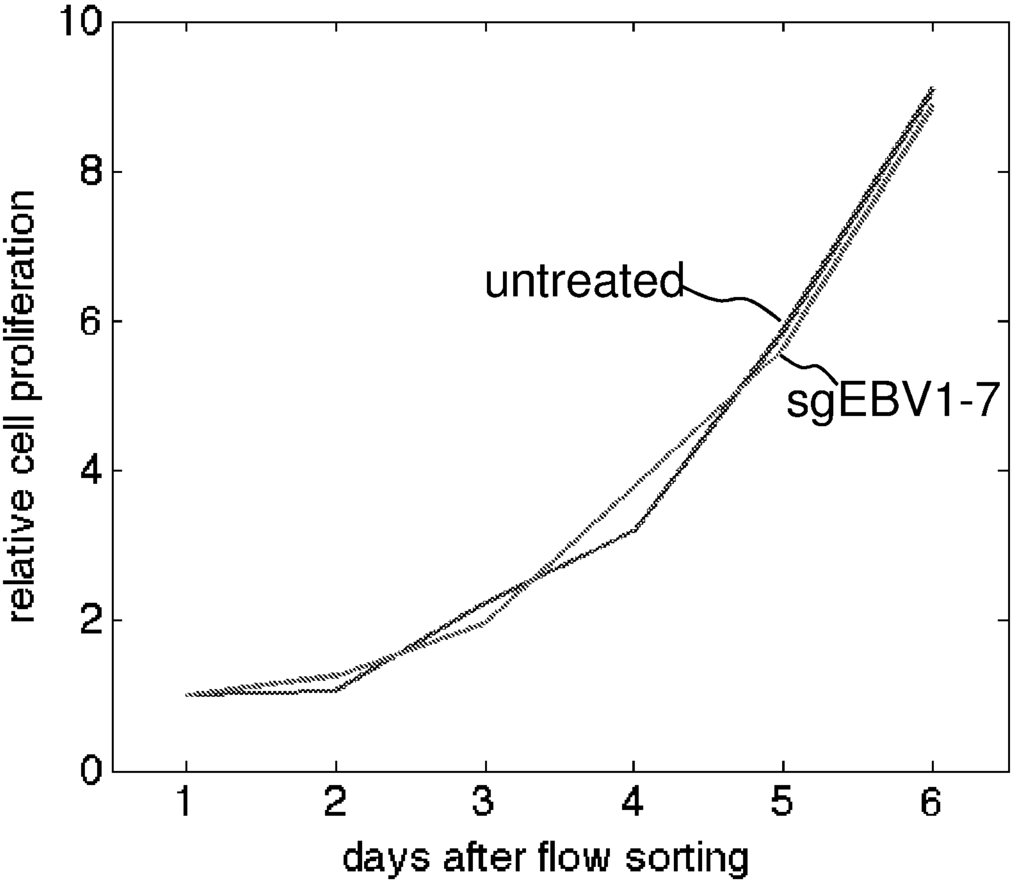
10
8
6
4
2
0
relative cell proliferation
1
2
3
4
5
6
days after flow sorting
untreated
sgEBV1-7

