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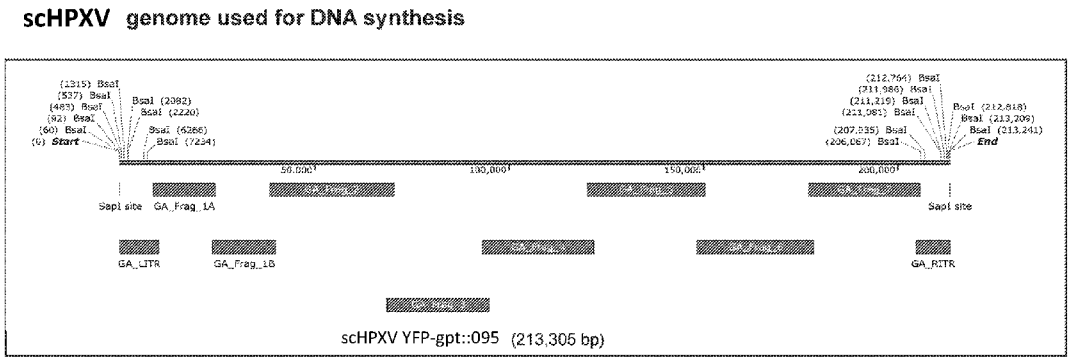
(54) Title: SYNTHETIC CHIMERIC POXVIRUSES

Figure 1B



(57) Abstract: The invention relates, in general, to synthetic chimeric poxviruses, compositions comprising such viruses, and the development and use of systems and methods for producing such synthetic chimeric poxviruses. The synthetic chimeric poxviruses are well suited for live virus vaccines and pharmaceutical formulations.

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SYNTHETIC CHIMERIC POXVIRUSES

BACKGROUND OF THE INVENTION

[0001] Poxviruses (members of the *Poxviridae* family) are double-stranded DNA viruses that can infect both humans and animals. Poxviruses are divided into two subfamilies based on host range. The *Chordopoxviridae* subfamily, which infects vertebrate hosts, consists of eight genera, of which four genera (*Orthopoxvirus*, *Parapoxvirus*, *Molluscipoxvirus*, and *Yatapoxvirus*) are known to infect humans. Smallpox is caused by infection with variola virus (VARV), a member of the genus *Orthopoxvirus* (OPV). The OPV genus comprises a number of genetically related and morphologically identical viruses, including camelpox virus (CMLV), cowpox virus (CPXV), ectromelia virus (ECTV, "mousepox agent"), horsepox virus (HPXV), monkeypox virus (MPXV), rabbitpox virus (RPXV), raccoonpox virus, skunkpox virus, Taterapox virus, Uasin Gishu disease virus, vaccinia virus (VACV), variola virus (VARV) and volepox virus (VPV). Other than VARV, at least three other OPVs, including VACV, MPXV and CPXV, are known to infect humans. So far, vaccination with "live" VACV is the only proven protection against smallpox. An aggressive program of vaccination led to the eradication of small pox in 1980 and routine smallpox vaccination of the public was stopped. However, a need remains to find new safe and effective means of vaccinating individuals against VARV and other OPVs.

[0002] A variety of preparations of VACV have been used as smallpox vaccines. Most of these comprised of a number of related viruses (e.g. Dryvax), and one comprises a single molecular clone, ACAM2000. However, like Dryvax and other VACV vaccines, even ACAM2000 is associated with serious side effects including cardiomyopathy and pericarditis. To reduce risks, the ACAM2000 vaccine, like other live vaccines, has numerous contraindications that preclude individuals with cancer, immunodeficiencies, organ transplant recipients, patients with atopic dermatitis, eczema, psoriasis, heart conditions, and patients on immunosuppressants. It is estimated that 15-50% of the US population would

fall under one of these categories, therefore confirming the need for the development of a safer vaccine or vaccination protocol (Kennedy et al., 2007 Kennedy R, Poland GA. 2007. T-Cell epitope discovery for variola and vaccinia viruses. Rev Med Viroll7: 93-113). Therefore, there is a need for the development of a vaccine that is equivalent in efficacy to Dryvax or ACAM2000™, but that is safer.

[0003] The present invention provides chimeric poxviruses assembled and replicated from chemically synthesized DNA. Because chemical genome synthesis is not dependent on a natural template, a plethora of structural and functional modifications of the viral genome are possible. Chemical genome synthesis is particularly useful when a natural template is not available for genetic replication or modification by conventional molecular biology methods.

SUMMARY OF THE INVENTION

[0004] The present invention provides synthetic chimeric poxviruses (e.g. synthetic chimeric OPV or scOPV), methods for producing such viruses and the use of such viruses, for example, as immunogens, in immunogenic formulations, in *in vitro* assays, as vehicles for heterologous gene expression, or as oncolytic agents. The synthetic chimeric poxviruses of the invention are characterized by one or more modifications relative to a wildtype poxvirus.

[0005] In part, the present invention relates to the discovery that a synthetic chimeric poxvirus (e.g. scOPV) can be produced from chemically synthesized overlapping fragments of the poxviral genome. Accordingly, the present invention in part, provides synthetic chimeric poxviruses (e.g. scOPV) replicated and assembled from chemically synthesized nucleic acids. The disclosure also provides compositions comprising such viruses. The disclosure further provides methods of using the poxviruses produced according to the methods of the disclosure.

[0006] In another aspect, the invention provides a method for protecting individual humans and populations of humans against the consequences of

infection with smallpox, pseudotypes of smallpox virus and other OPVs using the synthetic chimeric poxviruses of the invention. In another aspect, the invention is a method for protecting individual humans and populations of humans against the consequences of infection with smallpox (VARV) and pseudotypes of smallpox virus by using the synthetic chimeric poxviruses of the invention, with less toxicity, morbidity and mortality than available VACV-based vaccines. In certain aspects, the invention provides a synthetic chimeric poxvirus (scPV) that is replicated and reactivated from DNA derived from synthetic DNA, the viral genome of said virus differing from a wild type genome of said virus in that it is characterized by one or more modifications, the modifications being derived from a group comprising chemically-synthesized DNA, cDNA or genomic DNA.

[0007] In some embodiments, the synthetic DNA is selected from one or more of: chemically synthesized DNA, PCR amplified DNA, engineered DNA and polynucleotides comprising nucleoside analogs. In some embodiments, the synthetic DNA is chemically synthesized DNA.

[0008] In some embodiments, the one or more modifications comprise one or more deletions, insertions, substitutions, or a combination thereof. In some embodiments, the one or more modifications comprise one or more modifications to introduce one or more unique restriction sites.

[0009] In some embodiments, the viral genome comprises heterologous terminal hairpin loops. In some embodiments, the viral genome comprises terminal hairpin loops derived from vaccinia virus. In some embodiments, the left and right terminal hairpin loops a) comprise the slow form and the fast form of the vaccinia virus terminal hairpin loop, respectively, b) comprise the fast form and the slow form of the vaccinia virus terminal hairpin loop, respectively, c) both comprise the slow form of the vaccinia virus terminal hairpin loop, or d) both comprise the fast form of the vaccinia virus terminal loop.

[0010] In some embodiments, the virus is replicated and reactivated from overlapping chemically-synthesized DNA fragments that correspond to substantially all of the viral genome of the scPV.

[0011] In some embodiments, the virus is replicated and reactivated from 1-14 overlapping fragments. In some embodiments, the virus is replicated and reactivated from 8-12 overlapping fragments. In some embodiments, the virus is replicated and reactivated from 10 overlapping fragments.

[0012] In some embodiments, the virus is reactivated using leporipox virus-catalyzed recombination and reactivation. In some embodiments, the leporipox virus is selected from the group consisting of: Shope fibroma virus (SFV), hare fibroma virus, rabbit fibroma virus, squirrel fibroma virus, and myxoma virus.

[0013] In certain aspects, the invention provides a synthetic chimeric orthopox virus (scOPV) that is replicated and reactivated from DNA derived from synthetic DNA, the viral genome of said virus differing from a wild type genome of said virus in that it is characterized by one or more modifications, the modifications being derived from a group comprising chemically-synthesized DNA, cDNA or genomic DNA.

[0014] In some embodiments, the synthetic DNA is selected from one or more of: chemically synthesized DNA, PCR amplified DNA, engineered DNA and polynucleotides comprising nucleoside analogs. In some embodiments, the synthetic DNA is chemically synthesized DNA.

[0015] In some embodiments, the OPV is selected from the group consisting of: camelpox (CMLV) virus, cowpox virus (CPXV), ectromelia virus (ECTV), horsepox virus (HPXV), monkeypox virus (MPXV), vaccinia virus (VACV), variola virus (VARV), rabbitpox virus (RPXV), raccoon poxvirus, skunkpox virus, Taterapox virus, Uasin Gishu disease virus, and volepox virus.

[0016] In some embodiments, the OPV is a VACV. In some embodiments, the viral genome is based on the genome of VACV strain ACAM2000 and differs from the ACAM2000 genome in that it is characterized by one or more modifications. In some embodiments, the viral genome is based on the genome of VACV strain IOC and differs from the IOC genome in that it is characterized by one or more modifications. In some embodiments, the viral genome is based on

the genome of VACV strain MVA and differs from the MVA genome in that it is characterized by one or more modifications. In some embodiments, the viral genome is based on the genome of VACV strain MVA-BN and differs from the MVA-BN genome in that it is characterized by one or more modifications. In some embodiments, the wild type VACV genome is the genome of a strain selected from the group consisting of: Western Reserve, Clone 3, Tian Tian, Tian Tian clone TT9, Tian Tian clone TP3, NYCBH, Wyeth, Copenhagen, Lister 107, Lister-LO, IHD-W, LC16m18, , Lederle, Tashkent clone TKT3, Tashkent clone TKT4, USSR, Evans, Praha, LIVP, Ikeda, EM-63, Malbran, Duke, 3737, CV-1, Connaught Laboratories, Serro 2, CM-01, Dryvax clone DPP13, Dryvax clone DPP15, Dryvax clone DPP20, Dryvax clone DPP17, Dryvax clone DPP21, and chorioallantois vaccinia virus Ankara.

[0017] In some embodiments, the one or more modifications comprise one or more deletions, insertions, substitutions, or a combination thereof.

[0018] In some embodiments, the one or more modifications comprise one or more modifications to introduce one or more unique restriction sites. In some embodiments, the one or more modifications comprise one or more modifications to eliminate one or more restriction sites. In some embodiments, the one or more modifications comprise one or more modifications to eliminate one or more *AarI* restriction sites. In some embodiments, the one or more modifications comprise one or more modifications to eliminate all *AarI* restriction sites. In some embodiments, the one or more modifications comprise one or more modifications to eliminate one or more *BsaI* restriction sites.

[0019] In some embodiments, the viral genome comprises heterologous terminal hairpin loops. In some embodiments, the viral genome comprises terminal hairpin loops derived from vaccinia virus. In some embodiments, the left and right terminal hairpin loops a) comprise the slow form and the fast form of the vaccinia virus terminal hairpin loop, respectively, b) comprise the fast form and the slow form of the vaccinia virus terminal hairpin loop, respectively, c) both comprise the slow form of the vaccinia virus terminal hairpin loop, or d) both comprise the fast form of the vaccinia virus terminal loop. In some embodiments, the slow form

comprises a nucleotide sequence that is at least 85% identical to the nucleotide sequence of SEQ ID NO: 11 and the fast form comprises a nucleotide sequence that is at least 85% identical to the nucleotide sequence of SEQ ID NO: 12. In some embodiments, the slow form comprises a nucleotide sequence that is at least 90% identical to the sequence of SEQ ID NO: 11 and the fast form comprises a nucleotide sequence that is at least 90% identical to the nucleotide sequence of SEQ ID NO: 12. In some embodiments, the slow form comprises a nucleotide sequence that is at least 95% identical to the nucleotide sequence of SEQ ID NO: 11 and the fast form comprises a nucleotide sequence that is at least 95% identical to the nucleotide sequence of SEQ ID NO: 12. In some embodiments, the slow form consists of the nucleotide sequence of SEQ ID NO: 11 and the fast form consists of the nucleotide sequence of SEQ ID NO: 12.

[0020] In some embodiments, the virus is replicated and reactivated from overlapping chemically-synthesized DNA fragments that correspond to substantially all of the viral genome of the OPV.

[0021] In some embodiments, the virus is replicated and reactivated from 1-14 overlapping fragments. In some embodiments, the virus is replicated and reactivated from 8-12 overlapping fragments. In some embodiments, the virus is replicated and reactivated from 10 overlapping fragments.

[0022] In some embodiments, the virus is reactivated using leporipox virus-catalyzed recombination and reactivation. In some embodiments, the leporipox virus is selected from the group consisting of: Shope fibroma virus (SFV), hare fibroma virus, rabbit fibroma virus, squirrel fibroma virus, and myxoma virus.

[0023] In certain aspects, the invention provides a synthetic chimeric horsepox virus (scHPXV) that is replicated and reactivated from synthetic DNA, the viral genome differing from a wild type genome of HPXV in that it is characterized by one or more modifications, the modifications being derived from a group comprising chemically-synthesized DNA, cDNA or genomic DNA.

[0024] In some embodiments, the synthetic DNA is selected from one or more of: chemically synthesized DNA, PCR amplified DNA, engineered DNA and polynucleotides comprising nucleoside analogs. In some embodiments, the synthetic DNA is chemically synthesized DNA.

[0025] In some embodiments, the viral genome is based on the genome of HPXV strain MNR-76 and differs from the MNR-76 genome in that it is characterized by one or more modifications. In some embodiments, the one or more modifications comprise one or more deletions, insertions, substitutions, or a combination thereof.

[0026] In some embodiments, the one or more modifications comprise one or more modifications to introduce one or more unique restriction sites. In some embodiments, the one or more modifications are present in *HPXV044* or *HPXV095*. In some embodiments, the one or more modifications comprise one or more mutations listed in Table 3. In some embodiments, the one or more modifications comprise one or more modifications to eliminate one or more restriction sites. In some embodiments, the one or more modifications comprise one or more modifications to eliminate one or more *AarI* restriction sites. In some embodiments, the one or more modifications comprise one or more modifications to eliminate all *AarI* restriction sites. In some embodiments, the one or more modifications comprise one or more modifications to eliminate one or more *BsaI* restriction sites. In some embodiments, the one or more modifications comprise one or more mutations listed in Table 2.

[0027] In some embodiments, the viral genome comprises heterologous terminal hairpin loops. In some embodiments, the viral genome comprises terminal hairpin loops derived from vaccinia virus. In some embodiments, the left and right terminal hairpin loops a) comprise the slow form and the fast form of the vaccinia virus terminal hairpin loop, respectively, b) comprise the fast form and the slow form of the vaccinia virus terminal hairpin loop, respectively, c) both comprise the slow form of the vaccinia virus terminal hairpin loop, or d) both comprise the fast form of the vaccinia virus terminal loop. In some embodiments, the slow form comprises a nucleotide sequence that is at least 85% identical to the sequence of SEQ ID NO: 11 and the fast form comprises a nucleotide sequence that is at least

85% identical to the nucleotide sequence of SEQ ID NO: 12. In some embodiments, the slow form comprises a nucleotide sequence that is at least 90% identical to the sequence of SEQ ID NO: 11 and the fast form comprises a nucleotide sequence that is at least 90% identical to the nucleotide sequence of SEQ ID NO: 12. In some embodiments, the slow form comprises a nucleotide sequence that is at least 95% identical to the sequence of SEQ ID NO: 11 and the fast form comprises a nucleotide sequence that is at least 95% identical to the nucleotide sequence of SEQ ID NO: 12. In some embodiments, the slow form consists of the nucleotide sequence of SEQ ID NO: 11 and the fast form consists of the nucleotide sequence of SEQ ID NO: 12. In some embodiments, the viral genome comprises terminal hairpin loops derived from camelpox virus, cowpox virus, ectromelia virus, monkeypox virus, variola virus, rabbitpox virus, raccoon poxvirus, skunkpox virus, Taterapox virus, Uasin Gishu disease virus, or volepox virus.

[0028] In some embodiments, the virus is replicated and assembled from overlapping chemically-synthesized DNA fragments that correspond to substantially all of the viral genome of HPXV.

[0029] In some embodiments, the virus is replicated and reactivated from 1-14 overlapping fragments. In some embodiments, the virus is replicated and reactivated from 8-12 overlapping fragments. In some embodiments, the virus is replicated and reactivated from 10 overlapping fragments.

[0030] In some embodiments, the virus is reactivated using leporipox virus-catalyzed recombination and reactivation. In some embodiments, the leporipox virus is selected from the group consisting of: Shope fibroma virus (SFV), hare fibroma virus, rabbit fibroma virus, squirrel fibroma virus, and myxoma virus.

[0031] In certain aspects, the invention provides a method of producing a synthetic chimeric poxvirus (scPV) comprising the steps of: (i) chemically synthesizing overlapping DNA fragments that correspond to substantially all of the viral genome of the poxvirus; (ii) transfecting the overlapping DNA fragments into helper virus-infected cells; (iii) culturing said cells to produce a mixture of

helper virus and synthetic chimeric poxviral particles in said cells; and (iv) plating the mixture on host cells specific to the scPV to recover the scPV.

[0032] In some embodiments, the helper virus is a leporipox virus. In some embodiments, the leporipox virus is selected from the group consisting of: Shope fibroma virus (SFV), hare fibroma virus, rabbit fibroma virus, squirrel fibroma virus, and myxoma virus. In some embodiments, the leporipox virus is SFV.

[0033] In some embodiments, the helper virus is fowlpox virus.

[0034] In some embodiments,the helper virus is a psoralen-inactivated helper virus.

[0035] In some embodiments, the helper virus-infected cells are BGMK cells.

[0036] In some embodiments, step (i) further comprises chemically synthesizing terminal hairpin loops from a poxvirus and ligating them onto the fragments comprising the left and right termini of the viral genome.

[0037] In certain aspects, the invention provides a method of producing a synthetic chimeric orthopox virus (scOPV) comprising the steps of: (i) chemically synthesizing overlapping DNA fragments that correspond to substantially all of the viral genome of the OPV; (ii) transfecting the overlapping DNA fragments into helper virus-infected cells; (iii) culturing said cells to produce a mixture of helper virus and scOPV particles in said cells; and (iv) plating the mixture on OPV-specific host cells to recover the scOPV.

[0038] In some embodiments, the helper virus is a leporipox virus. In some embodiments, the leporipox virus is selected from the group consisting of: Shope fibroma virus (SFV), hare fibroma virus, rabbit fibroma virus, squirrel fibroma virus, and myxoma virus. In some embodiments, the leporipox virus is SFV.

[0039] In some embodiments, the helper virus is fowlpox virus.

[0040] In some embodiments, the helper virus is a psoralen-inactivated helper virus.

[0041] In some embodiments, the helper virus-infected cells are BGMK cells.

[0042] In some embodiments, the OPV-specific host cells are BSC-40 cells.

[0043] In some embodiments, the OPV is selected from the group consisting of: camelpox virus, cowpox virus, ectromelia virus, horsepox virus, monkeypox virus, vaccinia virus, variola virus, rabbitpox virus, raccoon poxvirus, skunkpox virus, Taterapox virus, Uasin Gishu disease virus, volepox virus.

[0044] In some embodiments, step (i) further comprises chemically synthesizing terminal hairpin loops from an OPV and ligating them onto the fragments comprising the left and right termini of the viral genome.

[0045] In certain aspects, the invention provides a method of producing a synthetic chimeric horsepox virus (scHPXV) comprising the steps of: (i) chemically synthesizing overlapping DNA fragments that correspond to substantially all of the HPXV genome; (ii) transfecting the overlapping DNA fragments into helper virus-infected cells; (iii) culturing said cells to produce a mixture of helper virus and scHPXV particles in said cells; and (iv) plating the mixture on HPXV-specific host cells to recover the scHPXV.

[0046] In certain aspects, the invention provides a method of producing a synthetic chimeric horsepox virus (scHPXV) comprising: (i) chemically synthesizing overlapping DNA fragments that correspond to substantially all of the HPXV genome; (ii) transfecting the overlapping DNA fragments into Shope fibroma virus (SFV)-infected cells; (iii) culturing said cells to produce a mixture of SFV and scHPXV particles in said cells; and (iv) plating the mixture on HPXV-specific host cells to recover the scHPXV.

[0047] In some embodiments, the helper virus is a leporipox virus. In some embodiments, the leporipox virus is selected from the group consisting of: Shope fibroma virus (SFV), hare fibroma virus, rabbit fibroma virus, squirrel fibroma virus, and myxoma virus. In some embodiments, the leporipox virus is SFV.