FIG. 33

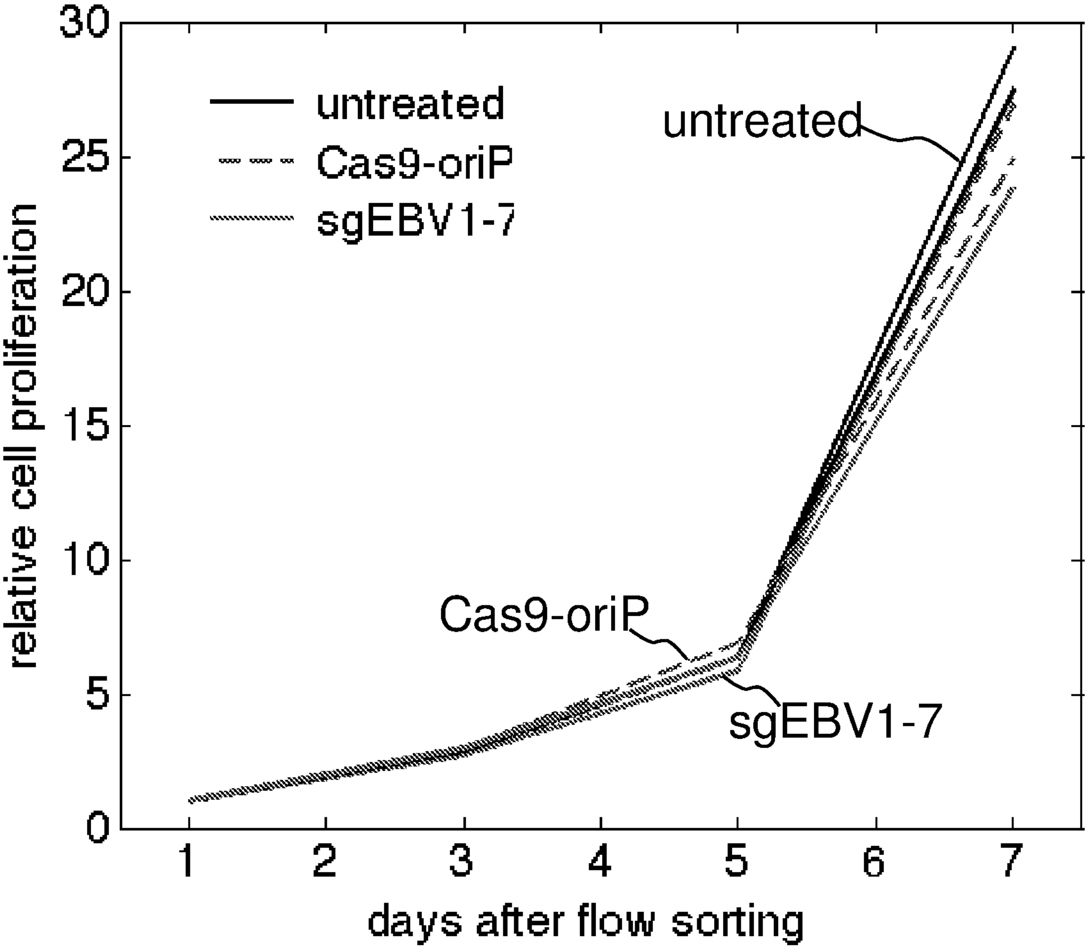
30
25
20
15
10
5
0
relative cell proliferation
1
2
3
4
5
6
7
days after flow sorting
untreated
Cas9-oriP
sgEBV1-7
untreated
Cas9-oriP
sgEBV1-7