[0048] In some embodiments, the helper virus is fowlpox virus.

[0049] In some embodiments, the helper virus is a psoralen-inactivated helper virus.

[0050] In some embodiments, the helper virus-infected cells are BGMK cells.

[0051] In some embodiments, the HPXV-specific host cells are BSC-40 cells.

[0052] In some embodiments, step (i) further comprises chemically synthesizing terminal hairpin loops from an OPV and ligating them onto the fragments comprising the left and right termini of the HPXV genome.

[0053] In some embodiments, the overlapping DNA fragments comprise: i) nucleotide sequences that are at least 85% identical to the sequences of SEQ ID NOs: 1 –10; ii) nucleotide sequences that are at least 90% identical to the sequences of SEQ ID NOs: 1 –10; (iii) nucleotide sequences that are at least 95% identical to the sequences of SEQ ID NOs: 1 –10; or (iv) nucleotide sequences that consist of the sequences of SEQ ID NOs: 1 –10.

[0054] In some embodiments, the SFV-infected cells are BGMK cells.

[0055] In some embodiments, the HPXV-specific host cells are BSC-40 cells.

[0056] In certain aspects, the invention provides a synthetic chimeric poxvirus (scPV) generated by the methods of the disclosure.

[0057] In certain aspects, the invention provides a synthetic chimeric orthopox virus (scOPV) generated by the methods of the disclosure.

[0058] In certain aspects, the invention provides a synthetic chimeric horsepox virus (scHPXV) generated by methods of the disclosure.

[0059] In certain aspects, the invention provides compositions comprising a pharmaceutically acceptable carrier and an scPV of the disclosure.

[0060] In certain aspects, the invention provides compositions comprising a pharmaceutically acceptable carrier and an scOPV of the disclosure.

[0061] In certain aspects, the invention provides a method of triggering or boosting an immune response against variola virus, comprising administering to a subject in need thereof a composition comprising an scOPV of the disclosure.

[0062] In certain aspects, the invention provides a method of triggering or boosting an immune response against vaccinia virus, comprising administering to a subject in need thereof a composition comprising an scOPV of the disclosure.

[0063] In certain aspects, the invention provides a method of triggering or boosting an immune response against monkeypox virus, comprising administering to a subject in need thereof a composition comprising an scOPV of the disclosure.

[0064] In certain aspects, the invention provides a method of immunizing a human subject to protect said subject from variola virus infection, comprising administering to said subject a composition comprising an scOPV of the disclosure.

[0065] In certain aspects, the invention provides a method of treating a variola virus infection, comprising administering to a subject in need thereof a composition comprising an scOPV of the disclosure.

[0066] In certain aspects, the invention provides a composition comprising a pharmaceutically acceptable carrier and an scHPXV of the disclosure.

[0067] In certain aspects, the invention provides a method of triggering or boosting an immune response against variola virus, comprising administering to a subject in need thereof a composition comprising an scHPXV of the disclosure.

[0068] In certain aspects, the invention provides a method of triggering or boosting an immune response against vaccinia virus, comprising administering to a subject in need thereof a composition comprising an scHPXV of the disclosure.

[0069] In certain aspects, the invention provides a method of triggering or boosting an immune response against monkeypox virus, comprising administering to a subject in need thereof a composition comprising an scHPXV of the disclosure.

[0070] In certain aspects, the invention provides a method of immunizing a human subject to protect said subject from variola virus infection, comprising administering to said subject a composition comprising an scHPXV of the disclosure.

[0071] In certain aspects, the invention provides a method of treating a variola virus infection, comprising administering to a subject in need thereof a composition comprising an scHPXV of the disclosure.

[0072] In certain aspects, the invention provides a kit comprising a composition comprising an scPV of the disclosure.

[0073] In certain aspects, the invention provides a kit comprising a composition comprising an OPV of the disclosure.

[0074] In certain aspects, the invention provides a kit comprising a composition comprising the scHPXV of the disclosure.

[0075] In certain aspects, a composition of the invention is administered in a poxvirus treatment facility. In certain aspects, a poxvirus treatment facility is a facility wherein subjects in need of immunization or treatment with a composition or method of the invention may be immunized or treated in an environment such that they are sequestered from other subjects not intended to be immunized or treated or who might be potentially infected by the treated subject (e.g., caregivers and household members). In some embodiments, the subjects not intended to be immunized or potentially infected by the treated subject, include HIV patients, patients undergoing chemotherapy, patients undergoing treatment for cancer, rheumatologic disorders, or autoimmune disorders, patients who are undergoing or have received an organ or tissue transplant, patients with immune deficiencies, children, pregnant women, patients with atopic dermatitis, eczema, psoriasis, heart conditions, and patients on immunosuppressants etc. In some embodiments, the poxvirus treatment facility is an orthopoxvirus treatment facility. In some embodiments, the poxvirus treatment facility is a smallpox treatment facility.

[0076] In certain aspects, a composition of the invention is administered by a specialist in smallpox adverse events. In some embodiments, the smallpox adverse events include but are not limited to eczema vaccinatum, progressive vaccinia, postvaccinal encephalitis, myocarditis, and dilated cardiomyopathy.

BRIEF DESCRIPTION OF THE DRAWINGS

[0077] The foregoing summary, as well as the following detailed description of the invention, will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention there are shown in the drawings embodiment(s) which are presently preferred. It should be understood, however, that the invention is not limited to the precise arrangements and instrumentalities shown.

[0078] **Figures 1A and 1B**. Schematic representation of the linear dsDNA HPXV genome (strain MNR; Genbank Accession DQ792504. A. Figure 1A illustrates the unmodified genome sequence of HPXV genome with individual HPXV genes (purple) and the naturally occurring *Aar*I and *Bsa*I sites indicated. B. Figure 1B depicts the modified synthetic chimeric HPXV (scHPXV) genome that was chemically synthesized using the overlapping genomic DNA fragments (shown in red). The engineered *Sap*I restriction sites that were used to ligate the VACV terminal hairpin loops onto the ITRs, along with the unmodified *Bsa*I sites in the left and right ITR fragments are also shown. The *Sap*I sites were located in plasmid vector sites immediately to the left and right ends of the Left Inverted Terminal Repeat (LITR) and Right Inverted Terminal Repeat (RITR), respectively.

[0079] **Figures 2A-2C**. Detailed schematic representation of the modified scHPXV YFP-gpt::095 genome and VACV (WR strain) terminal hairpin loops. A. Figure 2A depicts the modified scHPXV YFP-gpt::095 genome. The unmodified *Bsa*I sites are shown as blue lines on the genome. The novel *Ava*I and *Stu*I restriction sites that were created in HPXV044 (the VACV F4L homolog) are also marked (green lines). The location of the selectable marker yellow fluorescent protein/guanosine phosphoribosyl transferase (yfp/gpt) in the HPXV095 locus (the VACV J2R homolog) of Frag_3 is also shown (yellow). B. Figure 2B depicts the

nucleotide sequence of the S (SEQ ID NO: 11) and F (SEQ ID NO: 12) forms of the terminal hairpin loop, and the color coding is explained in (C). C. Figure 2C depicts the secondary structure predictions of the F and S forms of terminal hairpin loops that are covalently attached to the terminal ends of the linear dsDNA genomes of VACV. The terminal loop sequence is highlighted in green. The concatamer resolution sequence is boxed in red.

[0080] Figures 3A and 3B. The ~70bp VACV terminal hairpin can be ligated to the left and right HPXV ITR fragments. A. Figure 3A depicts a schematic diagram of the left and right HPXV ITR fragments marking the locations of the *Sap*I and *Pvu*II recognition sites. The predicted fragment sizes of the DNA following digestion with *Sap*I and *Pvu*II are shown. B. Figure 3 B depicts agarose gel electrophoresis of the left and right ITR fragments following ligation of the ~70bp terminal hairpin to the 1472bp ITR fragment cut with *Sap*I. The ligated DNAs were subsequently cut with *Pvu*II to facilitate detection of the small change in size cause by the addition of the hairpins.

[0081] Figures 4A-4C. PCR analysis and restriction digestion of scHPXV YFP-gpt::095 genomes confirm successful reactivation of scHPXV YFP-gpt::095. A. Figure 4A depicts the results of PCR analysis of scHPXV YFP-gpt::095 clones. Primers that flank conserved *Bsa*I restriction sites in both VACV and scHPXV YFP-gpt::095 were used to amplify a series of ~1kbp products. The PCR products were subsequently digested with *Bsa*I and the resulting DNA fragments were separated by agarose gel electrophoresis. VACV is cut, but all the *Bsa*I sites have been deleted from two different scHPXV YFP-gpt::095 clones. B. Figure 4B depicts pulse field gel electrophoresis (PFGE) of VACV-WR and scHPXV YFP-gpt::095 genomic DNAs. Virus DNAs was digested with *Bsa*I, *Hind*III, or left untreated, and were then separated on a 1% Seakem gold agarose gel for 14 h at 14˚C at 5.7V/cm with a switch time of 1 to 10 seconds. A slight difference in size between the intact VACV and scHPXV YFP-gpt::095 genomes was observed. The faint bands marked with an asterisk (*) are either incomplete DNA digestion products or could be cut mitochondrial DNA fragments that often contaminate VACV virion preparations. C. Figure 4C depicts conventional agarose gel

electrophoresis of VACV-WR and scHPXV YFP-gpt::095 genomic DNA digested with *Bsa*I or *Hind*III. DNA fragments were visualized by staining gels with SybrGold DNA stain.

[0082] Figures 5A and 5B. VACV terminal hairpins were successfully ligated onto the scHPXV YFP-gpt::095 ITR fragments. **A**. Illumina sequence reads were mapped to the scHPXV YFP-gpt::095 reference sequence. The *Sap*I site (in the vector, top strand sequence) was cut to create the point for ligating the telomeric hairpins onto the left and right ITR fragments. The location of the terminal loop of the VACV-derived terminal hairpin is shown, along with the concatamer resolution site. The first A in the concatamer resolution site is also the first nucleotide reported for HBXV (NCBI DQ792504), the remainder of the sequence to the left of that "A" was designed using VACV as a reference. Note that the "C's" were simply added as a scaffold to avoid the truncation of the reads by the assembly program. **B**. A subset comprising the longest Illumina sequencing reads, extending beyond the known HPXV sequence, are shown aligned against a polydC template. These reads span the entire length of an unfolded hairpin as was originally provided using synthetic oligonucleotides. Because of the inverted terminal duplications in poxviruses, all of the reads from the left and right ends "pile up" together and any original orientation or distribution is lost. It is apparent that both F- and S-forms of the VACV hairpin are detected and the ratio of F- to S-reads in this region was 1.03±0.01 (SEM) in eight different virus-sequencing reactions.

[0083] Figures 6A-6C. The *Bsa*I sites in scHPXV YFP-gpt::095 region 96,050 to 96,500 were correctly mutated. A. Figure 6A depicts Illumina sequence reads that mapped to one region of the HPXV (DQ792504) genome. Only a small fraction of the reads are shown. The conflicts in the sequencing reads at pos. 96,239 and pos. 96437 are highlighted in blue and yellow, respectively. B. Figure 6B depicts the magnification of the Illumina sequencing reads from scHPXV YFP-gpt::095 that mapped to HPXV (DQ792504) near pos. 96239. A nucleotide substitution (T96239C) was detected (refer to Table 2). C. Figure 6C depicts the magnification of the Illumina sequencing reads from scHPXV YFP-gpt::095 that mapped to HPXV (DQ792504) at pos. 96437. A nucleotide substitution

(A96437C) was detected (refer to Table 2). These mutations were introduced into the clones used to assemble scHPXV YFP-gpt::095 so as to delete undesirable *Bsa*I recognition sites (GGTCTC).

[0084] Figures 7A-7C. Nucleotide changes in the HPXV044 gene of scHPXV YFP-gpt::095 that were used to create the unique restriction sites *Ava*I and *Stu*I. A. Figure 7A depicts Illumina sequence reads that mapped to the HPXV044 gene in the HPXV (DQ792504) genome. Only a small fraction of the reads are shown. HPXV044 encodes the HPXV homolog of VACV F4L. The sites where the sequence reads do not match to the original HPXV (DQ792504) sequence are highlighted in yellow vertical lines. B. Figure 7B depicts the magnification of the Illumina sequencing reads from scHPXV YFP-gpt::095 that mapped to HPXV (DQ792504) at pos. 44,512. A nucleotide substitution (T44512G) was identified which creates an *Ava*I site (CYCGRG; refer to Table 2). C. Figure 7C depicts the magnification of the Illumina sequencing reads from scHPXV YFP-gpt::095 that mapped to HPXV (DQ792504) at pos. 45,061. A nucleotide substitution (T45061G) was detected that creates a StuI site (AGGCCT; refer to Table 2).

[0085] Figures 8A-8C. ScHPXV YFP-gpt::095 grows like other Orthopoxviruses but exhibits a small plaque phenotype in BSC-40 cells. A. Figure 8A illustrates the multi-step growth of VACV-WR, DPP15, CPXV, and scHPXV YFP-gpt::095 in BSC-40 (top left panel), HeLa (top middle panel), primary HEL (top right panel), and Vero (bottom left panel) cell lines. B. Figure 8B illustrates plaque size comparisons between VACV-WR, DPP15, CPXV, and scHPXV YFP-gpt::095. BSC-40 cells were infected with the indicated viruses and at 48 h post infection the cells were fixed and stained. The areas (in arbitrary units [A.U.]) of 24 plaques over three independent experiments were measured for each virus. Data are expressed as the mean plaque diameter. **, $P < 0.01$; ****, $P < 0.0001$. C. Figure 8C depicts plaque morphology of BSC-40 cells infected with the indicated viruses for 72h. Cells were fixed, stained, and scanned for visualization.

[0086] Figure 9. Schematic representation of the linear dsDNA genome of VACV (strain ACAM2000; Genbank Accession AY313847). The unmodified genome sequence of VACV ACAM2000 is illustrated with naturally occurring

AarI and *Bsa*I recognition sites marked. The overlapping DNA fragments are depicted in blue. The left (LITR_ACAM2000) and right (RITR_ACAM2000) fragments are shown in orange.

[0087] **Figure 10**. A graphical representation of the % weight loss over time after administration of various compositions and doses to mice. The depicted data are generated from groups of 5 female BALB/c mice that are inoculated with the indicated dose of scHPXV YFP-gpt::095 (also designated as scHPXV(ΔHPXV_095/J2R) or scHPXV (yfp/gpt)), scHPXV (wt), Dryvax DPP15, or VACV WR in 10µl of PBS. Mice are weighed daily for 28 days and any that lost >25% of their initial weight are euthanized. Data points represent mean scores, and error bars represent standard deviation.

[0088] **Figures 11A and 11B**. Graphical representations of the % weight loss over time after administration of various compositions and doses to mice. The depicted data are generated from mice that are previously vaccinated (Figure 10) and who are then challenged with a lethal dose of VACV WR (10^6 PFU) intranasally. **Figure 11A** shows the weight changes and **Figure 11B** shows the clinical scores in mice recorded daily for 13 days. Any mice that lost >25% of their initial weight are euthanized. Mice are assigned a clinical score based upon the appearance of ruffled fur, hunched posture, difficulty breathing, and decreased mobility. Data points represent mean differences in weights or scores, and error bars represent standard deviation. † indicate the number of mice that succumb to the VACV infection on a given day.

[0089] **Figure 12**. Graphical representation of the % survival over time after administration of various compositions and doses to mice. The depicted data are generated from mice that are previously vaccinated (Figure 10) and who are then challenged with a lethal dose of VACV WR (10^6 PFU) intranasally. Figure 12 shows survival curves of mice who are challenged intranasally with a lethal dose of VACV WR (10^6 PFU). † indicate the number of mice that succumb to the VACV infection on the indicated day.

[0090] Figures 13A and 13B. Characterization of VACV-HPXV hybrid viruses. A. HPXV inserts in VACV strain WR. Virus genomes were sequenced using an Illumina platform, assembled, and LAGAN[32] and "Base-by-Base"[33] software were used to align and generate the maps shown. Places where VACV sequences (white) have been replaced by HPXV sequences are color coded according to the difference. The first hybrid virus ("VACV/HPXV + fragment 3") was obtained by co-transfecting VACV DNA with HPXV Fragment_3 into SFV-infected cells. The green-tagged insertion encodes the YFP-gpt selection marker. Clones 1-3 were obtained by purifying the DNA from this first hybrid genome and transfecting it again, along with HPXV fragments 2, 4, 5, and 7, into SFV-infected cells. **B.** A PCR-based screening approach for identifying hybrid and reactivated viruses. PCR primers designed to target both HPXV and VACV and used to amplify DNA segments spanning the *Bsa*I sites that were mutated in the synthetic HPXV clones. Following PCR amplification, the products were digested with *Bsa*I to differentiate VACV sequences (which cut) from HPXV (which do not cut). The VACV/HPXV hybrids exhibit a mix of *Bsa*I sensitive and resistant sites whereas the reactivated scHPXV YFP-gpt::095 clone is fully *Bsa*I resistant.

[0091] Figures 14A-14C. Growth properties of scHPXV *versus* scHPXV YFP-gpt::095. A. Plaque size measurements. Homologous recombination was used to replace the YFP-gpt locus in scHPXV YFP-gpt::095 with thymidine kinase gene sequences. This produced a virus with a fully wild-type complement of HPXV genes (scHPXV). BSC-40 cells were infected with the indicated viruses and cultured for three days. The dishes were stained and the plaque areas measured using a scanned digital image. Statistically significant differences are noted ****$P< 0.0001$). **B.** Plaque images. **C.** Multi-step virus growth in culture. The indicated cell lines were infected with scHPXV or scHPXV YFP-gpt::095 at a multiplicity of infection of 0.01, the virus harvested at the indicated times, and titrated on BSC-40 cells in triplicate. No significant differences in the growth of these viruses were detected in these *in vitro* assays.

DETAILED DESCRIPTION OF THE INVENTION

General Techniques

[0092] Unless otherwise defined herein, scientific and technical terms used in this application shall have the meanings that are commonly understood by those of ordinary skill in the art. Generally, nomenclature used in connection with, and techniques of, pharmacology, cell and tissue culture, molecular biology, cell and cancer biology, neurobiology, neurochemistry, virology, immunology, microbiology, genetics and protein and nucleic acid chemistry, described herein, are those well known and commonly used in the art. In case of conflict, the present specification, including definitions, will control.

[0093] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, Molecular Cloning: A Laboratory Manual, second edition (Sambrook et al., 1989) Cold Spring Harbor Press; Oligonucleotide Synthesis (M.J. Gait, ed., 1984); Methods in Molecular Biology, Humana Press; Cell Biology: A Laboratory Notebook (J.E. Cellis, ed., 1998) Academic Press; Animal Cell Culture (R.I. Freshney, ed., 1987); Introduction to Cell and Tissue Culture (J.P. Mather and P.E. Roberts, 1998) Plenum Press; Cell and Tissue Culture: Laboratory Procedures (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., 1993-1998) J. Wiley and Sons; Methods in Enzymology (Academic Press, Inc.); Gene Transfer Vectors for Mammalian Cells (J.M. Miller and M.P. Calos, eds., 1987); Current Protocols in Molecular Biology (F.M. Ausubel et al., eds., 1987); PCR: The Polymerase Chain Reaction, (Mullis et al., eds., 1994); Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd. ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (2001); Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, NY (2002); Harlow and Lane Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1998);

Coligan et al., Short Protocols in Protein Science, John Wiley & Sons, NY (2003); Short Protocols in Molecular Biology (Wiley and Sons, 1999).

[0094] Enzymatic reactions and purification techniques are performed according to manufacturer's specifications, as commonly accomplished in the art or as described herein. The nomenclatures used in connection with, and the laboratory procedures and techniques of, analytical chemistry, biochemistry, immunology, molecular biology, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard techniques are used for chemical syntheses, and chemical analyses.

[0095] Throughout this specification and embodiments, the word "comprise," or variations such as "comprises" or "comprising," will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

[0096] It is understood that wherever embodiments are described herein with the language “comprising,” otherwise analogous embodiments described in terms of “consisting of” and/or “consisting essentially of” are also provided.

[0097] The term “including” is used to mean “including but not limited to.” “Including” and “including but not limited to” are used interchangeably.

[0098] Any example(s) following the term “e.g.” or “for example” is not meant to be exhaustive or limiting.

[0099] Unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[0100] The articles "a", "an" and “the” are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element. Reference to “about” a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter per se. For example, description referring to

"about X" includes description of "X." Numeric ranges are inclusive of the numbers defining the range.

[0101] Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements. Moreover, all ranges disclosed herein are to be understood to encompass any and all subranges subsumed therein. For example, a stated range of "1 to 10" should be considered to include any and all subranges between (and inclusive of) the minimum value of 1 and the maximum value of 10; that is, all subranges beginning with a minimum value of 1 or more, e.g., 1 to 6.1, and ending with a maximum value of 10 or less, e.g., 5.5 to 10.

[0102] Where aspects or embodiments of the invention are described in terms of a Markush group or other grouping of alternatives, the present invention encompasses not only the entire group listed as a whole, but each member of the group individually and all possible subgroups of the main group, and also the main group absent one or more of the group members. The present invention also envisages the explicit exclusion of one or more of any of the group members in the embodimented invention.

[0103] Exemplary methods and materials are described herein, although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention. The materials, methods, and examples are illustrative only and not intended to be limiting.

Definitions

The following terms, unless otherwise indicated, shall be understood to have the following meanings:

[0104] As used herein, the terms "wild type virus", " wild type genome", "wild type protein," or "wild type nucleic acid" refer to a sequence of amino or nucleic

acids that occurs naturally within a certain population (e.g., a particular viral species, etc.).

[0105] The terms "chimeric" or "engineered" or "modified" (e.g., chimeric poxvirus, engineered polypeptide, modified polypeptide, engineered nucleic acid, modified nucleic acid) or grammatical variations thereof are used interchangeably herein to refer to a non-native sequence that has been manipulated to have one or more changes relative a native sequence.

[0106] As used herein, "synthetic virus" refers to a virus initially derived from synthetic DNA (e.g. chemically synthesized DNA, PCR amplified DNA, engineered DNA, polynucleotides comprising nucleoside analogs, etc. or combinations thereof) and includes its progeny, and the progeny may not necessarily be completely identical (in morphology or in genomic DNA complement) to the original parent synthetic virus due to natural, accidental, or deliberate mutation. In some embodiments, the synthetic virus refers to a virus where substantially all of the viral genome is initially derived from chemically synthesized DNA.

[0107] As outlined elsewhere herein, certain positions of the viral genome can be altered. By "position" as used herein is meant a location in the genome sequence. Corresponding positions are generally determined through alignment with other parent sequences.

[0108] As used herein, "residue" refers to a position in a protein and its associated amino acid identity.

[0109] As known in the art, "polynucleotide," or "nucleic acid," as used interchangeably herein, refer to chains of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a chain by DNA or RNA polymerase. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after

assembly of the chain. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications include, for example, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, alpha- or beta-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S("thioate"), P(S)S ("dithioate"), (O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl.

Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

[0110] The terms "polypeptide", "oligopeptide", "peptide" and "protein" are used interchangeably herein to refer to chains of amino acids of any length. The chain may be linear or branched, it may comprise modified amino acids, and/or may be interrupted by non-amino acids. The terms also encompass an amino acid chain that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art. It is understood that the polypeptides can occur as single chains or associated chains.

[0111] "Homologous," in all its grammatical forms and spelling variations, refers to the relationship between two proteins that possess a "common evolutionary origin," including proteins from superfamilies in the same species of organism, as well as homologous proteins from different species of organism. Such proteins (and their encoding nucleic acids) have sequence homology, as reflected by their sequence similarity, whether in terms of percent identity or by the presence of specific residues or motifs and conserved positions.

[0112] However, in common usage and in the instant application, the term "homologous," when modified with an adverb such as "highly," may refer to sequence similarity and may or may not relate to a common evolutionary origin.

[0113] The term "sequence similarity," in all its grammatical forms, refers to the degree of identity or correspondence between nucleic acid or amino acid sequences that may or may not share a common evolutionary origin.

[0114] "Percent (%) sequence identity" with respect to a reference polypeptide (or nucleotide) sequence is defined as the percentage of amino acid residues (or nucleic acids) in a candidate sequence that are identical with the amino acid

residues (or nucleic acids) in the reference polypeptide (nucleotide) sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

[0115] As used herein, a "host cell" includes an individual cell or cell culture that can be or has been a recipient for vector(s) for incorporation of polynucleotide inserts. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in genomic DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation. A host cell includes cells transfected and/or transformed *in vivo* with a nucleic acid of this invention.

[0116] As used herein, "vector" means a construct, which is capable of delivering, and, preferably, expressing, one or more gene(s) or sequence(s) of interest in a host cell. Examples of vectors include, but are not limited to, viral vectors, naked DNA or RNA expression vectors, plasmid, cosmid or phage vectors, DNA or RNA expression vectors associated with cationic condensing agents, DNA or RNA expression vectors encapsulated in liposomes, and certain eukaryotic cells, such as producer cells.

[0117] As used herein, "expression control sequence" means a nucleic acid sequence that directs transcription of a nucleic acid. An expression control sequence can be a promoter, such as a constitutive or an inducible promoter, or an enhancer. The expression control sequence is operably linked to the nucleic acid sequence to be transcribed.

[0118] As used herein, "isolated molecule" (where the molecule is, for example, a polypeptide, a polynucleotide, or fragment thereof) is a molecule that by virtue of its origin or source of derivation (1) is not associated with one or more naturally associated components that accompany it in its native state, (2) is substantially free of one or more other molecules from the same species (3) is expressed by a cell from a different species, or (4) does not occur in nature. Thus, a molecule that is chemically synthesized, or expressed in a cellular system different from the cell from which it naturally originates, will be "isolated" from its naturally associated components. A molecule also may be rendered substantially free of naturally associated components by isolation, using purification techniques well known in the art. Molecule purity or homogeneity may be assayed by a number of means well known in the art. For example, the purity of a polypeptide sample may be assayed using polyacrylamide gel electrophoresis and staining of the gel to visualize the polypeptide using techniques well known in the art. For certain purposes, higher resolution may be provided by using HPLC or other means well known in the art for purification.

[0119] As used herein, the term "isolated", in the context of viruses, refers to a virus that is derived from a single parental virus. A virus can be isolated using routine methods known to one of skill in the art including, but not limited to, those based on plaque purification and limiting dilution.

[0120] As used herein, the phrase "multiplicity of infection" or "MOI" is the average number of virus per infected cell. The MOI is determined by dividing the number of viruses added (ml added×plaque forming units (PFU)) by the number of cells added (ml added×cells/ml).

[0121] As used herein, "purify," and grammatical variations thereof, refers to the removal, whether completely or partially, of at least one impurity from a mixture containing the polypeptide and one or more impurities, which thereby improves the level of purity of the polypeptide in the composition (i.e., by decreasing the amount (ppm) of impurity(ies) in the composition). As used herein "purified" in the context of viruses refers to a virus which is substantially free of cellular material and culture media from the cell or tissue source from which the virus is

derived. The language "substantially free of cellular material" includes preparations of virus in which the virus is separated from cellular components of the cells from which it is isolated or recombinantly produced. Thus, virus that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, or 5% (by dry weight) of cellular protein (also referred to herein as a "contaminating protein"). The virus is also substantially free of culture medium, i.e., culture medium represents less than about 20%, 10%, or 5% of the volume of the virus preparation. A virus can be purified using routine methods known to one of skill in the art including, but not limited to, chromatography and centrifugation.

[0122] As used herein, “substantially pure” refers to material which is at least 50% pure (i.e., free from contaminants), more preferably, at least 90% pure, more preferably, at least 95% pure, yet more preferably, at least 98% pure, and most preferably, at least 99% pure.

[0123] The terms “patient”, “subject”, or “individual” are used interchangeably herein and refer to either a human or a non-human animal. These terms include mammals, such as humans, primates, livestock animals (including bovines, porcines, camels, etc.), companion animals (e.g., canines, felines, etc.) and rodents (e.g., mice and rats).

[0124] As used herein, the terms "prevent", "preventing" and "prevention" refer to the prevention of the recurrence or onset of, or a reduction in one or more symptoms of a disease (e.g., a poxviral infection) in a subject as a result of the administration of a therapy (e.g., a prophylactic or therapeutic agent). For example, in the context of the administration of a therapy to a subject for an infection, "prevent", "preventing" and "prevention" refer to the inhibition or a reduction in the development or onset of an infection (e.g., a poxviral infection or a condition associated therewith), or the prevention of the recurrence, onset, or development of one or more symptoms of an infection (e.g., a poxviral infection or a condition associated therewith), in a subject resulting from the administration of a therapy (e.g., a prophylactic or therapeutic agent), or the administration of a combination of therapies (e.g., a combination of prophylactic or therapeutic agents).

[0125] "Treating" a condition or patient refers to taking steps to obtain beneficial or desired results, including clinical results. With respect to infections (e.g., a poxviral infection), treatment refers to the eradication or control of the replication of an infectious agent (e.g., a poxvirus), the reduction in the numbers of an infectious agent (e.g., the reduction in the titer of poxvirus), the reduction or amelioration of the progression, severity, and/or duration of an infection (e.g., a poxviral infection or a condition or symptoms associated therewith), or the amelioration of one or more symptoms resulting from the administration of one or more therapies (including, but not limited to, the administration of one or more prophylactic or therapeutic agents). With respect to cancer, treatment refers to the eradication, removal, modification, or control of primary, regional, or metastatic cancer tissue that results from the administration of one or more therapeutic agents of the invention. In certain embodiments, such terms refer to the minimizing or delaying the spread of cancer resulting from the administration of one or more therapeutic agents of the invention to a subject with such a disease. In other embodiments, such terms refer to elimination of disease causing cells.

[0126] "Administering" or "administration of" a substance, a compound or an agent to a subject can be carried out using one of a variety of methods known to those skilled in the art. For example, a compound or an agent can be administered sublingually or intranasally, by inhalation into the lung or rectally. Administering can also be performed, for example, once, a plurality of times, and/or over one or more extended periods. In some aspects, the administration includes both direct administration, including self-administration, and indirect administration, including the act of prescribing a drug. For example, as used herein, a physician who instructs a patient to self-administer a drug, or to have the drug administered by another and/or who provides a patient with a prescription for a drug is administering the drug to the patient.

[0127] Each embodiment described herein may be used individually or in combination with any other embodiment described herein.

Overview

[0128] Poxviruses are large (~200 kbp) DNA viruses that replicate in the cytoplasm of infected cells. The *Orthopoxvirus* (OPV) genus comprises a number of poxviruses that vary greatly in their ability to infect different hosts. Vaccinia virus (VACV), for example, can infect a broad group of hosts, whereas variola virus (VARV), the causative agent of smallpox, only infects humans. A feature common to many, if not all poxviruses, is their ability to non-genetically "reactivate" within a host. Non-genetic reactivation refers to a process wherein cells infected by one poxvirus can promote the recovery of a second "dead" virus (for example one inactivated by heat) that would be non-infectious on its own.

[0129] Purified poxvirus DNA is not infectious because the virus life cycle requires transcription of early genes via the virus-encoded RNA polymerases that are packaged in virions. However, this deficiency can be overcome if virus DNA is transfected into cells previously infected with a helper poxvirus, providing the necessary factors needed to transcribe, replicate, and package the transfected genome *in trans* (Sam CK, Dumbell KR. Expression of poxvirus DNA in coinfected cells and marker rescue of thermosensitive mutants by subgenomic fragments of DNA. Ann Virol (Inst Past). 1981;132:135-50). Although this produces mixed viral progeny, the problem can be overcome by performing the reactivation reaction in a cell line that supports the propagation of both viruses, and then eliminating the helper virus by plating the mixture of viruses on cells that do not support the helper virus' growth (Scheiflinger F, Dorner F, Falkner FG. Construction of chimeric vaccinia viruses by molecular cloning and packaging. Proceedings of the National Academy of Sciences of the United States of America. 1992;89(21):9977-81).

[0130] Previously, a method where the high-frequency recombination reactions catalyzed by a *Leporipoxvirus*, Shope fibroma virus (SFV), can be coupled with an SFV-catalyzed reactivation reaction, to rapidly assemble recombinant VACV strains using multiple overlapping fragments of viral DNA (Yao XD, Evans DH.

High-frequency genetic recombination and reactivation of orthopoxviruses from DNA fragments transfected into leporipoxvirus-infected cells. Journal of virology. 2003;77(13):7281-90). For the first time, the reactivation and characterization of a functional poxvirus (synthetic chimeric horsepox virus [scHPXV]) using chemically synthesized, overlapping double-stranded DNA fragments is described. The principles can be analogously applied and extrapolated to other poxviruses, including but not limited to camelpox virus (CMLV), cowpox virus (CPXV), ectromelia virus (ECTV, "mousepox agent"), horsepox (HPXV), monkeypox virus (MPXV), rabbitpox virus (RPXV), raccoonpox virus, skunkpox virus, Taterapox virus, Uasin Gishu disease virus, vaccinia virus (VACV), and volepox virus (VPV).

[0131] It is further shown here that one embodiment of a synthetic chimeric poxvirus of the invention (e.g. a synthetic chimeric horsepox virus), can infect and immunize mice against a lethal VACV challenge and can do so without causing any disease during the initial immunization step.

Synthetic Chimeric Poxviruses of the Invention

[0132] The invention provides functional synthetic chimeric poxviruses (scPVs) that are initially replicated and assembled from chemically synthesized DNA. The viruses that may be produced in accordance with the methods of the invention can be any poxvirus whose genome has been sequenced in large part or for which a natural isolate is available. An scPV of the invention may be based on the genome sequences of naturally occurring strains, variants or mutants, mutagenized viruses or genetically engineered viruses. The viral genome of an scPV of the invention comprises one or more modifications relative to the wild type genome or base genome sequence of said virus. The modifications may include one or more deletions, insertions, substitutions, or combinations thereof. It is understood that the modifications may be introduced in any number of ways commonly known in the art. The modified portions of the genome may be derived from chemically synthesized DNA, cDNA or genomic DNA.

[0133] Chemical genome synthesis is particularly useful when a natural template is not available for genetic modification, amplification, or replication by conventional molecular biology methods. For example, a natural isolate of horsepox virus (HPXV) is not readily available to obtain template DNA but the genome sequence for HPXV (strain MNR-76) has been described. The HPXV genome sequence, however, is incomplete. The sequence of the terminal hairpin loops was not determined. In a surprising result, a functional synthetic chimeric HPXV (scHPXV) was generated by using terminal hairpin loops based on VACV telomeres in lieu of HPXV terminal hairpin loop sequences. In some embodiments, the poxvirus belongs to the *Chordopoxvirinae* subfamily. In some embodiments, the poxvirus belongs to a genus of *Chordopoxvirinae* subfamily selected from *Avipoxvirus, Capripoxvirus, Cervidpoxvirus, Crocodylipoxvirus, Leporipoxvirus, Molluscipoxvirus, Orthopoxvirus, Parapoxvirus, Suipoxvirus, or Yatapoxvirus.* In some embodiments, the poxvirus is an *Orthopoxvirus*. In some embodiments, the *Orthopoxvirus* is selected from camelpox virus (CMLV), cowpox virus (CPXV), ectromelia virus (ECTV, "mousepox agent"), HPXV, monkeypox virus (MPXV), rabbitpox virus (RPXV), raccoonpox virus, skunkpox virus, Taterapox virus, Uasin Gishu disease virus, vaccinia virus (VACV), variola virus (VARV) and volepox virus (VPV). In a preferred embodiment, the poxvirus is an HPXV. In another preferred embodiment, the poxvirus is a VACV. In some embodiments, the poxvirus is a *Parapoxvirus*. In some embodiments, the *Parapoxvirus* is selected from orf virus (ORFV), pseudocowpox virus (PCPV), bovine popular stomatitis virus (BPSV), squirrel parapoxvirus (SPPV), red deer parapoxvirus, Ausdyk virus, Chamois contagious ecythema virus, reindeer parapoxvirus, or sealpox virus. In some embodiments, the poxvirus is a *Molluscipoxvirus*. In some embodiments, the *Molluscipoxvirus* is molluscum contagiousum virus (MCV). In some embodiments, the poxvirus is a *Yatapoxvirus*. In some embodiments, the *Yatapoxvirus* is selected from Tanapox virus or Yaba monkey tumor virus (YMTV). In some embodiments, the poxvirus is a *Capripoxvirus*. In some embodiments, the *Capripoxvirus* is selected from sheepox, goatpox, or lumpy skin disease virus. In some embodiments, the poxvirus is a *Suipoxvirus*. In some embodiments, the *Suipoxvirus* is swinepox

virus. In some embodiments, the poxvirus is a *Leporipoxvirus*. In some embodiments, the *Leporipoxvirus* is selected from myxoma virus, Shope fibroma virus (SFV), squirrel fibroma virus, or hare fibroma virus. New poxviruses (e.g. Orthopoxviruses) are still being constantly discovered. It is understood that an scPV of the invention may be based on such a newly discovered poxvirus.

[0134] Chemical viral genome synthesis also opens up the possibility of introducing a large number of useful modifications to the resulting genome or to specific parts of it. The modifications may improve ease of cloning to generate the virus, provide sites for introduction of recombinant gene products, improve ease of identifying reactivated viral clones and/or confer a plethora of other useful features (e.g. introducing a desired antigen, producing an oncolytic virus, etc.). In some embodiments, the modifications may include the attenuation or deletion of one or more virulence factors. In some embodiments, the modifications may include the addition or insertion of one or more virulence regulatory genes or gene-encoding regulatory factors.

[0135] Traditionally, the terminal hairpins of poxviruses have been difficult to clone and sequence, hence, it is not surprising that some of the published genome sequences (e.g., VACV, ACAM 2000 and HPXV MNR-76) are incomplete. The published sequence of the HPXV genome is likewise incomplete, probably missing ~60 bp from the terminal ends. Thus, the HPXV hairpins cannot be precisely replicated and prior to this invention, it was not known whether HPXV could be replicated and assembled from polynucleotides based on only the known portion of the HPXV genome. Nor was it known that hairpins from one virus would be operable in another. In an exemplary embodiment, 129 nt ssDNA fragments were chemically synthesized using the published sequence of the VACV telomeres as a guide and ligated onto dsDNA fragments comprising left and right ends of the HPXV genome. In some embodiments, the terminal hairpins of an scPV of the invention are derived from VACV. In some embodiments, the terminal hairpins are derived from CMLV, CPXV, ECTV, HPXV, MPXV, RPXV, raccoonpox virus, skunkpox virus, Taterapox virus, Uasin Gishu disease virus or VPV. In some embodiments, the terminal hairpins are based on the terminal hairpins of any

poxvirus whose genome has been completely sequenced or a natural isolate of which is available for genome sequencing.