FIG. 34

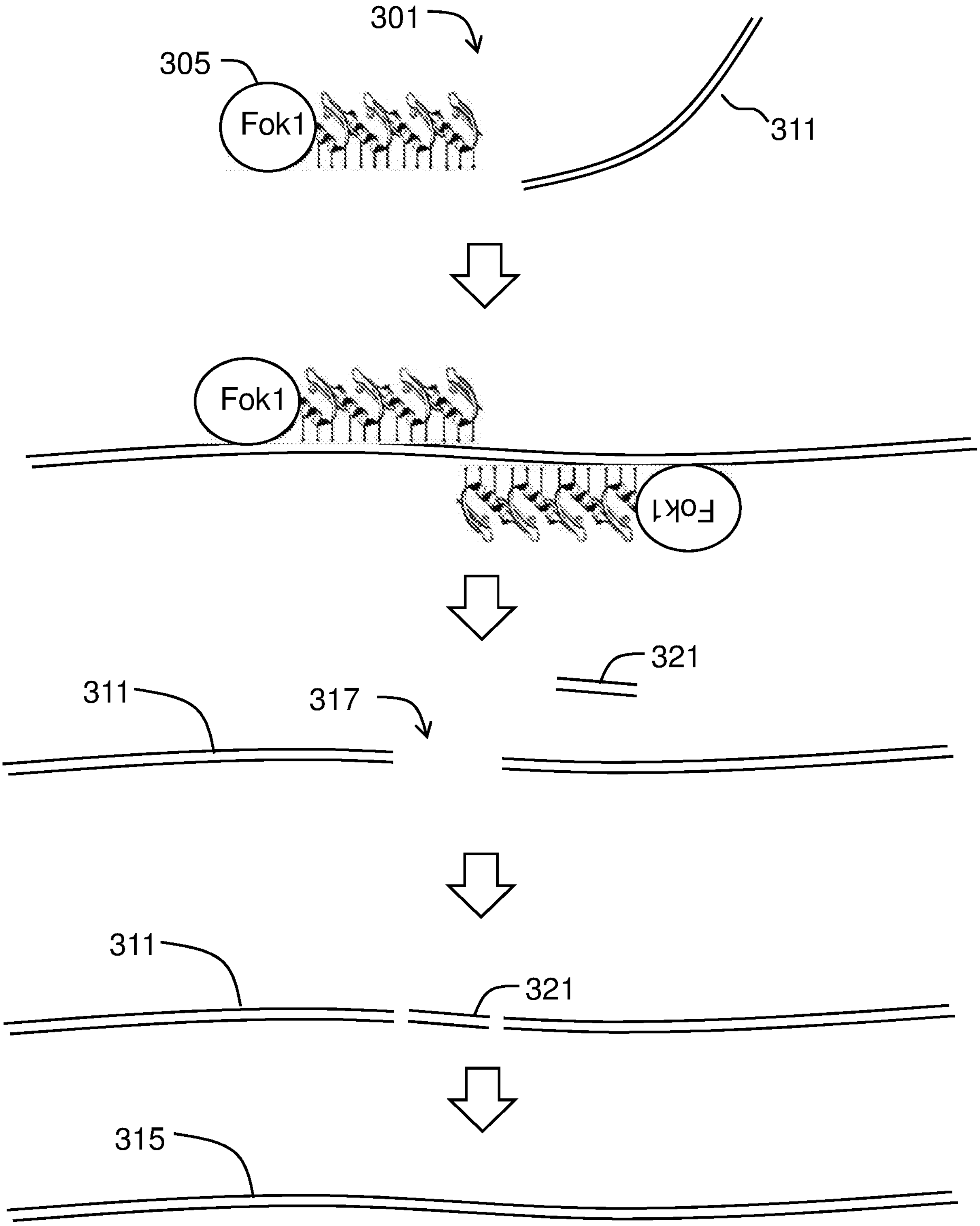

FIG. 35

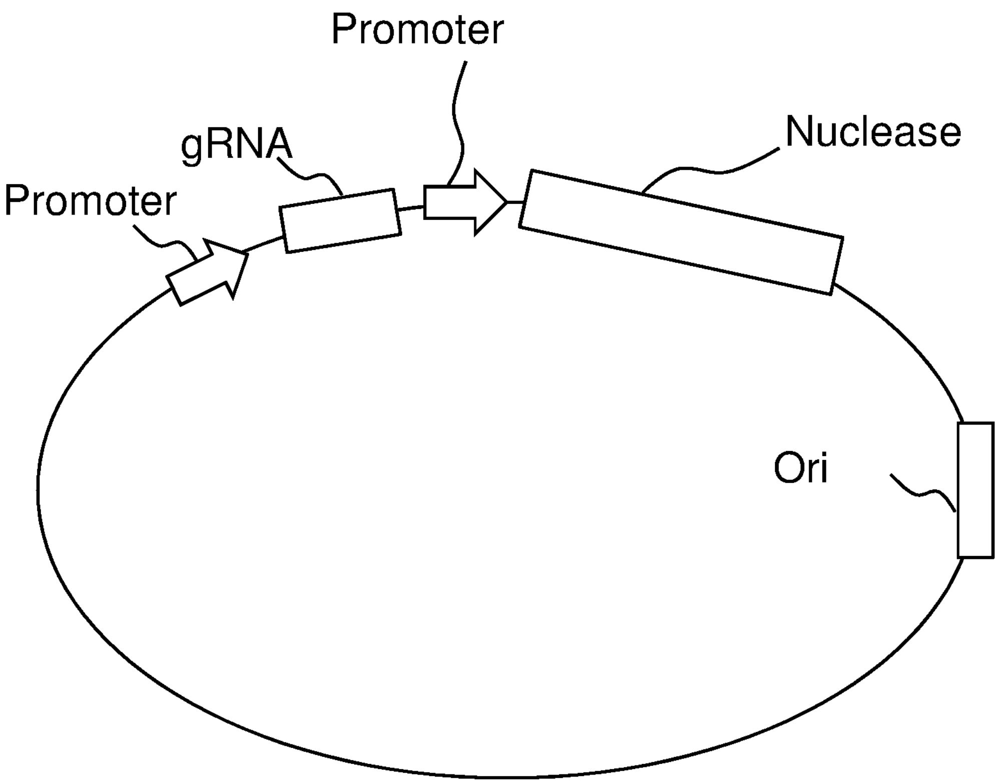

FIG. 36

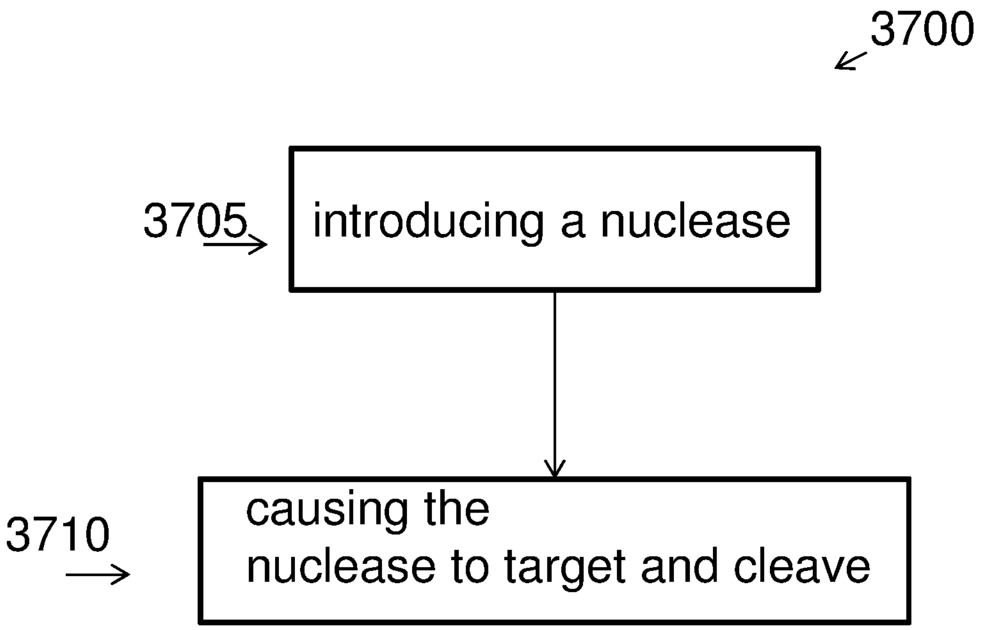
3700
3705
introducing a nuclease
3710