[0136] In some embodiments, the modifications may include the deletion of one or more restriction sites. In some embodiments, the modifications may include the introduction of one or more restriction sites. In some embodiments, the restriction sites to be deleted from the genome or added to the genome may be selected from one or more of restriction sites such as but not limited to *AanI, AarI, AasI, AatI, AatII, AbaSI, AbsI, Acc65I , AccI, AccII, AccIII, AciI, AclI, AcuI, AfeI, AflII, AflIII, AgeI, AhdI, AleI, AluI, AlwI, AlwNI, ApaI, ApaLI, ApeKI, ApoI, AscI, AseI, AsiSI, AvaI, AvaII, AvrII, BaeGI, BaeI, BamHI BanI, BanII, BbsI, BbvCI, BbvI, BccI, BceAI, BcgI, BciVI, BclI, BcoDI, BfaI, BfuAI, BfuCI, BglI, BglII, BlpI, BmgBI, BmrI, BmtI, BpmI, Bpu10I, BpuEI, BsaAI, BsaBI, BsaHI, BsaI, BsaJI, BsaWI, BsaXI, BseRI, BseYI, BsgI, BsiEI, BsiHKAI, BsiWI, BslI, BsmAI, BsmBI, BsmFI, BsmI, BsoBI, Bsp1286I, BspCNI, BspDI, BspEI, BspHI, BspMI, BspQI, BsrBI, BsrDI, BsrFαI, BsrGI, BsrI, BssHII, BssSαI, BstAPI, BstBI, BstEII, BstNI, BstUI, BstXI, BstYI, BstZ17I, Bsu36I, BtgI, BtgZI, BtsαI, BtsCI, BtsIMutI, Cac8I, ClaI, CspCI, CviAII, CviKI-1, CviQI, DdeI, DpnI, DpnII, DraI, DrdI, EaeI, EagI, EarI, EciI, Eco53kI, EcoNI, EcoO109I, EcoP15I, EcoRI, EcoRV, FatI, FauI, Fnu4HI, FokI, FseI, FspEI, FspI, HaeII, HaeIII, HgaI, HhaI, HincII, HindIII, HinfI, HinP1I, HpaI, HpaII, HphI, Hpy166II, Hpy188I, Hpy188III, Hpy99I, HpyAV, HpyCH4III, HpyCH4IV, HpyCH4V, I-CeuI, I-SceI, KasI, KpnI, LpnPI, MboI, MboII, MfeI, MluCI, MluI, MlyI, MmeI, MnlI, MscI, MseI, MslI, MspA1I, MspI, MspJI, MwoI, NaeI, NarI, NciI, NcoI, NdeI, NgoMIV, NheI, NlaIII, NlaIV, NmeAIII, NotI, NruI, NsiI, NspI, PacI, PaeR7I, PciI, PflFI, PflMI, PleI, PluTI, PmeI, PmlI, PpuMI, PshAI, PsiI, PspGI, PspOMI, PspXI, PstI, PvuI, PvuII, RsaI, RsrII, SacI, SacII, SalI, SapI, Sau3AI, Sau96I, SbfI, ScrFI, SexAI, SfaNI, SfcI, SfiI, SfoI, SgrAI, SmaI, SmlI, SnaBI, SpeI, SphI, SrfI, SspI, StuI, StyD4I, StyI, SwaI, TaqαI, TfiI, TseI, Tsp45I, TspMI, TspRI, Tth111I, XbaI, XcmI, XhoI, XmaI, XmnI, or ZraI*. It is understood that any desired restriction site(s) or combination of restriction sites may be inserted into the genome or mutated and/or eliminated from the genome. In some embodiments, one or more *AarI* sites are deleted from the viral genome. In some embodiments, one or more *Bsa*I sites are deleted from the

viral genome. In some embodiments, one or more restriction sites are completely eliminated from the genome (e.g. all the *AarI* sites in the viral genome may be eliminated). In some embodiments, one or more *AvaI* restriction sites are introduced into the viral genome. In some embodiments, one or more *StuI* sites are introduced into the viral genome. In some embodiments, the one or more modifications may include the incorporation of recombineering targets including but not limited to *loxP* or FRT sites.

[0137] In some embodiments, the modifications may include the introduction of fluorescence markers such as but not limited to green fluorescent protein (GFP), enhanced GFP, yellow fluorescent protein (YFP), cyan/blue fluorescent protein (BFP), red fluorescent protein (RFP), or variants thereof, etc.; selectable markers such as but not limited to drug resistance markers (e.g. E. coli xanthine-guanine phosphoribosyl transferase gene (*gpt), Streptomyces alboniger* puromycin acetyltransferase gene (*pac*), neomycin phosphotransferase I gene (*nptI*), neomycin phosphotransferase gene II (*nptII*), hygromycin phosphotransferase (*hpt*), sh *ble* gene, etc.; protein or peptide tags such as but not limited to MBP (maltose-binding protein), CBD (cellulose-binding domain), GST (glutathione-S-transferase), poly(His), FLAG, V5, c-Myc, HA (hemagglutinin), NE-tag, CAT (chloramphenicol acetyl transferase), DHFR (dihydrofolate reductase), HSV (Herpes simplex virus), VSV-G (Vesicular stomatitis virus glycoprotein), luciferase, protein A, protein G, streptavidin, T7, thioredoxin, Yeast 2-hybrid tags such as B42, GAL4, LexA, or VP16; localization tags such as an NLS-tag, SNAP-tag, Myr-tag, etc. It is understood that other selectable markers and/or tags known in the art may be used. In some embodiments, the modifications include one or more selectable markers to aid in the selection of reactivated clones (e.g. a fluorescence marker such as YFP, a drug selection marker such as *gpt*, etc.) to aid in the selection of reactivated viral clones. In some embodiments, the one or more selectable markers are deleted from the reactivated clones after the selection step.

[0138] The scPVs of the invention can be used as vaccines to protect against pathogenic poxviral infections (e.g. VARV, MPXV, MCV, ORFV, Ausdyk virus, BPSV, sealpox virus etc.), as therapeutic agents to treat or prevent pathogenic

poxviral infections (e.g. VARV, MPXV, MCV, ORFV, Ausdyk virus, BPSV, sealpox virus etc.), as vehicles for heterologous gene expression, or as oncolytic agents. In some embodiments, the scPVs of the invention can be used as vaccines to protect against VARV infection. In some embodiments, the scPVs of the invention can be used to treat or prevent VARV infection.

Methods of Producing Synthetic Chimeric Poxviruses

[0139] The invention provides systems and methods for synthesizing, reactivating and isolating functional synthetic chimeric poxviruses (scPVs) from chemically synthesized overlapping double-stranded DNA fragments of the viral genome. Recombination of overlapping DNA fragments of the viral genome and reactivation of the functional scPV is carried out in cells previously infected with a helper virus. Briefly, overlapping DNA fragments that encompass all or substantially all of the viral genome of the scPV are chemically synthesized and transfected into helper virus-infected cells. The transfected cells are cultured to produce mixed viral progeny comprising the helper virus and reactivated scPV. Next, the mixed viral progeny are plated on host cells that do not support the growth of the helper virus but allow the synthetic chimeric poxvirus to grow, in order to eliminate the helper virus and recover the synthetic chimeric poxvirus. In some embodiments, the helper virus does not infect the host cells. In some embodiments, the helper virus can infect the host cells but grows poorly in the host cells. In some embodiments, the helper virus grows more slowly in the host cells compared to the scPV.

[0140] In some embodiments, substantially all of the synthetic chimeric poxviral genome is derived from chemically synthesized DNA. In some embodiments, about 40%, about 50%, about 60%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, over 99%, or 100% of the synthetic chimeric poxviral genome is derived from chemically synthesized DNA. In some embodiments, the poxviral genome is derived from a combination of chemically synthesized DNA and naturally occurring DNA.

[0141] The number of overlapping DNA fragments used in the methods of the invention will depend on the size of the poxviral genome. Practical considerations such as reduction in recombination efficiency as the number of fragments increases on the one hand and difficulties in synthesizing very large DNA fragments as the number of fragments decreases on the other hand will also inform the number of overlapping fragments used in the methods of the invention. In some embodiments, the synthetic chimeric poxviral genome may be synthesized as a single fragment. In some embodiments, the synthetic chimeric poxviral genome is assembled from 2-14 overlapping DNA fragments. In some embodiments, the synthetic chimeric poxviral genome is assembled from 4-12 overlapping DNA fragments. In some embodiments, the synthetic chimeric poxviral genome is assembled from 6-10 overlapping DNA fragments. In some embodiments, the synthetic chimeric poxviral genome is assembled from 8-12 overlapping DNA fragments. In some embodiments, the synthetic chimeric poxviral genome is assembled from 8-10, 10-12, or 10-14 overlapping DNA fragments. In some embodiments, the synthetic chimeric poxviral genome is assembled from 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 overlapping DNA fragments. In some embodiments, the synthetic chimeric poxviral genome is assembled from 10 overlapping DNA fragments. In an exemplary embodiment of the disclosure, a synthetic chimeric horsepox virus (scHPXV) is reactivated from 10 chemically synthesized overlapping double-stranded DNA fragments. In some embodiments, terminal hairpin loops are synthesized separately and ligated onto the fragments comprising the left and right ends of the poxviral genome. In some embodiments, terminal hairpin loops may be derived from a naturally occurring template. In some embodiments, the terminal hairpins of an scPV of the invention are derived from VACV. In some embodiments, the terminal hairpins are derived from CMLV, CPXV, ECTV, HPXV, MPXV, RPXV, raccoonpox virus, skunkpox virus, Taterapox virus, Uasin Gishu disease virus or VPV. In some embodiments, the terminal hairpins are based on the terminal hairpins of any poxvirus whose genome has been completely sequenced or a natural isolate of which is available for genome sequencing. In some embodiments, all of the fragments encompassing the poxviral genome are chemically synthesized. In some embodiments, one or more

of the fragments are chemically synthesized and one or more of the fragments are derived from naturally occurring DNA (e.g. by PCR amplification or by well-established recombinant DNA techniques).

[0142] The size of the overlapping fragments used in the methods of the invention will depend on the size of the poxviral genome. It is understood that there can be wide variations in fragment sizes and various practical considerations such as the ability to chemically synthesize very large DNA fragments, will inform the choice of fragment sizes. In some embodiments, the fragments range in size from about 2,000 bp to about 50,000 bp. In some embodiments, the fragments range in size from about 3,000 bp to about 45,000 bp. In some embodiments, the fragments range in size from about 4,000 bp to 40,000 bp. In some embodiments, the fragments range in size from about 5,000 bp to 35,000 bp. In some embodiments, the largest fragments are about 20,000 bp, 21,000 bp, 22,000 bp, 23,000 bp, 24, 000 bp, 25,000 bp, 26,000 bp, 27,000 bp, 28,000 bp, 29,000 bp, 30,000 bp, 31,000 bp, 32,000 bp, 33,000 bp, 34,000 bp, 35,000 bp, 36,000 bp, 37,000 bp, 38,000 bp, 39,000 bp, 40,000 bp, 41,000 bp, 42,000 bp, 43,000 bp, 44, 000 bp, 45,000 bp, 46,000 bp, 47,000 bp, 48,000 bp, 49,000 bp, or 50,000 bp. In an exemplary embodiment of the disclosure, an scHPXV is reactivated from 10 chemically synthesized overlapping double-stranded DNA fragments ranging in size from about 8,500 bp to about 32,000 bp (Table 1).

[0143] The helper virus may be any poxvirus that can provide the trans-acting enzymatic machinery needed to reactivate a poxvirus from transfected DNA. The helper virus may have a different or narrower host cell range than an scPV to be produced (e.g. Shope fibroma virus (SFV) has a very narrow host range compared to Orthopoxviruses such as vaccinia virus (VACV) or HPXV). The helper virus may have a different plaque phenotype compared to the scPV to be produced. In some embodiments, the helper virus is a *Leporipoxvirus*. In some embodiments, the *Leporipoxvirus* is an SFV, hare fibroma virus, rabbit fibroma virus, squirrel fibroma virus, or myxoma virus. In some embodiments, the helper virus is an SFV. In some embodiments, the helper virus is an *Orthopoxvirus*. In some embodiments, the *Orthopoxvirus* is a camelpox virus (CMLV), cowpox virus

(CPXV), ectromelia virus (ECTV, "mousepox agent"), HPXV, monkeypox virus (MPXV), rabbitpox virus (RPXV), raccoonpox virus, skunkpox virus, Taterapox virus, Uasin Gishu disease virus, VACV and volepox virus (VPV). In some embodiments, the helper virus is an *Avipoxvirus*, *Capripoxvirus*, *Cervidpoxvirus*, *Crocodylipoxvirus*, *Molluscipoxvirus*, *Parapoxvirus*, *Suipoxvirus*, or *Yatapoxvirus.* In some embodiments, the helper virus is a fowlpox virus. In some embodiments, the helper virus is an *Alphaentomopoxvirus*, *Betaentomopoxvirus*, or *Gammaentomopoxvirus*. In some embodiments, the helper virus is a psoralen-inactivated helper virus. In an exemplary embodiment of the disclosure, an scHPXV is reactivated from overlapping DNA fragments transfected into SFV-infected BGMK cells. The SFV is then eliminated by plating the mixed viral progeny on BSC-40 cells.

[0144] The skilled worker will understand that appropriate host cells to be used for the reactivation of the scPV and the selection and/or isolation of the scPV will depend on the particular combination of helper virus and chimeric poxvirus being produced by the methods of the invention. Any host cell that supports the growth of both the helper virus and the scPV may be used for the reactivation step and any host cell that does not support the growth of the helper virus may be used to eliminate the helper virus and select and/or isolate the scPV. In some embodiments, the helper virus is a *Leporipoxvirus* and the host cells used for the reactivation step may be selected from rabbit kidney cells (e.g., LLC-RK1, RK13, etc.), rabbit lung cells (e.g. R9ab), rabbit skin cells (e.g., SF1Ep, DRS, RAB-9), rabbit cornea cells (e.g., SIRC), rabbit carcinoma cells (e.g., Oc4T/cc), rabbit skin/carcinoma cells (e.g., CTPS), monkey cells (e.g., Vero, BGMK, etc.) or hamster cells (e.g., BHK-21, etc.). In some embodiments, the helper virus is SFV.

[0145] The scPVs of the present invention can be propagated in any substrate that allows the virus to grow to titers that permit the uses of the scPVs described herein. In one embodiment, the substrate allows the scPVs to grow to titers comparable to those determined for the corresponding wild-type viruses. The scPVs of the invention may be grown in cells (e.g. avian cells, bat cells, bovine cells, camel cells, canary cells, cat cells, deer cells, equine cells, fowl cells, gerbil

cells, goat cells, human cells, monkey cells, pig cells, rabbit cells, raccoon cells, seal cells, sheep cells, skunk cells, vole cells, etc.) that are susceptible to infection by the poxviruses. Such methods are well-known to those skilled in the art. Representative mammalian cells include, but are not limited to BHK, BGMK, BRL3A, BSC-40, CEF, CEK, CHO, COS, CVI, HaCaT, HEL, HeLa cells, HEK293, human bone osteosarcoma cell line 143B, MDCK, NIH/3T3, Vero cells, etc.). For virus isolation, the scPV is removed from cell culture and separated from cellular components, typically by well known clarification procedures, e.g., such as gradient centrifugation and column chromatography, and may be further purified as desired using procedures well known to those skilled in the art, e.g., plaque assays.

Polynucleotides of the invention

[0146] The invention provides polynucleotides (e.g. double stranded DNA fragments) for producing functional synthetic chimeric poxviruses (scPVs). The invention provides methods for producing functional scPVs from synthetic DNA (e.g. chemically synthesized DNA, PCR amplified DNA, engineered DNA, polynucleotides comprising nucleoside analogs, etc.). In some embodiments, the invention provides methods for producing functional scPVs from chemically synthesized overlapping double-stranded DNA fragments of the viral genome. The polynucleotides of the invention may be designed based on publicly available genome sequences. Where natural isolates of a poxvirus are readily available, the viral genome may be sequenced prior to selecting and designing the polynucleotides of the invention. Alternatively, where partial DNA sequences of a poxvirus are available, for example from a clinical isolate, from a forensic sample or from PCR amplified DNA from material associated with an infected person, the partial viral genome may be sequenced prior to selecting and designing the polynucleotides of the invention. An scPV of the invention, and thus, the polynucleotides of the invention, may be based on the genome sequences of naturally occurring strains, variants or mutants, mutagenized viruses or genetically engineered viruses.

[0147] The invention provides isolated polynucleotides including a nucleotide sequence that is at least 90% identical (e.g., at least 91%, 92%, 93%, or 94% identical), at least 95% identical (e.g., at least 96%, 97%, 98%, or 99% identical), or 100% identical to all or a portion of a reference poxviral genome sequence or its complement. The isolated polynucleotides of the invention may include at least 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 15000, 20000, 25000, 30000, 35000, 40000, 45000 bp or more contiguous or non-contiguous nucleotides of a reference polynucleotide molecule (e.g., a reference poxviral genome or a fragment thereof). One of ordinary skill in the art will appreciate that nucleic acid sequences complementary to the nucleic acids, and variants of the nucleic acids are also within the scope of this invention. In further embodiments, the nucleic acid sequences of the invention can be isolated, recombinant, and/or fused with a heterologous nucleotide sequence, or in a DNA library.

[0148] In some aspects, the invention provides polynucleotides for producing scPVs wherein the poxvirus is selected from the genus *Avipoxvirus, Capripoxvirus, Cervidpoxvirus, Crocodylipoxvirus, Leporipoxvirus, Molluscipoxvirus, Orthopoxvirus, Parapoxvirus, Suipoxvirus, or Yatapoxvirus.* In some embodiments, the poxvirus is an *Orthopoxvirus*. In some embodiments, the *Orthopoxvirus* is selected from camelpox virus (CMLV), cowpox virus (CPXV), ectromelia virus (ECTV, "mousepox agent"), HPXV, monkeypox virus (MPXV), rabbitpox virus (RPXV), raccoonpox virus, skunkpox virus, Taterapox virus, Uasin Gishu disease virus, VACV, variola virus (VARV) and volepox virus (VPV). In a preferred embodiment, the poxvirus is an HPXV. In another preferred embodiment, the poxvirus is a VACV. In another preferred embodiment, the poxvirus is the ACAM2000 clone of VACV. In another preferred embodiment, the poxvirus is the VACV strain IOC (VACV-IOC) (Genbank Accession KT184690 and KT184691). In another preferred embodiment, the scVACV genome is based on Modified Vaccinia virus Ankara (Genbank Acccession U94848; Genbank Accession AY603355). In yet another preferred embodiment, the scVACV genome is based on MVA-BN (Genbank Accession DQ983238). In some embodiments, the poxvirus is a *Parapoxvirus*. In some embodiments, the

Parapoxvirus is selected from orf virus (ORFV), pseudocowpox virus (PCPV), bovine popular stomatitis virus (BPSV), squirrel parapoxvirus (SPPV), red deer parapoxvirus, Ausdyk virus, Chamois contagious ecythema virus, reindeer parapoxvirus, or sealpox virus. In some embodiments, the poxvirus is a *Molluscipoxvirus*. In some embodiments, the *Molluscipoxvirus* is molluscum contagiousum virus (MCV). In some embodiments, the poxvirus is a *Yatapoxvirus*. In some embodiments, the *Yatapoxvirus* is selected from Tanapox virus or Yaba monkey tumor virus (YMTV). In some embodiments, the poxvirus is a *Capripoxvirus*. In some embodiments, the *Capripoxvirus* is selected from sheepox, goatpox, or lumpy skin disease virus. In some embodiments, the poxvirus is a *Suipoxvirus*. In some embodiments, the *Suipoxvirus* is swinepox virus. In some embodiments, the poxvirus is a *Leporipoxvirus*. In some embodiments, the *Leporipoxvirus* is selected from myxoma virus, Shope fibroma virus (SFV), squirrel fibroma virus, or hare fibroma virus. New poxviruses (e.g. Orthopoxviruses) are still being constantly discovered. It is understood that an scPV of the invention may be based on such a newly discovered poxvirus.

[0149] In some aspects, the scPV is a CMLV whose genome is based on a published genome sequence (e.g. strain CMS (Genbank Accession AY009089.1)). In some aspects, the scPV is a CPXV whose genome is based on a published genome sequence (e.g. strain Brighton Red (Genbank Accession AF482758), strain GRI-90 (Genbank Accession X94355)). In some aspects, the scPV is a ECTV whose genome is based on a published genome sequence (e.g. strain Moscow (Genbank Accession NC_004105)). In some aspects, the scPV is a MPXV whose genome is based on a published genome sequence (e.g. strain Zaire-96-1-16 (Genbank Accession AF380138)). In some aspects, the scPV is a RPXV whose genome is based on a published genome sequence (e.g. strain Utrecht (Genbank Accession AY484669)). In some aspects, the scPV is a Taterapox virus whose genome is based on a published genome sequence (e.g. strain Dahomey 1968 (Genbank Accession NC_008291)).

[0150] In one aspect, the invention provides polynucleotides for producing a synthetic chimeric horsepox virus (scHPXV). In a specific embodiment, the

scHPXV genome may be based on the genome sequence described for HPXV strain MNR-76 (SEQ ID NO: 49) (Tulman ER, Delhon G, Afonso CL, Lu Z, Zsak L, Sandybaev NT, et al. Genome of horsepox virus. Journal of virology. 2006;80(18):9244-58). This genome sequence is incomplete and appears not to include the sequence of the terminal hairpin loops. It is shown here that terminal hairpin loops from vaccinia virus (VACV) can be ligated onto the ends of the HPXV genome to produce functional scHPXV particles using the methods of the invention. The HPXV genome may be divided into 10 overlapping fragments as described in the working examples of the disclosure and shown in Table 1. In some embodiments, the genome may be divided into 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 overlapping fragments. In some embodiments, the entire genome may be provided as one fragment. The genomic locations of the exemplary overlapping fragments and fragment sizes are shown in Table 1. Table 2 shows some of the modifications that may be made in these fragments relative to the base sequence. The polynucleotides of the invention comprise nucleic acids sequences that are at least 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NOs: 1-10. In some embodiments, an isolated polynucleotide of the invention comprises a variant of these sequences, wherein such variants can include missense mutations, nonsense mutations, duplications, deletions, and/or additions. SEQ ID NO: 11 and SEQ ID NO: 12 depict the nucleotide sequences of VACV (WR strain) terminal hairpin loops. In some embodiments, the terminal hairpin loops comprise nucleic acid sequences that are at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 11 or SEQ ID NO: 12.