causing the nuclease to target and cleave

FIG. 37

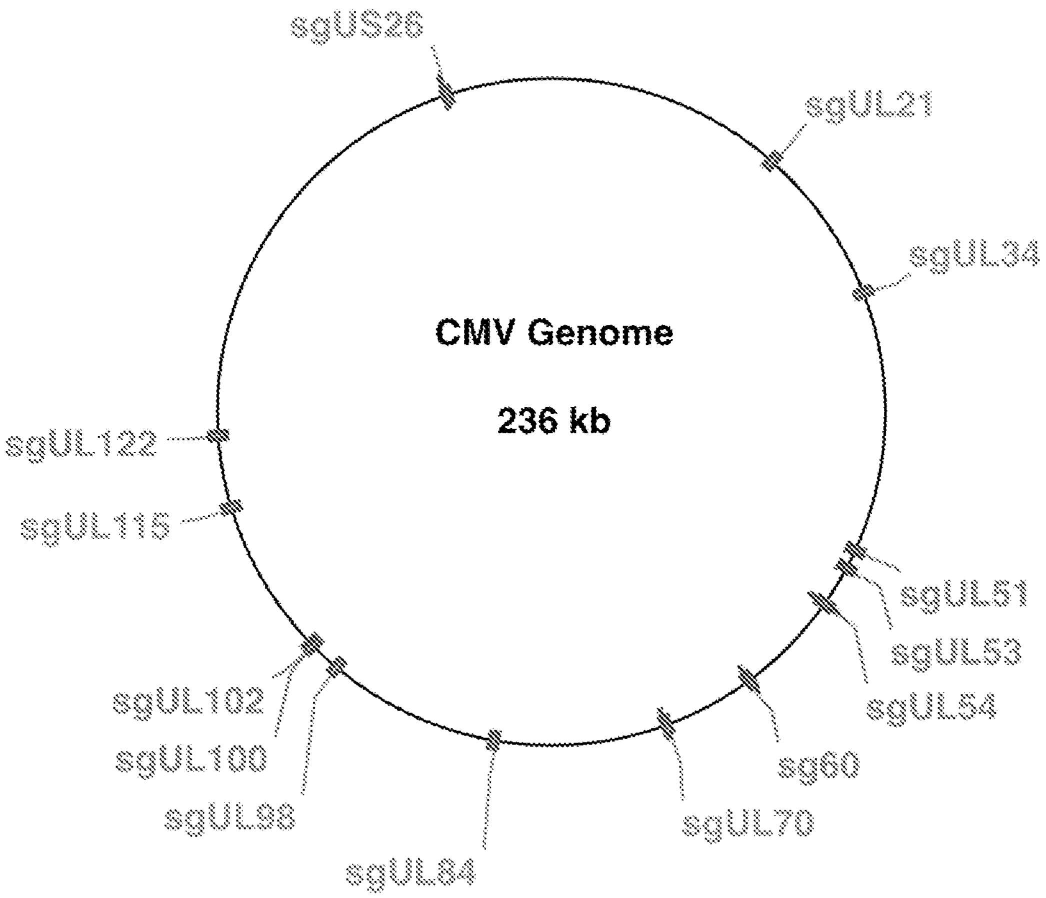
CMV Genome
236 kb
sgUS26
sgUL21
sgUL34
sgUL51
sgUL53
sgUL54
sg60
sgUL70
sgUL84
sgUL98
sgUL100
sgUL102
sgUL115
sgUL122

FIG. 38

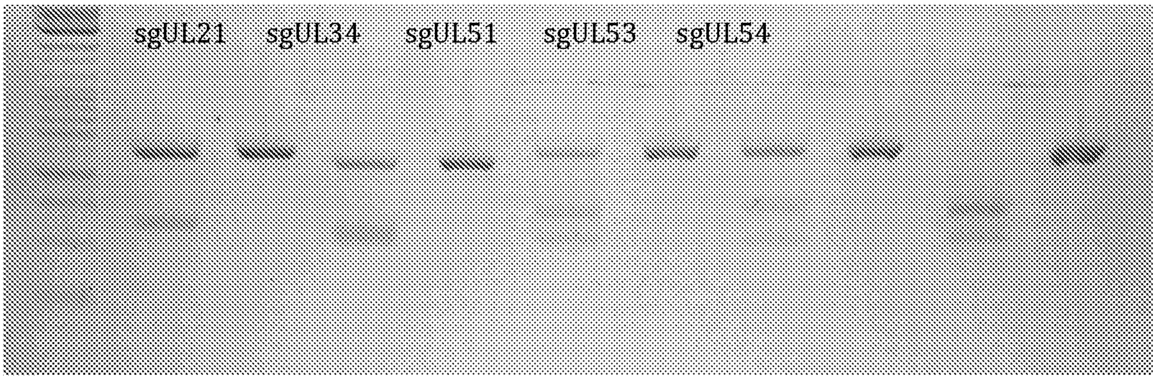
sgUL21
sgUL34
sgUL51
sgUL53
sgUL54

FIG. 39

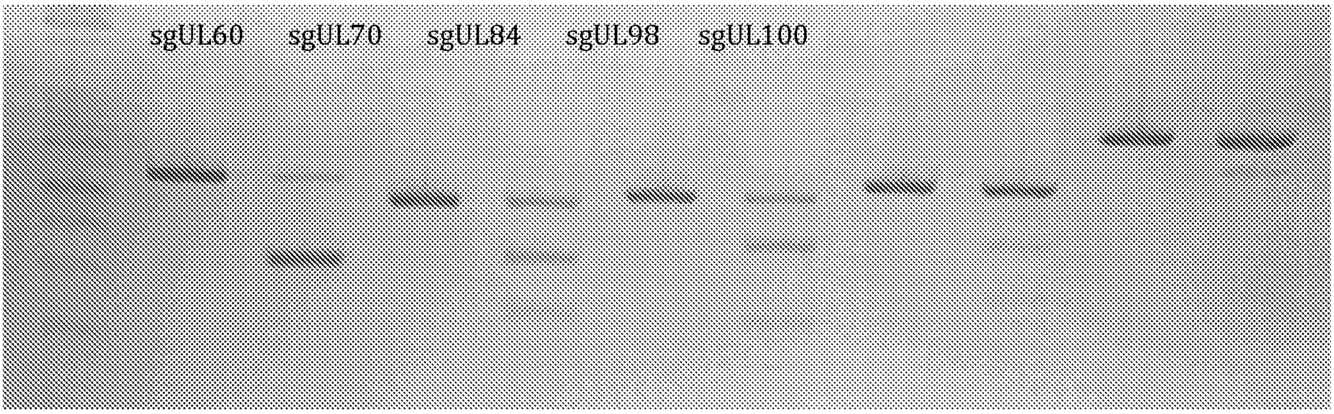
sgUL60
sgUL70
sgUL84
sgUL98
sgUL100

FIG. 40