[0151] The invention provides isolated polynucleotides including a nucleotide sequence that is at least 90% identical (e.g., at least 91%, 92%, 93%, or 94% identical), at least 95% identical (e.g., at least 96%, 97%, 98%, or 99% identical), or 100% identical to all or a portion of a reference HPXV genome sequence (e.g. SEQ ID NO: 49). In some embodiments, an isolated polynucleotide of the invention comprises a variant of the reference sequences, wherein such variants can include missense mutations, nonsense mutations, duplications, deletions,

and/or additions. The isolated polynucleotides of the invention may include at least 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 15000, 20000, 25000, 30000, 35000, 40000, 45000 bp or more contiguous or non-contiguous nucleotides of a reference polynucleotide molecule (e.g., a reference HPXV genome including but not limited to SEQ ID NO: 49, or a portion thereof).

[0152] In another aspect, the invention provides polynucleotides for producing a synthetic chimeric VACV (scVACV). In a specific embodiment, the scVACV genome is based on a published VACV genome. In a specific embodiment, the scVACV genome is based on strain ACAM2000; Genbank Accession AY313847). In a specific embodiment, the scVACV genome is based on VACV-IOC (Genbank Accession KT184690 and KT184691). In a specific embodiment, the scVACV genome is based on Modified Vaccinia virus Ankara (Genbank Acccession U94848; Genbank Accession AY603355). In a specific embodiment, the scVACV genome is based on MVA-BN (Genbank Accession DQ983238). The VACV genome may be divided into 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 overlapping fragments. In some embodiments, the entire genome may be provided as one fragment. In an specific embodiment, the VACV genome is divided into the nine overlapping fragments as shown in Table 7. The polynucleotides of the invention comprise nucleic acids sequences that are at least 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NOs: 50-58. In some embodiments, an isolated polynucleotide of the invention comprises a variant of these sequences, wherein such variants can include missense mutations, nonsense mutations, duplications, deletions, and/or additions. In other embodiments, the scVACV genome is based on a VACV strain selected from Western Reserve (Genbank Accession NC 006998; Genbank Accession AY243312), CL3 (Genbank Accession AY313848), Tian Tian (Genbank Accession AF095689.1), Tian Tian clones TT9 (JX489136), TP3 (Genbank Accession KC207810) and TP5 (Genbank Accession KC207811), NYCBH, Wyeth, Copenhagen (Genbank Accession M35027), Lister 107 (Genbank Accession DQ121394) Lister-LO (Genbank Accession AY678276), Modified Vaccinia virus Ankara (MVA) (Genbank Acccession U94848; Genbank

Accession AY603355), MVA-BN (Genbank Accession DQ983238), Lederle, Tashkent clones TKT3 (Genbank Accession KM044309) and TKT4 (KM044310), USSR, Evans, Praha, LIVP, Ikeda, IHD-W (Genbank Accession KJ125439), LC16m8 (AY678275), EM-63, IC, Malbran, Duke (Genbank Accession DQ439815), 3737 (Genbank Accession DQ377945), CV-1, Connaught Laboratories, CVA (Genbank Accession AM501482), Serro 2 virus (Genbank Accession KF179385), Cantaglo virus isolate CM-01 (Genbank Accession KT013210), Dryvax clones DPP15 (Genbank Accession JN654981), DPP20 (Genbank Accession JN654985), DPP13 (Genbank Accession JN654980), DPP17(Genbank Accession JN654983), DPP21 (Genbank Accession JN654986). The VACV genome may be divided into 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 overlapping fragments. In some embodiments, the entire genome may be provided as one fragment.

[0153] The invention provides in one embodiment isolated polynucleotides including a nucleotide sequence that is at least 90% identical (e.g., at least 91%, 92%, 93%, or 94% identical), at least 95% identical (e.g., at least 96%, 97%, 98%, or 99% identical), or 100% identical to all or a portion of a reference genome sequence or its complement (e.g., VACV). In some embodiments, an isolated polynucleotide of the invention comprises a variant of the reference sequences, wherein such variants can include missense mutations, nonsense mutations, duplications, deletions, and/or additions. The isolated polynucleotides of the invention may include at least 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 15000, 20000, 25000, 30000, 35000, 40000, 45000 bp or more contiguous or non-contiguous nucleotides of a reference genome or portion thereof.

[0154] Polynucleotides complementary to any of the polynucleotide sequences disclosed herein are also encompassed by the present invention. Polynucleotides may be single-stranded (coding or antisense) or double-stranded, and may be DNA (genomic or synthetic) or RNA molecules. RNA molecules include mRNA molecules. Additional coding or non-coding sequences may, but need not, be

present within a polynucleotide of the present invention, and a polynucleotide may, but need not, be linked to other molecules and/or support materials.

[0155] Two polynucleotide or polypeptide sequences are said to be "identical" if the sequence of nucleotides or amino acids in the two sequences is the same when aligned for maximum correspondence as described below. Comparisons between two sequences are typically performed by comparing the sequences over a comparison window to identify and compare local regions of sequence similarity. A "comparison window" as used herein, refers to a segment of at least about 20 contiguous positions, usually 30 to about 75, or 40 to about 50, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Polynucleotides or variants may also, or alternatively, be substantially homologous to a polynucleotide provided herein. Such polynucleotide variants are capable of hybridizing under moderately stringent conditions to a polynucleotide of the invention (or its complement).

[0156] Suitable “moderately stringent conditions” include prewashing in a solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 50°C-65°C, 5 X SSC, overnight; followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1 % SDS.

[0157] As used herein, "highly stringent conditions" or "high stringency conditions" are those that: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt’s solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1

x SSC containing EDTA at 55°C. The skilled artisan will recognize how to adjust the temperature, ionic strength, etc. as necessary to accommodate factors such as probe length and the like.

[0158] The polynucleotides of this invention can be obtained using chemical synthesis, recombinant methods, or PCR. Methods of chemical polynucleotide synthesis are well known in the art and need not be described in detail herein. One of skill in the art can use the sequences provided herein and a commercial DNA synthesizer to produce a desired DNA sequence.

[0159] For preparing polynucleotides using recombinant methods, a polynucleotide comprising a desired sequence can be inserted into a suitable vector, and the vector in turn can be introduced into a suitable host cell for replication and amplification, as further discussed herein. Polynucleotides may be inserted into host cells by any means known in the art. Cells are transformed by introducing an exogenous polynucleotide by direct uptake, endocytosis, transfection, F-mating or electroporation. Once introduced, the exogenous polynucleotide can be maintained within the cell as a non-integrated vector (such as a plasmid) or integrated into the host cell genome. The polynucleotide so amplified can be isolated from the host cell by methods well known within the art. See, e.g., Sambrook et al., 1989.

[0160] Alternatively, PCR allows reproduction of DNA sequences. PCR technology is well known in the art and is described in U.S. Patent Nos. 4,683,195, 4,800,159, 4,754,065 and 4,683,202, as well as PCR: The Polymerase Chain Reaction, Mullis et al. eds., Birkauswer Press, Boston, 1994.

[0161] RNA can be obtained by using the isolated DNA in an appropriate vector and inserting it into a suitable host cell. When the cell replicates and the DNA is transcribed into RNA, the RNA can then be isolated using methods well known to those of skill in the art, as set forth in Sambrook et al., 1989, supra, for example.

[0162] In other embodiments, nucleic acids of the invention also include nucleotide sequences that hybridize under highly stringent conditions to the

nucleotide sequences set forth in SEQ ID NOs: 1-10, or 50-58, or sequences complementary thereto. One of ordinary skill in the art will readily understand that appropriate stringency conditions which promote DNA hybridization can be varied. For example, one could perform the hybridization at 6.0 x sodium chloride/sodium citrate (SSC) at about 45 °C, followed by a wash of 2.0 x SSC at 50 °C. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50 °C to a high stringency of about 0.2 x SSC at 50 °C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22 °C, to high stringency conditions at about 65 °C. Both temperature and salt may be varied, or temperature or salt concentration may be held constant while the other variable is changed. In one embodiment, the invention provides nucleic acids which hybridize under low stringency conditions of 6 x SSC at room temperature followed by a wash at 2 x SSC at room temperature.

[0163] Isolated nucleic acids which differ due to degeneracy in the genetic code are also within the scope of the invention. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC are synonyms for histidine) may result in "silent" mutations which do not affect the amino acid sequence of the protein. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-5% of the nucleotides) of the nucleic acids encoding a particular protein may exist among members of a given species due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of this invention.

[0164] The present invention further provides recombinant cloning vectors and expression vectors that are useful in cloning a polynucleotide of the present invention. The present invention further provides transformed host cells comprising a polynucleotide molecule or recombinant vector of the invention, and novel strains or cell lines derived therefrom.

[0165] A host cell may be a bacterial cell, a yeast cell, a filamentous fungal cell, an algal cell, an insect cell, or a mammalian cell. In some embodiments, the host cell is *E. coli*. A variety of different vectors have been developed for specific use in each of these host cells, including phage, high copy number plasmids, low copy number plasmids, and shuttle vectors, among others, and any of these can be used to practice the present invention.

[0166] Suitable cloning vectors may be constructed according to standard techniques, or may be selected from a large number of cloning vectors available in the art. While the cloning vector selected may vary according to the host cell intended to be used, useful cloning vectors will generally have the ability to self-replicate, may possess a single target for a particular restriction endonuclease, and/or may carry genes for a marker that can be used in selecting clones containing the vector. Suitable examples include plasmids and bacterial viruses, e.g., pBAD18, pUC18, pUC19, Bluescript (e.g., pBS SK+) and its derivatives, mp18, mp19, pBR322, pMB9, ColE1, pCR1, RP4, phage DNAs, and shuttle vectors such as pSA3 and pAT28. These and many other cloning vectors are available from commercial vendors such as BioRad, Strategene, and Invitrogen.

[0167] To aid in the selection of host cells transformed or transfected with cloning vectors of the present invention, the vector can be engineered to further comprise a coding sequence for a reporter gene product or other selectable marker. Such a coding sequence is preferably in operative association with the regulatory element coding sequences, as described above. Reporter genes that are useful in the invention are well-known in the art and include those encoding green fluorescent protein, luciferase, xylE, and tyrosinase, among others. Nucleotide sequences encoding selectable markers are well known in the art, and include those that encode gene products conferring resistance to antibiotics or anti-metabolites, or that supply an auxotrophic requirement. Examples of such sequences include those that encode resistance to ampicillin, erythromycin, thiostrepton or kanamycin, among many others.

[0168] The vectors containing the polynucleotides of interest and/or the polynucleotides themselves, can be introduced into the host cell by any of a

number of appropriate means, including electroporation, transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; and infection (e.g., where the vector is an infectious agent such as vaccinia virus). The choice of introducing vectors or polynucleotides will often depend on features of the host cell.

[0169] The present invention further provides transformed host cells comprising a polynucleotide molecule or recombinant vector of the invention, and novel strains or cell lines derived therefrom. In some embodiments, host cells useful in the practice of the invention are *E. coli* cells. A strain of E. coli can typically be used, such as e.g., *E. coli* TOP10, or *E. coli* BL21(DE3), DH5α, etc., available from the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Va. 20110, USA and from commercial sources. In some embodiments, other prokaryotic cells or eukaryotic cells may be used. In some embodiments, the host cell is a member of a genus selected from: *Clostridium*, *Zymomonas*, *Escherichia*, *Salmonella*, *Serratia*, *Erwinia*, *Klebsiella, Shigella*, *Rhodococcus*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Enterococcus, Alcaligenes*, *Paenibacillus*, *Arthrobacter*, *Corynebacterium*, *Brevibacterium, Schizosaccharomyces*, *Kluyveromyces*, *Yarrowia*, *Pichia*, *Candida*, *Pichia, or Saccharomyces*. Such transformed host cells typically include but are not limited to microorganisms, such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA vectors, or yeast transformed with recombinant vectors, among others. Preferred eukaryotic host cells include yeast cells, although mammalian cells or insect cells can also be utilized effectively. Suitable host cells include prokaryotes (such as *E. coli*, *B. subtillis*, *S. lividans*, or *C. glutamicum*) and yeast (such as *S. cerevisae*, *S. pombe*, *P. pastoris*, or *K. lactis*).

[0170] In one aspect, the invention also includes the genome of the scPV, its recombinants, or functional parts thereof. A functional part of the viral genome may be a portion of the genome that encodes a protein or portion thereof (e.g. domain, epitope, etc.), a portion that comprises regulatory elements or components of regulatory elements such as a promoter, enhancer, cis- or trans-acting elements, etc. Such viral sequences can be used to identify or isolate the virus or its

recombinants, e.g., by using PCR, hybridization technologies, or by establishing ELISA assays.

Exemplary Uses

Prevention or Treatment of Pathogenic Poxviral Infections

[0171] The synthetic chimeric poxviruses (scPVs) of the invention can be used in immunization of a subject against a pathogenic poxviral infection. The scPVs of the invention can be used to prevent, manage, or treat one or more pathogenic poxviral infections in a subject. In some embodiments, the pathogenic poxvirus is an *Orthopoxvirus* (e.g., camelpox virus (CMLV), cowpox virus (CPXV), ectromelia virus (ECTV, "mousepox agent"), HPXV, monkeypox virus (MPXV), rabbitpox virus (RPXV), raccoonpox virus, skunkpox virus, Taterapox virus, Uasin Gishu disease virus, vaccinia virus (VACV), variola virus (VARV) and volepox virus (VPV)). In some embodiments, the pathogenic poxvirus is a *Parapoxvirus* (e.g.,orf virus (ORFV), pseudocowpox virus (PCPV), bovine popular stomatitis virus (BPSV), squirrel parapoxvirus (SPPV), red deer parapoxvirus, Ausdyk virus, Chamois contagious ecythema virus, reindeer parapoxvirus, or sealpox virus). In some embodiments, the pathogenic poxvirus is a *Molluscipoxvirus* (e.g., molluscum contagiousum virus (MCV)). In some embodiments, the pathogenic poxvirus is a *Yatapoxvirus* (e.g., Tanapox virus or Yaba monkey tumor virus (YMTV)). In some embodiments, the pathogenic poxvirus is a *Capripoxvirus* (e.g., sheepox, goatpox, or lumpy skin disease virus). In some embodiments, the poxvirus is a *Suipoxvirus* (e.g., swinepox virus). In some embodiments, the pathogenic poxvirus is a *Leporipoxvirus* (e.g. myxoma virus, Shope fibroma virus (SFV), squirrel fibroma virus, or hare fibroma virus). In some embodiments, the pathogenic poxvirus is VARV. In some embodiments, the pathogenic poxvirus is MPXV. In some embodiments, the pathogenic poxvirus is MCV. In some embodiments, the pathogenic poxvirus is ORFV. In some embodiments, the pathogenic poxvirus is CPXV. The pathogenic poxvirus may be a poxvirus pseudotype or chimera. In some embodiments, the subject is a human subject. In some embodiments, the subject is an animal subject. New poxviruses (e.g. Orthopoxviruses) are still being constantly discovered. It is understood that an

scPV of the invention can be used in immunization of a subject against a newly discovered pathogenic poxvirus or in the prevention, management, or treatment of an infection by a newly discovered pathogenic poxvirus.

[0172] The scPVs of the invention can be used in immunogenic formulations, e.g., vaccine formulations. The formulations may be used to prevent, manage, neutralize, treat and/or ameliorate a pathogenic poxviral infection. The immunogenic formulations may comprise either a live or inactivated scPV of the invention. The scPV can be inactivated by methods well known to those of skill in the art. Common methods use formalin and heat for inactivation. In some embodiments, the immunogenic formulation comprises a live vaccine. Production of such live immunogenic formulations may be accomplished using conventional methods involving propagation of the scPV in cell culture followed by purification. For example, the scPV can be cultured in BHK, BGMK, BRL3A, BSC-40, CEF, CEK, CHO, COS, CVI, HaCaT, HEL, HeLa cells, HEK293, human bone osteosarcoma cell line 143B, MDCK, NIH/3T3, Vero cells, etc., as can be determined by the skilled worker.

[0173] In one aspect, the scPVs of the invention can be used to prevent, manage, or treat smallpox. The scPVs of the invention (e.g. a synthetic chimeric HPXV (scHPXV) or a synthetic chimeric VACV (scVACV)) can be used as a vaccine for the prevention of smallpox in individuals or populations that have been exposed, potentially exposed, or are at risk of exposure to smallpox. The scPVs of the invention can be used to create a new national stockpile of smallpox vaccine (e.g. an scHPXV or scVACV of the invention). In some embodiments, the scPVs of the invention can be prophylactically administered to defense personnel, first responders, etc.

[0174] In one embodiment, a composition comprising a scHPXV of the invention is used as a smallpox vaccine. It is shown here that a scHPXV produced according to the methods of the invention has a small plaque phenotype. In general, a small plaque phenotype is considered to reflect attenuation. Accordingly, a scHPXV produced according to the methods of the invention provides a safe alternative to the existing smallpox vaccines. In some embodiments, the vaccine may be safe for

administration to immunosuppressed subjects (e.g., HIV patients, patients undergoing chemotherapy, patients undergoing treatment for cancer, rheumatologic disorders, or autoimmune disorders, patients who are undergoing or have received an organ or tissue transplant, patients with immune deficiencies, children, pregnant women, patients with atopic dermatitis, eczema, psoriasis, heart conditions, and patients on immunosuppressants etc.) who may suffer from severe complications from an existing smallpox vaccine and are thus contraindicated for an existing smallpox vaccine. In some embodiments the vaccine may be used in combination with one or more anti-viral treatments to suppress viral replication. In some embodiments the vaccine may be used in combination with brincidofovir treatment to suppress viral replication. In some embodiments the vaccine may be used in combination with tecovirimat/SIGA-246 treatment to suppress viral replication. In some embodiments, the vaccine may be used in combination with acyclic nucleoside phosphonates (cidofovir), oral alkoxyalkyl prodrugs of acyclic nucleoside or phosphonates (brincidofovir or CMX001). In some embodiments, the vaccine may be used in combination with Vaccinia Immune Globulin (VIG). In some embodiments the vaccine may be used in subjects who have been previously immunized with peptide or protein antigens derived from VACV, VARV or HPXV. In some embodiments the vaccine may be used in subjects who have been previously immunized with killed or inactivated VACV. In some embodiments the vaccine may be used in subjects who have been previously immunized with the replication-deficient/defective VACV virus strain, MVA (modified virus Ankara). A vaccine formulation comprising a scHPXV of the invention may comprise either a live or inactivated scHPXV.

[0175] In one embodiment, a composition comprising a scVACV of the invention is used as a smallpox vaccine. The scVACV may be based on a VACV strain selected from ACAM2000 (Genbank Accession AY313847), Western Reserve (Genbank Accession NC 006998; Genbank Accession AY243312), CL3 (Genbank Accession AY313848), Tian Tian (Genbank Accession AF095689.1), Tian Tian clones TT9 (JX489136), TP3 (Genbank Accession KC207810) and TP5 (Genbank Accession KC207811), NYCBH, Wyeth, Copenhagen (Genbank Accession M35027), Lister 107 (Genbank Accession DQ121394) Lister-LO

(Genbank Accession AY678276), Modified Vaccinia virus Ankara (MVA) (Genbank Acccession U94848; Genbank Accession AY603355), MVA-BN (Genbank Accession DQ983238), Lederle, Tashkent clones TKT3 (Genbank Accession KM044309) and TKT4 (KM044310), USSR, Evans, Praha, LIVP, Ikeda, IHD-W (Genbank Accession KJ125439), LC16m8 (AY678275), EM-63, IC, Malbran, Duke (Genbank Accession DQ439815), 3737 (Genbank Accession DQ377945), CV-1, Connaught Laboratories, CVA (Genbank Accession AM501482), Serro 2 virus (Genbank Accession KF179385), Cantaglo virus isolate CM-01 (Genbank Accession KT013210), Dryvax clones DPP15 (Genbank Accession JN654981), DPP20 (Genbank Accession JN654985), DPP13 (Genbank Accession JN654980), DPP17(Genbank Accession JN654983), DPP21 (Genbank Accession JN654986) and IOC (Genbank Accession KT184690 and KT184691). In one embodiment, the scVACV to be used as a smallpox vaccine is based on strain ACAM2000 (Genbank Accession AY313847). In one embodiment, the scVACV to be used as a smallpox vaccine is based on strain VACV-IOC (Genbank Accession KT184690 and KT184691). In one embodiment, the scVAVC to be used as a smallpox vaccine is based on strain MVA(Genbank Acccession U94848; Genbank Accession AY603355). In one embodiment, the scVACV to be used as a smallpox vaccine is based on strain MVA-BN (Genbank Accession DQ983238). A vaccine formulation comprising a scPV of the invention may comprise either a live or inactivated scVACV.