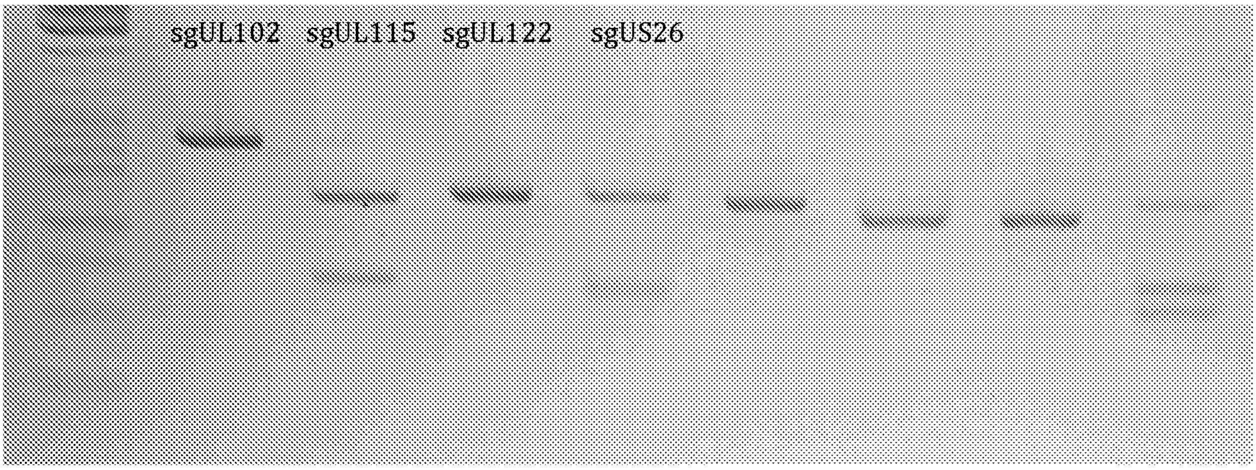

FIG. 41

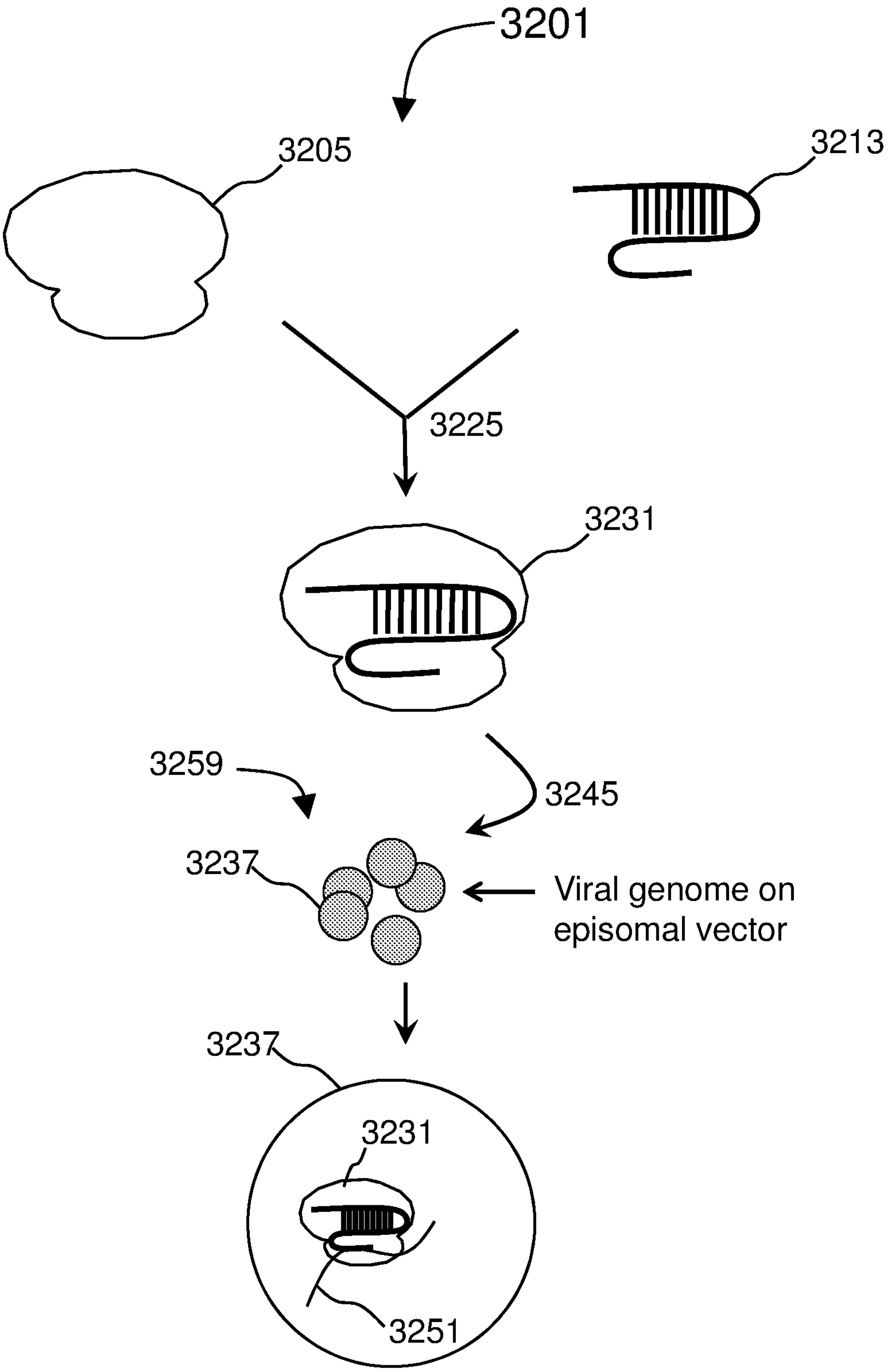
3201
3205
3213
3225
3231
3259
3245
3237
Viral genome on episomal vector
3237
3231
3251