[0176] In some embodiments, a composition comprising a scPV of the invention (e.g. a scHPXV or a scVACV) is used as a vaccine against a VACV infection, a MPXV infection or a CPXV infection.

[0177] In some embodiments, a scPV of the invention may be designed to express heterologous antigens or epitopes and can be used as vaccines against the source organisms of such antigens and/or epitopes.

[0178] The immunogenic formulations of the present invention (e.g. vaccines) comprise an effective amount of a scPV of the invention, and a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S.

Pharmacopeia or other generally recognized pharmacopeiae for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the pharmaceutical composition (e.g., immunogenic or vaccine formulation) is administered. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. The formulation should suit the mode of administration. The particular formulation may also depend on whether the scPV is live or inactivated. Purified scPVs of the invention may be lyophilized for later use or can be immediately prepared in a pharmaceutical solution. The scPVs may also be diluted in a physiologically acceptable solution such as sterile saline, with or without an adjuvant or carrier.

[0179] The immunogenic formulations (e.g. vaccines) of the invention may be administered to patients by scarification. The vaccines may also be administered by any other standard route of administration. Many methods may be used to introduce the immunogenic formulations (e.g., vaccines), these include but are not limited to intranasal, intratracheal, oral, intradermal, intramuscular, intraperitoneal, intravenous, conjunctival and subcutaneous routes. In birds, the methods may further include choanal inoculation. As an alternative to parenteral administration, the invention also encompasses routes of mass administration for agricultural purposes such as via drinking water or in a spray. Alternatively, it may be preferable to introduce an scPV of the invention via its natural route of infection. In some embodiments, the immunogenic formulations of the invention are administered as an injectable liquid, a consumable transgenic plant that expresses the vaccine, a sustained release gel or an implantable encapsulated composition, a solid implant or a nucleic acid. The immunogenic formulation may also be administered in a cream, lotion, ointment, skin patch, lozenge, or oral liquid such as a suspension, solution and emulsion (oil in water or water in oil).

[0180] In certain embodiments, an immunogenic formulation of the invention (e.g., vaccine) does not result in complete protection from an infection, but results in a lower titer or reduced number of the pathogen (e.g., pathogenic poxvirus) compared to an untreated subject. In certain embodiments, administration of the immunogenic formulations of the invention results in a 0.5 fold, 1 fold, 2 fold, 4 fold, 6 fold, 8 fold, 10 fold, 15 fold, 20 fold, 25 fold, 50 fold, 75 fold, 100 fold, 125 fold, 150 fold, 175 fold, 200 fold, 300 fold, 400 fold, 500 fold, 750 fold, or 1,000 fold or greater reduction in titer of the pathogen relative to an untreated subject. Benefits of a reduction in the titer, number or total burden of pathogen include, but are not limited to, less severity of symptoms of the infection and a reduction in the length of the disease or condition associated with the infection.

[0181] In certain embodiments, an immunogenic formulation of the invention (e.g., vaccine) does not result in complete protection from an infection, but results in a lower number of symptoms or a decreased intensity of symptoms, or a decreased morbidity or a decreased mortality compared to an untreated subject.

[0182] In various embodiments, the immunogenic formulations of the invention (e.g. vaccines) or antibodies generated by the scPVs of the invention are administered to a subject in combination with one or more other therapies (e.g. antiviral or immunomodulatory therapies) for the prevention of an infection (e.g. a pathogenic poxviral infection). In other embodiments, the immunogenic formulations of the invention or antibodies generated by the scPVs of the invention are administered to a subject in combination with one or more other therapies (e.g. antiviral or immunomodulatory therapies) for the treatment of an infection (e.g. a pathogenic poxviral infection). In yet other embodiments, the immunogenic formulations of the invention or antibodies generated by the scPVs of the invention are administered to a subject in combination with one or more other therapies (e.g. antiviral or immunomodulatory therapies) for the management and/or amelioration of an infection (e.g. a pathogenic poxviral infection). In a specific embodiment, the immunogenic formulations of the invention or antibodies generated by the scPVs of the invention are administered to a subject in combination with one or more other therapies (e.g. antiviral or immunomodulatory therapies) for the

prevention of smallpox. In another specific embodiment, the immunogenic formulations of the invention or antibodies generated by the scPVs of the invention are administered to a subject in combination with one or more other therapies (e.g. antiviral or immunomodulatory therapies) for the treatment of smallpox. In some embodiments the vaccine may be used in combination with one or more anti-viral treatments to suppress viral replication. In some embodiments the vaccine may be used in combination with brincidofovir treatment to suppress viral replication. In some embodiments the vaccine may be used in combination with tecovirimat/SIGA-246 treatment to suppress viral replication. In some embodiments, the vaccine may be used in combination with acyclic nucleoside phosphonates (cidofovir), oral alkoxyalkyl prodrugs of acyclic nucleoside or phosphonates (brincidofovir or CMX001). In some embodiments, the vaccine may be used in combination with Vaccinia Immune Globulin (VIG). In some embodiments the vaccine may be used in subjects who have been previously immunized with peptide or protein antigens derived from VACV, VARV or HPXV. In some embodiments the vaccine may be used in subjects who have been previously immunized with killed or inactivated VACV. In some embodiments the vaccine may be used in subjects who have been previously immunized with the replication-deficient/defective VACV virus strain, MVA (modified virus Ankara).

[0183] Any anti-viral agent well-known to one of skill in the art can be used in the formulations (e.g., vaccine formulations) and the methods of the invention. Non-limiting examples of anti-viral agents include proteins, polypeptides, peptides, fusion proteins antibodies, nucleic acid molecules, organic molecules, inorganic molecules, and small molecules that inhibit and/or reduce the attachment of a virus to its receptor, the internalization of a virus into a cell, the replication of a virus, or release of virus from a cell. In particular, anti-viral agents include but are not limited to antivirals that blocks extracellular virus maturation (tecovirimat/SIGA-246), acyclic nucleoside phosphonates (cidofovir), oral alkoxyalkyl prodrugs of acyclic nucleoside phosphonates (brincidofovir or CMX001) or Vaccinia Immune Globulin (VIG). In some embodiments, anti-viral agents include, but are not limited to, nucleoside analogs (e.g., zidovudine, acyclovir, gangcyclovir,

vidarabine, idoxuridine, trifluridine, and ribavirin), foscarnet, amantadine, rimantadine, saquinavir, indinavir, ritonavir, alpha-interferons and other interferons, and AZT.

[0184] Doses and dosing regimens can be determined by one of skill in the art according to the needs of a subject to be treated. The skilled worker may take into consideration factors such as the age or weight of the subject, the severity of the disease or condition being treated, and the response of the subject to treatment. A composition of the invention can be administered, for example, as needed or on a daily basis. Dosing may take place over varying time periods. For example, a dosing regimen may last for 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, or longer. In some embodiments, a dosing regimen will last 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 12 months, or longer.

[0185] The scPVs of the invention can also be used to produce antibodies useful for passive immunotherapy, diagnostic or prognostic immunoassays, etc. Methods of producing antibodies are well-known in the art. The antibodies may be further modified (e.g., chimerization, humanization, etc.) prior to use in immunotherapy.

Oncolytic Agents

[0186] The synthetic chimeric poxviruses (scPVs) of the invention can be used as oncolytic agents that selectively replicate in and kill cancer cells. Cells that are dividing rapidly, such as cancer cells, are generally more permissive for poxviral infection than non-dividing cells. Many features of poxviruses, such as safety in humans, ease of production of high-titer stocks, stability of viral preparations, and capacity to induce antitumor immunity following replication in tumor cells make poxviruses desirable oncolytic agents. The scPVs produced according to the methods of the invention may comprise one or modifications that render them suitable for the treatment of cancer. Accordingly, in one aspect, the disclosure provides a method of inducing death in a cancer cells, the method comprising contacting the cells with an isolated scPV or pharmaceutical composition

comprising an scPV of the invention. In one aspect, the disclosure provides a method of treating cancer, the method comprising administering to a patient in need thereof, a therapeutically effective amount of an scPV of the invention. Another aspect includes the use of an scPV or a composition described herein to induce death in a neoplastic disorder cell such as a cancer cell or to treat a neoplastic disorder such as cancer. In some embodiments, the poxvirus oncolytic therapy is administered in combination with one or more conventional cancer therapies (e.g., surgery, chemotherapy, radiotherapy, thermotherapy, and biological/immunological therapy). In specific embodiments, the oncolytic virus is a synthetic chimeric VACV (scVACV) of this invention. In some embodiments, the oncotyic virus is a synthetic chimeric myxoma virus of this invention. In some embodiments, the oncolytic virus is a synthetic chimeric HPXV (scHPXV) of this invention. In some embodiments, the oncolytic virus is a synthetic chimeric raccoonpox virus of this invention. In some embodiments, the oncolytic virus is a synthetic chimeric yaba-like disease virus of this invention.

[0187] Using the methods of this invention, one or more desirable genes can be easily introduced and one or more undesirable genes can be easily deleted from the synthetic chimeric poxviral genome. In some embodiments, the scPVs of the invention for use as oncolytic agents are designed to express transgenes to enhance their immunoreactivity, antitumor targeting and/or potency, cell-to-cell spread and/or cancer specificity. In some embodiments, an scPV of the invention is designed or engineered to express an immunomodulatory gene (e.g., GM-CSF, or a viral gene that blocks TNF function). In some embodiments, an scPV of the invention is designed to include a gene that expresses a factor that attenuates virulence. In some embodiments, an scPV of the invention is designed or engineered to express a therapeutic agent (e.g. hEPO, BMP-4, antibodies to specific tumor antigens or portions thereof, etc.). In some embodiments, the scPVs of the invention have been modified for attenuation. In some embodiments, the scPV of the invention is designed or engineered to lack the viral TK gene. In some embodiments, an scVACV of the invention is designed or engineered to lack vaccinia growth factor gene. In some embodiments, an scVACV of the invention is designed or engineered to lack the hemagglutinin gene.

[0188] The scPVs of the invention are useful for treating a variety of neoplastic disorder and/or cancers. In some embodiments, the type of cancer includes but is not limited to bone cancer, breast cancer, bladder cancer, cervical cancer, colorectal cancer, esophageal cancer, gliomas, gastric cancer, gastrointestinal cancer, head and neck cancer, hepatic cancer such as hepatocellular carcinoma, leukemia, lung cancer, lymphomas, ovarian cancer, pancreatic cancer, prostate cancer, renal cancer, skin cancer such as melanoma, testicular cancer, etc. or any other tumors or pre-neoplastic lesions that may be treated.

[0189] In another embodiment, the method further comprises detecting the presence of the administered scPV, in the neoplastic disorder or cancer cell and/or in a sample from a subject administered an isolated or recombinant virus or composition described herein. For example, the subject can be tested prior to administration and/or following administration of the scPV or composition described herein to assess for example the progression of the infection. In some embodiments, an scPV of the disclosure comprises a detection cassette and detecting the presence of the administered chimeric poxvirus comprises detecting the detection cassette encoded protein. For example, wherein the detection cassette encodes a fluorescent protein, the subject or sample is imaged using a method for visualizing fluorescence.

[0190] The oncolytic formulations of the present invention comprise an effective amount of an scPV of the invention, and a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeiae for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the pharmaceutical composition (e.g., oncolytic formulation) is administered. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. Examples of suitable

pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. The formulation should suit the mode of administration.

Viral Vectors for Recombinant Gene Expression

[0191] The synthetic chimeric poxviruses (scPVs) of the invention may be engineered to carry heterologous sequences. The heterologous sequences may be from a different poxvirus species or from any non-poxviral source. In one aspect, the heterologous sequences are antigenic epitopes that are selected from any non-poxviral source. In some embodiments, the recombinant virus may express one or more antigenic epitopes from a non-poxviral source including but not limited to *Plasmodium falciparum*, mycobacteria, *Bacillus anthracis*, *Vibrio cholerae*, MRSA, rhabdovirus, influenza virus, viruses of the family of flaviviruses, paramyxoviruses, hepatitis viruses, human immunodeficiency viruses, or from viruses causing hemorrhagic fever, such as hantaviruses or filoviruses, i.e., ebola or marburg virus. In another aspect, the heterologous sequences are antigenic epitopes from a different poxvirus species. These viral sequences can be used to modify the host spectrum or the immunogenicity of the scPV.

[0192] In some embodiments, an scPV of the invention may code for a heterologous gene/nucleic acid expressing a therapeutic nucleic acid (e.g. antisense nucleic acid) or a therapeutic peptide (e.g. peptide or protein with a desired biological activity).

[0193] In some embodiments, the expression of a heterologous nucleic acid sequence is preferably, but not exclusively, under the transcriptional control of a poxvirus promoter. In some embodiments, the heterologous nucleic acid sequence is preferably inserted into a non-essential region of the virus genome. Methods for inserting heterologous sequences into the poxviral genome are known to a person skilled in the art. In some embodiments, the heterologous nucleic acid is introduced by chemical synthesis. In an exemplary embodiment, a heterologous nucleic acid may be cloned into the HPXV095/J2R or HPXV044 locus of an scHPXV of the invention.

[0194] An scPV of the present invention may be used for the introduction of a heterologous nucleic acid sequence into a target cell, the sequence being either homologous or heterologous to the target cell. The introduction of a heterologous nucleic acid sequence into a target cell may be used to produce *in vitro* heterologous peptides or polypeptides, and/or complete viruses encoded by the sequence. This method comprises the infection of a host cell with the scPV; cultivation of the infected host cell under suitable conditions; and isolation and/or enrichment of the peptide, protein and/or virus produced by the host cell.

[0195] It is to be understood that the embodiments of the present invention which have been described are merely illustrative of some of the applications of the principles of the present invention. Numerous modifications may be made by those skilled in the art based upon the teachings presented herein without departing from the true spirit and scope of the invention.

[0196] The following examples are set forth as being representative of the present invention. These examples are not to be construed as limiting the scope of the invention as these and other equivalent embodiments will be apparent in view of the present disclosure, figures, and accompanying embodiments.

Examples

Example 1. Selection and Design of overlapping fragments of the viral genome

Materials and Methods

Synthetic chimeric HPXV (scHPXV) genome design

[0197] Design of the scHPXV genome is based on the previously described genome sequence for HPXV (strain MNR-76; **Figure 1A**) [GenBank accession DQ792504] (Tulman ER, Delhon G, Afonso CL, Lu Z, Zsak L, Sandybaev NT, et al. Genome of horsepox virus. Journal of virology. 2006;80(18):9244-58). The 212,633 bp genome is divided into 10 overlapping fragments (**Figure 1B**). These fragments were designed so that they shared at least 1.0 kbp of overlapping sequence (i.e. homology) with each adjacent fragment, to provide sites where

homologous recombination will drive the assembly of full-length genomes (**Table 1**). These overlapping sequences will provide sufficient homology to accurately carry out recombination between the co-transfected fragments (Yao XD, Evans DH. High-frequency genetic recombination and reactivation of orthopoxviruses from DNA fragments transfected into leporipoxvirus-infected cells. Journal of virology. 2003;77(13):7281-90). It is possible that shorter or longer overlaps will serve a similar purpose. The terminal 40bp from the HPXV genome sequence (5'-TTTATTAAATTTTACTATTTATTTAGTGTCTAGAAAAAAA-3') (SEQ ID NO: 59) is not included in the synthesized inverted terminal repeat (ITR) fragments. Instead, a *Sap*I restriction site is added at the 5'-terminus (GA_LITR) and 3'-terminus (GA_RITR) of the ITR fragments followed by a TGT sequence. These *Sap*I restriction sites are used to ligate the VACV terminal hairpins onto the ITR fragments (described below).

[0198] Each fragment is chemically synthesized and subcloned into a plasmid using terminal *Sfi*I restriction sites on each fragment. To assist with sub-cloning these fragments, *Aar*I and *Bsa*I restriction sites are silently mutated in all the fragments, except for the two ITR-encoding fragments (**Table 2**). The *Bsa*I restriction sites in the two ITR-encoding fragments are not mutated, in case these regions contain nucleotide sequence-specific recognition sites that are important for efficient DNA replication and concatamer resolution.

[0199] A yfp/gpt cassette under the control of a poxvirus early late promoter is introduced into the HPXV095/J2R locus within GA_Fragment_3) so that reactivation of HPXV (scHPXV YFP-gpt::095) will be easy to visualize under a fluorescence microscope. The gpt locus also provides a potential tool for selecting reactivated viruses using drug selection. HPXV095 encodes the HPXV homolog of the non-essential VACV J2R gene and by co-transfecting Fragment_3 and other HPXV clones into SFV-infected BGMK cells, along with VACV DNA, a variety of hybrid viruses are recovered, validating the selection strategy (Figures 13A and13B). Silent mutations are also introduced into the HPXV044 ($VACV^{WR}$F4L) sequence (GA_Fragment_2) to create two unique restrictions sites within GA_Fragment_2 (**Table 3**). In some embodiments, these unique restriction sites

may be used to rapidly introduce recombinant gene products (such as but not limited to, selectable markers, fluorescent proteins, antigens, etc.) into GA_Fragment_2 prior to reactivation of HPXV.

Table 1: The HPXV genome fragments used in this study. The size of each fragment and location within the HPXV genome are indicated.

Fragment Name	**Size (bp)**	**Location within HPXV [DQ792504] (bp)**
GA_Left ITR (SEQ ID NO: 1)	10,095	41 – 10,135
GA_Fragment 1A (SEQ ID NO: 2)	16,257	8505 – 24,761
GA_Fragment 1B (SEQ ID NO: 3)	16,287	23764 – 40,050
GA_Fragment 2 (SEQ ID NO: 4)	31,946	38,705 – 70,650
GA_Fragment 3 (SEQ ID NO: 5)	25,566	68,608 – 94,173
GA_Fragment 4 (SEQ ID NO: 6)	28,662	92,587 – 121,248
GA_Fragment 5 (SEQ ID NO: 7)	30,252	119,577 – 149,828
GA_Fragment 6 (SEQ ID NO: 8)	30,000	147,651 – 177,650
GA_Fragment 7 (SEQ ID NO: 9)	28,754	176,412 – 205,165
GA_Right ITR (SEQ ID NO: 10)	8,484	204,110 – 212,593

Table 2: Silent mutations created in scHPXV YFP-gpt::095 fragments to remove *Aar*I and *Bsa*I restriction sites from HPXV genome.

GA_HPXV Fragment	**Restriction endonuclease recognition site removed**	**Nucleotide change in coding strand of HPXV genome**	**HPXV Gene**	**Location in HPXV genome [DQ792504]**	**Mutation verified by whole genome sequencing**
GA_Frag_1A	*Bsa*I	A to G	HPXV011a	11,228	√
GA_Frag_1B	*Bsa*I	A to G	HPXV025	27,845	√
GA_Frag_2	*Bsa*I	A to G	HPXV040	41,232	√
	*Bsa*I	G to A	HPXV059	56,775	√
	*Bsa*I	G to A	HPXV066	67,836	√
GA_Frag_3	*Bsa*I	G to A	HPXV083	84,361	√
	*Aar*I	T to C	HPXV091	89,368	√
GA_Frag_4	*Bsa*I	T to C	HPXV099	96,239	√
	*Bsa*I	A to G	HPXV099	96,437	√
	*Bsa*I	A to G	HPXV110	109,492	√
	*Bsa*I	A to G	HPXV111	110,661	√
	*Bsa*I	G to A	HPXV111	110,840	√

GA_Frag_4 GA_Frag_5	*Bsa*I	C to T	HPXV119	120,933	√
GA_Frag_5	*Bsa*I	A to G	HPXV123	123,035	√
	*Bsa*I	T to C	HPXV145	144,834	√
GA_Frag_5 GA_Frag_6	*Bsa*I	T to C	HPXV146d	149,727	√
GA_Frag_6	*Bsa*I	G to A	HPXV178b	175,070	√
GA_Frag_7	*Bsa*I	G to A	HPXV182	180,573	√
	*Bsa*I	A to G	HPXV192	187,476	√
	*Aar*I	G to A	HPXV193	188,761	√
	*Bsa*I	C to T	HPXV197	195,680	√
	*Aar*I	T to C	HPXV200	199,873	√

Table 3: Introduction of silent nucleotide mutations in the HPXV044 (VACV F4L) gene to create unique restriction endonuclease sites in GA_Fragment_2.