FIG. 42

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/034606

A. CLASSIFICATION OF SUBJECT MATTER

A61K 48/00(2006.01)I, A61K 9/08(2006.01)I

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K 48/00; C12N 15/63; C12N 15/10; A61K 9/08

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: Cas9, guide RNA, HSV, CMV, herpes

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015-053995 A1 (ELWHA LLC) 16 April 2015 See pages 17-19; and figure 3.	1-10,21-25,27,29-35
A	SUROVAYA, A. N. et al., 'Complex of the herpes simplex virus initiator protein UL9 with DNA as a platform for the design of a new type of antiviral drugs' Biophysics, 2010, Vol. 55, No. 2, pp. 204-214 See the whole document.	1-10,21-25,27,29-35
A	WANG, J. et al., 'RNA-guided endonuclease provides a therapeutic strategy to cure latent herpesviridae infection' Proceedings of the National Academy of Sciences, 2014, Vol. 111, No. 36, pp. 13157-13162 See the whole document.	1-10,21-25,27,29-35
A	RUSSELL, T. A. et al., 'Engineering herpes simplex viruses by infection-transfection methods including recombination site targeting by CRISPR/Cas9 nucleases' Journal of Virological Methods, e.pub. 03 December 2014, Vol. 213, pp. 18-25 See the whole document.	1-10,21-25,27,29-35

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search 06 September 2016 (06.09.2016)	Date of mailing of the international search report **06 September 2016 (06.09.2016)**
Name and mailing address of the ISA/KR International Application Division Korean Intellectual Property Office 189 Cheongsa-ro, Seo-gu, Daejeon, 35208, Republic of Korea Facsimile No. +82-42-481-8578	Authorized officer PARK, Jung Min Telephone No. +82-42-481-3516

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YUAN, M. et al., 'Efficiently editing the vaccinia virus genome by using the CRISPR-Cas9 system' Journal of Virology, Accepted manuscript posted 04 March 2015, Vol. 89, No. 9, pp. 5176-5179 See the whole document.	1-10,21-25,27,29-35

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

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

1. ☒ Claims Nos.: 11-20,36-55
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 11-20 and 36-55 pertain to methods for treatment of the human body by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv), to search.

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

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

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

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

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

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

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

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

Remark on Protest

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

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2016/034606

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2015-053995 A1	16/04/2015	US 2015-0098954 A1	09/04/2015