HPXV gene	**Restriction endonuclease site created**	**Nucleotide change in the HPXV coding strand**	**Location in HPXV genome**
HPXV044	AvaI	A to C	44,512
	StuI	A to C	45,061

Synthesis of the Slow (S) and Fast (F) forms of the terminal hairpin loops from VACV (strain WR)

[0200] The Slow (S) and Fast (F) forms of the terminal hairpin loops from VACV (strain WR) are synthesized as 157nt ssDNA fragments (Integrated DNA Technologies; **Figure 2B**). Through DNA synthesis, a 5' overhang comprised of three nucleotides is left at the end of each hairpin (5'-ACA; **Figure 2C**). The concatamer resolution site from the HPXV sequence [DQ792504] is also synthesized in the terminal hairpin loops (**Figure 2B**).

Digestion and purification of scHPXV YFP-gpt::095 fragments

[0201] Synthetic HPXV fragments are digested with *Sfi*I overnight at 50˚C. The scHPXV ITR fragments are individually digested with *Sap*I (ThermoFisher Scientific) for 1h, inactivated at 65˚C for 10 minutes, before digestion with *Sfi*I overnight at 50˚C. Approximately 1U of FastAP alkaline phosphatase is added to the scHPXV YFP-gpt::095 ITR digestions and incubated at 37˚C for an additional

1h. All scHPXV YFP-gpt::095 fragments are subsequently purified using a QiaexII DNA cleanup kit (Qiagen). All scHPXV YFP-gpt::095 fragments are eluted from the QiaexII suspension in 10mM Tris-HCl. DNA concentrations are estimated using a NanoDrop (ThermoFisher Scientific).

Results

[0202] Poxviruses catalyze very high-frequency homologous recombination reactions that are inextricably linked to the process of virus replication. Herein, it is demonstrated that large fragments of chemically synthesized HPXV duplex DNA can be joined to form a functional scHPXV genome using virus-catalyzed recombination and replication reactions.

[0203] Using the published sequence of the HPXV genome (strain WNR-76), the 212,633 bp genome is divided into 10-overlapping fragments (**Figure 2**). All of the *Bsa*I and *Aar*I sites in every fragment except the ITRs are mutated, in case sequence-specific sites within this region are unknowingly required for efficient genome replication and concatamer resolution. As described above, to facilitate the addition of the terminal hairpin loop structures from VACV onto the end of the ITRs, a *Sap*I recognition site is included next to the left- and right-terminal end of both LITR and RITR fragments, respectively (**Figure 2A**). These *Sap*I sites are embedded within the flanking vector sequences, and the *Sap*I enzyme cuts downstream of the site, outside of the recognition sequence and in the HPXV DNA. Thus when DNA is cut with *Sap*I, it leaves sticky ends within the DNA copied from the HPXV sequence and thus permits the assembly of a precise sequence copy (through a subsequent ligation), containing no extraneous restriction sites. The other ends of the LITR and RITR fragments (the internal ends with respect to the genome map) are each bounded by *Sfi*I recognition sites, as are both ends of the remaining HPXV fragments. All of these DNAs are supplied in a plasmid form for easy propagation. To prepare the internal fragments for transfection into SFV-infected cells, these plasmids are digested with *Sfi*I to release the plasmid from each scHPXV YFP-gpt::095 fragment (see below for how the LITR and RITR fragments are processed). Following digestion, each reaction is purified to remove any contaminating enzyme, but the plasmid is not removed

from the digestion and is co-transfected alongside each scHPXV YFP-gpt::095 fragment. This does not interfere with the reaction and is done to minimize the amount of DNA manipulation and possible fragmentation of these large DNA fragments.

[0204] While the reaction efficiency may be affected by the number of transfected fragments, greater than or less than 10 overlapping fragments may be used in the methods of the invention. Without being bound by theory, ~15 fragments may represent a practical upper limit without further optimization of the reactivation reaction. The ideal lower limit would be a single genome fragment, but in practice the telomeres are most easily manipulated as more modest-sized fragments (e.g. ~10 kb).

Example 2. Ligation of VACV F- and S-terminal hairpin loops onto scHPXV YFP-gpt::095 **left and right ITR fragments**

Materials and Methods

Ligation of the S- and F- forms of the terminal hairpin loops onto scHPXV YFP-gpt::095 ITR fragments

[0205] Approximately one microgram of each of the terminal VACV hairpin loops are incubated at 95˚C for 5 minutes followed by a "snap" cool on ice to form the hairpin structure. The hairpin loops are subsequently phosphorylated at their 5' end before ligation. Briefly, separate 20µl reactions containing 1µg of either VACV F-hairpin or VACV S-hairpin, 2µl of 10x T4 polynucleotide kinase buffer (ThermoFisher Scientific), 1mM ATP, and 10 units of T4 polynucleotide kinase (ThermoFisher Scientific) are incubated at 37˚C for 1 h. The reaction is terminated by heat inactivation at 75˚C.

[0206] Approximately one microgram of either left ITR or right ITR is incubated separately with a 20-fold molar excess of each terminal hairpin in the presence of 5% PEG-4000, and 5 units of T4 DNA ligase overnight at 16˚C. Each ligation reaction is heat-inactivated at 65˚C for 10 minutes followed by incubation on ice until ready to transfect into cells.

Results

[0207] Orthopoxviruses encode linear dsDNA genomes bearing variable length inverted terminal repeats (ITR) at each end of the genome. The two strands of the duplex genome are connected by hairpin loops to form a covalently continuous polynucleotide chain. The loops are A+T-rich, cannot form a completely base-paired structure, and exist in two forms that are inverted and complementary in sequence (Baroudy BM, Venkatesan S, Moss B. Incompletely base-paired flip-flop terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. Cell. 1982;28(2):315-24) (**Figure 2B**). They are called slow [S] and fast [F] forms based upon their electrophoretic properties and probably fold into partially duplex hairpin structures that cap the ends of the linear dsDNA genome (**Figure 2C**). The published sequence of the HPXV genome is incomplete, probably missing ~60 bp from the terminal ends, making it impossible to precisely replicate the HPXV hairpins. Instead, 157 nt ssDNA fragments were chemically synthesized using the published sequence of the VACV telomeres as a guide and leaving a 5' overhang comprised of three nucleotides at the end of each hairpin (5'-ACA; **Figure 2C**) (Baroudy BM, Venkatesan S, Moss B. Incompletely base-paired flip-flop terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. Cell. 1982;28(2):315-24). This overhang is complementary to the ends generated by cutting cloned LITR and RITR fragments with *Sap*I.

[0208] Sequences derived from VACV are used based upon data suggesting a close common ancestry between HPXV and VACV. It may be possible to use other terminal hairpins from other poxviruses since there are sequence features that are commonly conserved between the hairpin ends of different Chordopoxviruses. For example, the resolution sites in the hairpin ends are highly conserved in both sequence and functionality (they resemble late promoters).

[0209] These single-stranded oligonucleotides are heated to 95°C and then quickly chilled on ice to form the incompletely base-paired terminal hairpin (**Figure 2C**). Next, each oligonucleotide is phosphorylated and ligated separately at 20-fold molar excess with either the left or right ITR fragment previously

digested with both *Sap*I and *Sfi*I. Digestion of the ITRs with these enzymes results in a 5'-TGT overhang at the 5' termini of each ITR, which was complementary to the 5'-ACA overhang in the terminal hairpin loop structure. This produces a hairpin-terminated copy of each ITR.

[0210] To confirm that a hairpin-terminated structure is added to both ITR fragments, restriction digestion of the ITR fragments with *Pvu*II is performed. Since it is impossible to visualize the addition of a ~70bp terminal hairpin onto the terminus of a ~10kb ITR by gel electrophoresis, a small amount of each ligation is digested with *Pvu*II. If no terminal hairpin is ligated to the ITR, then digestion with *Pvu*II results in a 1472bp product (**Figure 3A and 3B, lanes 2 and 5**). If, however, the terminal hairpin loop is successfully added to the HPXV ITRs, then an increase in the size of the ITR fragment is seen on an agarose gel (**Figure 3B**, compare lane 2 with 3 and 4; compare lane 5 with 6 and 7). These data suggest that under these conditions almost all of the HPXV ITRs contain terminal hairpin loops at one end of the fragment.

Example 3. Reactivation of scHPXV YFP-gpt::095 from chemically synthesized dsDNA fragments

Materials and Methods

Viruses and cell culture

[0211] SFV strain Kasza and BSC-40 were originally obtained from the American Type Culture Collection. Buffalo green monkey kidney (BGMK) cells were obtained from G. McFadden (University of Florida). BSC-40 and BGMK cells are propagated at 37˚C in 5% CO_2 in minimal essential medium (MEM) supplemented with L-glutamine, nonessential amino acids, sodium pyruvate, antibiotics and antimycotics, and 5% fetal calf serum (FCS; ThermoFisher Scientific).

Reactivation of scHPXV YFP-gpt::095 in Shope Fibroma Virus-infected cells

[0212] Buffalo green monkey kidney (BGMK) cells are grown in MEM containing 60mm tissue-culture dishes until they reached approximately 80% confluency. Cells are infected with Shope Fibroma Virus (SFV) in serum-free MEM at a MOI of 0.5 for 1h at 37˚C. The inoculum is replaced with 3ml of warmed MEM containing 5% FCS and returned to the incubator for an additional hour. Meanwhile, transfection reactions are set up as follows. Lipofectamine complexes are prepared by mixing approximately 5 µg total synthetic HPXV DNA fragments in 1ml Opti-MEM with Lipofectamine2000 diluted in 1ml Opti-MEM at a ratio of 3:1 (Lipofectamine2000 to total DNA). A sample calculation to determine the relative amount of each HPXV fragment is shown in **Table 4**. The complexes are incubated at room temperature for 10 minutes and then added dropwise to the BGMK cells previously infected with SFV. Approximately 16h post infection, the media is replaced with fresh MEM containing 5% FCS. The cells are cultured for an additional 4d (total of 5d) at 37˚C. Virus particles were recovered by scraping the infected cells into the cell culture medium and performing three cycles of freezing and thawing. The crude extract is diluted 10^{-2} in serum-free MEM and 4ml of the inoculum is plated on 9 – 16 150mm tissue culture plates of BSC-40 cells to recover reactivated scHPXV YFP-gpt::095. One hour post infection, the inoculum is replaced with MEM containing 5% FCS and 0.9% Noble Agar. Yellow fluorescent plaques are visualized under an inverted microscope and individual plaques are picked for further analysis. ScHPXV YFP-gpt::095 plaques are plaque purified three times with yellow fluorescence selection.

Table 4: Sample calculation of the quantity of each GA_HPXV fragment transfected into SFV-infected BGMK cells.

fragment	**frag. length**	**Ratio (frag. length:genome length)**	**Amount of DNA to transfect (ng)**		
			~1 µg	**~3 µg**	**~5 µg**
GA_LITR + F-hairpin	10,165	0.05	50	150	250
GA_LITR + S-hairpin	10,165	0.05	50	150	250
GA_Frag_1A	16,257	0.08	80	240	400

GA_Frag_1B	16,287	0.08	80	240	400
GA_Frag_2	31,946	0.15	150	450	750
GA_Frag_3	25,566	0.12	120	450	600
GA_Frag_4	28,662	0.13	130	390	650
GA_Frag_5	30,252	0.14	140	420	700
GA_Frag_6	30,000	0.14	140	420	700
GA_Frag_7	28,754	0.13	130	390	650
GA_RITR + F-hairpin	8,554	0.04	40	120	200
GA_RITR + S-hairpin	8,554	0.04	40	120	200

Results

[0213] SFV-catalyzed recombination and reactivation of Orthopoxvirus DNA to assemble recombinant vaccinia viruses has previously been described (Yao XD, Evans DH. High-frequency genetic recombination and reactivation of orthopoxviruses from DNA fragments transfected into leporipoxvirus-infected cells. Journal of virology. 2003;77(13):7281-90; and Yao XD, Evans DH. Construction of recombinant vaccinia viruses using leporipoxvirus-catalyzed recombination and reactivation of orthopoxvirus DNA. Methods Mol Biol. 2004;269:51-64). Several biological features make this an attractive model system. First, SFV has a narrow host range, productively infecting rabbit cells and certain monkey cell lines, like BGMK. It can infect, but grows very poorly on cells like BSC-40. Second, it grows more slowly compared to Orthopoxviruses, taking approximately 4-5 days to form transformed "foci" in monolayers of cells, a characteristic that is very different from Orthopoxviruses, which produce plaques within 1-2 days in culture. This difference in growth between Leporipoxviruses and Orthopoxviruses allows one to differentiate these viruses by performing the reactivation assays in BGMK cells and plating the progeny on BSC-40 cells. In some embodiments, other helper viruses (such as but not limited to fowlpox virus) may be used. In some embodiments, different cell combinations may be used.

[0214] BGMK cells are infected with SFV at a MOI of 0.5 and then transfected with 5μg of digested GA_HPXV fragments (**Table 4**) 2 h later. Five days post transfection all of the infectious particles are recovered by cell lysis and re-plated on BSC-40 cells, which only efficiently support growth of HPXV (or other

Orthopoxviruses). The resulting reactivated scHPXV YFP-gpt::095 plaques are visualized under a fluorescence microscope. The visualization is enabled by the yfp/gpt selectable marker in the HPXV095/J2R locus within Frag_3 (**Figure 2A**). Virus plaques are detected in BSC-40 monolayers within 48 h of transfection. The efficiency of recovering scHPXV YFP-gpt::095 is dependent on a number of factors, including DNA transfection efficiency, but ranges up to a few PFU/μg of DNA transfected.

Example 4. Confirmation of scHPXV YFP-gpt::095 genome sequence by PCR and restriction fragment analysis

Materials and Methods

PCR and restriction digestion analysis of scHPXV

[0215] To rapidly confirm the presence of scHPXV YFP-gpt::095 in reactivated plaque picks, PCR primers are designed to flank individual *Bsa*I sites that were mutated in the scHPXV (**Table 5**). Genomic scHPXV YFP-gpt::095 DNA is isolated from BSC-40 cells infected with scHPXV YFP-gpt::095 and used as a template. Genomic DNA from VACV-infected BSC-40 cells is used as a control to confirm the presence of *Bsa*I sites within each PCR product. Following PCR amplification, reactions are subsequently digested with *Bsa*I for 1h at 37˚C. PCR reactions are separated on a 1% agarose gel containing SYBR® safe stain to visualize DNA bands.

[0216] Further analysis of scHPXV YFP-gpt::095 genomes by restriction digestion followed by pulse-field gel electrophoresis (PFGE) is carried out on genomic DNA isolated using sucrose gradient purification (Yao XD, Evans DH. Construction of recombinant vaccinia viruses using leporipoxvirus-catalyzed recombination and reactivation of orthopoxvirus DNA. Methods Mol Biol. 2004;269:51-64). Briefly, 100ng of purified viral genomic DNA is digested with 5U of *Bsa*I or *Hind*III for 2h at 37˚C. Digested DNA is run on a 1% Seakem Gold agarose gel cast and run in 0.5X tris-borate-EDTA electrophoresis (TBE) buffer [110mM tris; 90mM borate; 2.5mM EDTA]. The DNA is resolved on a CHEF

DR-III apparatus (BioRad) at 5.7V/cm for 9.5h at 14˚C, using a switching time gradient of 1 to 10s, a linear ramping factor, and a 120˚ angle. This program allows resolution of DNA species from 1kbp to >200kbp. To resolve fragments from 75bp to 5kbp, electrophoresis on 1.5% agarose gel cast and run in 1.0X TBE at 115V for 2h at room temperature is carried out. The DNA is visualized with SYBR® gold stain. The size of digested scHPXV YFP-gpt::095 DNA fragments is compared to control VACV genomic DNA.

Table 5: Primers that are used in this study to amplify regions within VACV and HPXV surrounding the *Bsa*I restriction sites found in GA_Fragment_1A, GA_Fragment_1B, GA_Fragment_2, GA_Fragment_3, GA_Fragment_4, GA_Fragment_5, GA_Fragment_6, and GA_Fragment_7.

Primer Name	**Primer sequence (5' to 3')**	**Position of *Bsa*I site in VACV [NC_006998]**	**Position of *Bsa*I site in HPXV [DQ792504]**
HPXV 1A – FWD (SEQ ID NO: 13)	CTGTATACCCATACTGAATTGATGAAC	16,756	27,849
HPXV 1A – REV (SEQ ID NO: 14)	GAGTTAATATAGACGACTTTACTAAAGTCATG		
HPXV 1B – FWD (SEQ ID NO: 15)	GGTTCTTTTTATTCTTTTAAACAGATCAATGG	23,076	N/A
HPXV 1B – REV (SEQ ID NO: 16)	TTCTTATTAAGACATTGAGCCCAGC		
HPXV 2A – FWD (SEQ ID NO: 17)	AGTCATCAATCATCATTTTTTCACC	30,073	41,225
HPXV 2A – REV (SEQ ID NO: 18)	ATATAACGGACATTTCACCACC		
HPXV 2B – FWD (SEQ ID NO: 19)	GTAACATATACAACTTTTATTATGGCGTC	45,485	56,778
HPXV 2B – REV (SEQ ID NO: 20)	CTAATCCACAAAAAATAGAATGTTTAGTTATTTTG		
HPXV 2C – FWD (SEQ ID NO: 21)	AGTGACTGTATCCTCAAACATCC	56,576	67,839
HPXV 2C – REV (SEQ ID NO: 22)	TTTATAAAGGGTTAACCTTTGTCACATC		
HPXV 3A – FWD (SEQ ID NO: 23)	TTGTGTAGCGCTTCTTTTTAGTC	60,981	N/A
HPXV 3A – REV (SEQ ID NO: 24)	AAACGGATCCATGGTAGAATATG		
HPXV 3B – FWD	TATTTGCATCTGCTGATAATCATC	84,916	84,353

(SEQ ID NO: 25)	C		
HPXV 3B – REV (SEQ ID NO: 26)	CGATGGATTCAAATGACTTGTTAATG		
HPXV 4A – FWD (SEQ ID NO: 27)	ATGCCTTTACAGTGGATAAAGTTAAAC	85,101	96,243 & 96,428
HPXV 4A – REV (SEQ ID NO: 28)	CTGGATCCTTAGAGTCTGGAAG		
HPXV 4B – FWD (SEQ ID NO: 29)	CGGAAAATGAAAAGGTACTAGATACG	98,134	109,485
HPXV 4B – REV (SEQ ID NO: 30)	TGAATAGCCGTTAAATAATCTATTTCGTC		
HPXV 4C – FWD (SEQ ID NO: 31)	TATGGATACATTGATAGCTATGAAACG	99,302 & 99,481	110,653 & 110,832
HPXV 4C – REV (SEQ ID NO: 32)	AATACATCTGTTAAAATTGTTTGACCCG		
HPXV 5A – FWD (SEQ ID NO: 33)	CATTTTATTTCTAGACGTTGCCAG	111,686	123,037
HPXV 5A – REV (SEQ ID NO: 34)	CGATATGAAACTTCAGGCGG		
HPXV 5B – FWD (SEQ ID NO: 35)	ACAAAACGATTTAATTACAGAGTTTTCAG	122,484	N/A
HPXV 5B – REV (SEQ ID NO: 36)	GTCCGGTATGAGACGACAG		
HPXV 5C – FWD (SEQ ID NO: 37)	TTAGGGATCACATGAATGAAATTCG	133,505	144,838
HPXV 5C – REV (SEQ ID NO: 38)	TATGGAAGTTCCGTTTCATCCG		
HPXV 5D – FWD (SEQ ID NO: 39)	GACTTGATAATCATATATTAAACACATTGGATC	138,306	149,718
HPXV 5D – REV (SEQ ID NO: 40)	AGATCTCCAGATTTCATAATATGATCAC		
HPXV 6A – FWD (SEQ ID NO: 41)	ATGATACGTACAATGATAATGATACAGTAC	163,521	175,062
HPXV 6A – REV (SEQ ID NO: 42)	TGATTTTTGCAATTGTCAGTTAACACAAG		
HPXV 7A – FWD (SEQ ID NO: 43)	TACTGTACCCACTATGAATAACGC	169,035	180,578
HPXV 7A – REV (SEQ ID NO: 44)	GATATCAACATCCACTGAAGAAGAC		
HPXV 7B – FWD (SEQ ID NO: 45)	ATCTTACCATGTCCTCAAATAAATACG	175,849	187,467
HPXV 7B – REV (SEQ ID NO: 46)	ATAGCTCTAGGTATAGTCTGCAAG		
HPXV 7C – FWD (SEQ ID NO: 47)	GCGAACTCCATTACACAAATATTTG	181,952	195,683
HPXV 7D – REV (SEQ ID NO: 48)	GATGTTTCTAAATATAGGTTCCGTAAGC		

Results

[0217] The genome sequence of virus isolated from plaques grown from the reactivation assay is confirmed by PCR, restriction digestion, and whole genome sequencing. The PCR analysis is based on the mutated *Bsa*I sites within all but the ITR HPXV fragments. Primer sets are designed to flank each *Bsa*I site in scHPXV YFP-gpt::095 (Table 5). It is confirmed that these primer sets would also amplify a similar region within VACV WR. After PCR amplification of an approximate 1kb region surrounding these mutated *Bsa*I sites within scHPXV YFP-gpt::095, each reaction is digested with *Bsa*I and the resulting DNA fragments are analyzed by gel electrophoresis. Since no *Bsa*I sites are mutated in VACV (wt), enzymatic digestion successfully digests each PCR product, resulting in a smaller DNA fragment (**Figure 4A, VACV**). The PCR products generated from scHPXV YFP-gpt::095 genomic DNA are resistant to *Bsa*I digestion, suggesting that the *Bsa*I recognition site is successfully mutated in these genomes (**Figure 4A, scHPXV YFP-gpt::095 (PP1) and scHPXV YFP-gpt::095 (PP3)**). The primer products for primer set 7C did not result in any amplification of DNA in the scHPXV YFP-gpt::095 PP1 and PP3 samples. To confirm whether this primer set was non-functional or if this area of Fragment 7 did not get assembled into the resulting scHPXV YFP-gpt::095 genome, PCR was performed on the original GA_Frag_7 plasmid DNA and this reaction was also unsuccessful in amplifying a product.

[0218] Genomic DNA is next isolated from sucrose-gradient purified scHPXV YFP-gpt::095 genomes, digested with *Bsa*I or *Hind*III, and separated by agarose gel electrophoresis to confirm that the majority of the *Bsa*I sites in scHPXV YFP-gpt::095 are successfully mutated. Interestingly, undigested genomic DNA from 3 different scHPXV YFP-gpt::095 clones run noticeably slower on a gel compared to VACV, confirming that the genome of scHPXV YFP-gpt::095 (213,305bp) is larger than VACV-WR (194,711bp) (**Figure 4B, compare lanes 2-4 with lane 5**). The scHPXV YFP-gpt::095 clones are resistant to *Bsa*I digestion, resulting in one large DNA fragment (~198000bp) and a smaller DNA fragment at around 4000 bp after separation by PFGE (**Figure 4A, lane 7-9**). This is in contrast to the VACV-

WR genome, which when digested with *Bsa*I, leads to a number of DNA fragments being separated on the gel (**Figure 4B, lane 10**). Since the expected DNA sizes following digestion of scHPXV YFP-gpt::095 genome with *Bsa*I are relatively small (**Table 6**), these digestion products are separated by conventional agarose gel electrophoresis and it is confirmed that the scHPXV YFP-gpt::095 generates the appropriately sized fragments (**Figure 4C, lanes 2-4**). It is also confirmed that scHPXV YFP-gpt::095 produces the correct size of DNA fragments following *Hind*III digestion, suggesting that these recognitions are maintained during synthesis of the large DNA fragments (**Table 6; Figure 4B, lanes 12-14; Figure 4C, lanes 6-8**). Overall, *in vitro* analysis of the scHPXV YFP-gpt::095 genome suggests that reactivation of HPXV from chemically synthesized DNA fragments is successful.

Table 6: Expected sizes of scHPXV YFP-gpt::095 DNA fragments digested with either *Bsa*I or *Hind*III.

Fragment #	**scHPXV YFP-gpt::095 digested with *Bsa*I (bp)**	**scHPXV YFP-gpt::095 digested with *Hind*III (bp)**
1	198,833	53,822
2	4046	24,848
3	4046	19,283
4	968	16,056
5	968	15,176
6	778	13,836
7	778	13,558
8	767	12,679
9	767	8877
10	391	8637
11	391	6493
12	138	5803
13	138	4631
14	64	4115
15	60	2216
16	54	1560
17	54	1442
18	32	273
19	32	

[0219] Since HPXV095 encodes the HPXV homolog of the non-essential VACV J2R gene, by co-transfecting Fragment_3 and other HPXV clones into SFV-infected BGMK cells, along with VACV DNA, a variety of hybrid viruses are recovered, validating the selection strategy (Figures 13A and13B). The first hybrid virus ("VACV/HPXV + fragment 3") is obtained by co-transfecting VACV DNA with HPXV Frag_3 (**Fig 1**) into SFV-infected cells. The green-tagged insertion encodes the YFP-gpt selection marker. Clones 1-3 are obtained by purifying the DNA from this first hybrid genome and transfecting it again, along with HPXV fragments 2, 4, 5, and 7, into SFV-infected cells. PCR primers were designed to target both HPXV and VACV (Table 5) are used to amplify DNA segments spanning the *Bsa*I sites that are mutated in the scHPXV clones. Following PCR amplification, the products are digested with *Bsa*I to differentiate VACV sequences (which cut) from HPXV (which do not cut). The VACV/HPXV hybrids exhibit a mix of *Bsa*I sensitive and resistant sites whereas the reactivated scHPXV YFP-gpt::095 clone is fully *Bsa*I resistant.

Example 5. Confirmation of scHPXV YFP-gpt::095 genome sequence by whole genome sequence analysis

Materials and Methods

Virus DNA isolation and sequencing

[0220] Stocks of HPXV YFP-gpt::095 clones (plaque pick [PP] 1.1, PP 2.1, and PP 3.1]) are prepared and purified over sucrose gradients. Viral DNAs are extracted from each purified virus preparation using proteinase K digestion followed by phenol-chloroform extraction. The amount of dsDNA is determined using a Qubit dsDNA HS assay kit (ThermoFisher Scientific). Each viral genome is sequenced at the Molecular Biology Facility (MBSU) at the University of Alberta. Sequencing libraries are generated using the Nextera Tagmentation system (Epicentre Biotechnologies). Approximately 50ng of each sample is sheared and library prepped for paired end sequencing (2x300bp) using an Illumina MiSeq platform with an average read depth of 3,100 reads·nt^{-1} across the genome and ~190 reads·nt^{-1} in the F- and S-hairpins.

Sequence assembly, analysis, and annotation

[0221] Raw sequencing reads are trimmed of low quality sequence scores and initially mapped to the HPXV reference sequence [GenBank Accession DQ792504] using CLC Genomics Workbench 8.5 software. All nucleotide insertions, deletions, and substitutions within the scHPXV YFP-gpt::095 sequence are verified against the HPXV reference sequence. The Genome Annotation Transfer Utility (GATU) (Tcherepanov V, Ehlers A, Upton C. Genome Annotation Transfer Utility (GATU): rapid annotation of viral genomes using a closely related reference genome. BMC Genomics. 2006;7:150. Epub 2006/06/15) is used to transfer the reference annotation to the scHPXV genome sequences.

Results

[0222] Purified scHPXV YFP-gpt::095 genomes are sequenced using a multiplex approach and an Illumina MiSeq sequencer. The sequence reads are mapped onto the wild-type HPXV (DQ792504) and scHPXV YFP-gpt::095 reference sequences to confirm the presence of specific modifications in the scHPXV YFP-gpt::095 genome. To confirm that the VACV terminal repeat sequences are correctly ligated onto the terminal end of the left ITR, sequencing reads in this area of the genome are analyzed. A string of Cs is added to the beginning of the scHPXV YFP-gpt::095 genome reference sequence to capture all of the sequence reads that mapped in this region (**Figures 5A and 5B**). This is done because the program used to assemble the sequence reads will otherwise truncate the display of sequences at the point where the scHPXV YFP-gpt::095 genome reference sequence ends.

[0223] It is clear from the mapped reads that although the *Sap*I recognition site is present in the scHPXV YFP-gpt::095 reference genome, all of the sequencing reads lack this sequence. This confirms that the approach described herein produces an authentic HPXV sequence at the site where the synthetic hairpin was ligated to the ends of the ITRs. The complete sequence of the VACV WR terminal hairpin loop is also successfully obtained, which proves to be identical to the sequence of the synthetic ssDNA that is ligated onto the TIR ends. Overall, these data suggest that the VACV-WR terminal hairpin loops are successfully ligated

onto the HPXV ITR sequences and recovered in the infectious viruses. Moreover, the 1:1 distribution of F- and S-reads in each of five viruses suggested that both ends are required to produce a virus (Figure 5B)

[0224] Next, it is verified that each nucleotide substitution to silently mutate the *Bsa*I sites has correctly been incorporated into the scHPXV YFP-gpt::095 genome (**Figure 6**). Sequencing reads are mapped to the HPXV (DQ792504) reference sequence. The overall Illumina sequencing read coverage in scHPXV YFP-gpt::095 from region 96,050 to 96,500 is shown in **Figure 6A**. It is clear from here that there are two conflicts in this region that do not align correctly with reference HPXV (**Figure 6A, blue and yellow vertical lines**). Upon magnification of these regions it is clear that at position 96,239 there is a T to C substitution (**Figure 6B**) and at position 96,437 then is an A to G substitution (**Figure 6C**) in the scHPXV YFP-gpt::095 genome. It was verified that all of the nucleotide substitutions that are introduced in order to mutate the selected *Bsa*I and *Aar*I recognition sites are created in the scHPXV YFP-gpt::095 genome (**Table 2**).

[0225] Finally, it is determined that the nucleotide substitutions in HPXV044, designed to create unique restriction sites in GA_Frag_2, are also incorporated into the scHPXV YFP-gpt::095 genome. The sequencing reads that map to HPXV044 (region 44,400 to 45,100) are shown in **Figure 7A**. Within this region there are two regions where the sequencing reads conflict with that of the sequence in the HPXV YFP-gpt::095 reference sequence (**Figure 7A, yellow vertical lines**). Upon magnification of these regions, it is clear that two T to G substitutions are introduced into the non-coding strand of HPXV044 at positions 44,512 and 45,061, thus creating *Ava*I and *Stu*I restriction sites in Frag_2 (**Figure 7B and 7C**). Overall, the sequencing data corroborates the *in vitro* genomic analysis data and confirms that scHPXV YFP-gpt::095 is successfully reactivated in SFV-infected cells.

Example 6. ScHPXV YFP-gpt::095 replicates more slowly in HeLa cells compared to other poxviruses

Materials and Methods

[0226] BSC-40, HeLa, and HEL fibroblasts were originally obtained from the American Type Culture Collection. BSC-40 cells are propagated at 37˚C in 5% CO_2 in minimal essential medium (MEM) supplemented with L-glutamine, nonessential amino acids, sodium pyruvate, antibiotics and antimycotics, and 5% fetal calf serum (FCS; ThermoFisher Scientific). HeLa and HEL cells are propagated at 37˚C in 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with L-glutamine, antibiotics and antimycotics, and 10% FCS.

Results

[0227] Multi-step growth curves and plaque size measurements are used to evaluate whether scHPXV YFP-gpt::095 replicated and spread *in vitro* similar to other Orthopoxviruses. Since a natural HPXV isolate is unavailable, the growth of scHPXV YFP-gpt::095 is compared to the prototypic poxvirus, VACV (strain WR), Cowpox virus (CPX), a poxvirus that is closely related to HPXV and a clone of Dryvax virus, DPP15. Monkey kidney epithelial cells (BSC-40), Vero cells, a human carcinoma cell line (HeLa), and primary human fibroblasts cells (HEL) are infected with VACV WR, CPX, DPP15, or scHPXV YFP-gpt::095 at a low MOI and infected cells are harvested over a 72 h time course. In BSC-40 cells, the rate of virus replication and spread is comparable among all viruses tested (**Figure 8A**). Importantly, scHPXV YFP-gpt::095 replicates as well as any of the other poxviruses tested. The virus grew to somewhat lower titers on HEL cells and Vero cells, and least well on HeLa cells. In HeLa cells, up to a 1.5-log decrease in virus production is seen compared to other Orthopoxviruses.

[0228] Next, the plaque size of scHPXV YFP-gpt::095 grown in BSC-40 cells is measured. A statistically significant decrease in plaque size of scHPXV YFP-gpt::095 compared to VACV WR and even cowpox virus (**Figure 8B**) is observed. Interestingly, in BSC-40 cells, scHPXV YFP-gpt::095 produces the smallest plaques when compared to all other Orthopoxviruses tested (**Figure 8C**). Also,

while different VACV strains produce extracellular viruses that form smaller secondary plaques, these are not produced by scHPXV YFP-gpt::095 (Figure 8C). Overall, these data suggest that reactivation of scHPXV YFP-gpt::095 using the system described herein does not introduce any obvious defects in virus replication and spread *in vitro* when compared to other Orthopoxviruses. Moreover, the plaque size of scHPXV YFP-gpt::095 is similar to that of cowpox virus (CPXV), suggesting that synthetic virus reactivation does not have any deleterious effects on the small plaque phenotype that has previously been observed with other HPXV-like clones (Medaglia ML, Moussatche N, Nitsche A, Dabrowski PW, Li Y, Damon IK, et al. Genomic Analysis, Phenotype, and Virulence of the Historical Brazilian Smallpox Vaccine Strain IOC: Implications for the Origins and Evolutionary Relationships of Vaccinia Virus. Journal of virology. 2015;89(23):11909-25).

Example 7. Removal of yfp/gpt selection marker

[0229] Following reactivation of the scHPXV YFP-gpt::095, the yfp/gpt selection marker in the HPXV095 locus is removed. To do this, a 1349 bp region of sequence corresponding to nucleotide positions 91573 to 92921 in HPXV (DQ792504) is synthesized (ThermoFisher Scientific) (SEQ ID NO: 60). This fragment included approximately 400 bp of homology flanking either side of the wt HPXV095/J2R gene. This sequence of DNA is cloned into a commercial vector provided by GeneArt. To replace the yfp/gpt cassette with the HPXV095 gene sequence, BSC-40 cells are infected with scHPXV YFP-gpt::095 at a MOI of 0.5 and then transfected, 2 hr later, with 2 μg of linearized plasmid containing the wtHPXV095 sequence using Lipofectamine 2000 (ThermoFisher Scientific). The virus recombinants are harvested 48 hr post infection and recombinant viruses (scHPXV (wt)) are isolated using three rounds of non-fluorescent plaque purification under agar. PCR is used to confirm the identity of the scHPXV (wt) using primers that flank the HPXV095 gene locus. The primers used to confirm the correct replacement of the HPXV095 gene are HPXV095_check-FWD 5'-CCTATTAGATACATAGATCCTCGTCG-3' (SEQ ID NO: 61) and

HPXV095_check-REV 5'-CGGTTTATCTAACGACACAACATC-3' (SEQ ID NO: 62).

Example 8. Growth properties of scHPXV (wt) versus scHPXV YFP-gpt::095

[0230] In experiments performed as described above in Example 6, scHPXV(wt) shows growth properties not significantly different from scHPXV YFP-gpt::095 *in vitro* (Figures 14A-C). A statistically significant decrease in plaque size of scHPXV(wt) compared to VACV WR is observed (Figure 14A). scHPXV (wt), like scHPXV YFP-gpt::095, does not produce extracellular viruses (Figure 14B) and there are no significant differences in the growth of scHPXV (wt) and scHPXV YFP-gpt::095 on BSC-40 cells, HEL cells, HeLA cells, and Vero cells (Figure 14C). The finding that scHPXV(wt) does not produce extracellular viruses is of relevance given that this property affects virulence.

Example 9. Determination of the virulence of scHPXV (wt) in a murine intranasal model

[0231] The toxicity effects of scHPXV (wt) are determined in this study. For this experiment, 6 groups of Balb/c mice are administered 3 different doses of scHPXV (ΔHPXV_095/J2R) or scHPXV (wt) described in Examples 1-7 and compared to a PBS control group as well as a VACV (WR) control group and a VACV (Dryvax strain DPP15) control group (9 treatment groups in total). There are 3 additional mice included in this experiment that do not receive any treatment for the duration of the study. All mice are sampled for blood at predetermined points throughout the experiment and the additional mice serve as a baseline for serum analysis.

[0232] Prior to inoculation of Balb/c mice, all virus strains are grown in BSC-40 cells (African green monkey kidney), harvested by trypsinization, washed in PBS, extracted from cells by dounce homogenization, purified through a 36% sucrose cushion by ultracentrifugation, resuspended in PBS, and titered such that the final concentrations are: 1) VACV (WR) – 5 x 10^5 PFU/ml; 2) VACV (DPP15) – 10^9 PFU/ml; 3) scHPXV (ΔHPXV_095/J2R) – 10^7 PFU/ml, 10^8 PFU/ml, and 10^9 PFU/ml and 4) scHPXV (wt) - 10^7 PFU/ml, 10^8 PFU/ml, and 10^9 PFU/ml.

[0233] The scHPXV doses chosen for this study (10^5 PFU/dose, 10^6 PFU/dose, and 10^7 PFU/dose) are based on previous studies using known vaccine strains of VACV, including Dryvax and IOC (Medaglia ML, Moussatche N, Nitsche A, Dabrowski PW, Li Y, Damon IK, et al. Genomic Analysis, Phenotype, and Virulence of the Historical Brazilian Smallpox Vaccine Strain IOC: Implications for the Origins and Evolutionary Relationships of Vaccinia Virus. Journal of virology. 2015;89(23):11909-25; Qin L, Favis N, Famulski J, Evans DH. Evolution of and evolutionary relationships between extant vaccinia virus strains. Journal of virology. 2015;89(3):1809-24).

[0234] Since weight loss is used as a measurement of virulence in mice, VACV (strain WR) is administered intranasally at a dose of 5 x 10^3 PFU, which leads to approximately 20 – 30% weight loss. The VACV Dryvax clone, DPP15, is also administered intranasally at 10^7 PFU/dose, so that the virulence of this well-known Smallpox vaccine can be directly compared to scHPXV (wt). Mice are purchased from Charles River Laboratories and once received, are acclimatized to their environment for at least one week prior to virus administration.

[0235] Each mouse receives a single dose of virus (~10ul) administered via the intranasal injection while under anesthesia. Mice are monitored for signs of infection, such as swelling, discharge, or other abnormalities every day for a period of 30 days. Each mouse is specifically monitored for weight loss every day after virus administration. Mice that lose more than 25% of their body weight in addition to other morbidity factors are subjected to euthanasia in accordance with our animal health care facility protocols at the University of Alberta.

[0236] Even at the highest doses of scHPXV tested, there may be no overt signs of illness in Balb/c mice. The VACV strains most closely related to scHPXV, old South American viruses, in some cases produced no disease at 10^7 PFU (Medaglia ML, Moussatche N, Nitsche A, Dabrowski PW, Li Y, Damon IK, et al. Genomic Analysis, Phenotype, and Virulence of the Historical Brazilian Smallpox Vaccine Strain IOC: Implications for the Origins and Evolutionary Relationships of Vaccinia Virus. Journal of virology. 2015;89(23):11909-25). It is impractical to

test much higher doses than this due to the difficulty of making purified stocks with titers in excess of 10^9 PFU/mL.

Example 10. Determine whether scHPXV confers immune protection against a lethal VACV-WR challenge

[0237] Mice that appear to have been unaffected by the initial virus administration described in Example 9 continue to gain weight normally throughout the experiment. Thirty days post virus inoculation, mice are subsequently challenged with a lethal dose of VACV-WR (10^6 PFU/dose) via intranasal inoculation. Mice are closely monitored for signs of infection as described above. Mice are weighed daily and mice that lose greater than 25% of their body weight in addition to other morbidity factors are subjected to euthanasia. We expect that mice inoculated with PBS prior to administration of a lethal dose of VACV-WR show signs of significant weight loss and other morbidity factors within 7-10 days post inoculation. Approximately 14 days post lethal challenge with VACV-WR all mice are euthanized and blood is collected to confirm the presence of VACV-specific neutralizing antibodies in the serum by standard plaque reduction assays.

Example 11. Construction of a synthetic chimeric VACV (strain ACAM2000) (scACAM2000) using SFV-catalyzed recombination and reactivation reactions

Design of overlapping fragments of the VACV (ACAM2000) genome

[0238] Using the published sequence of the VACV genome (strain ACAM2000; Genbank Accession AY313847), the genome is divided into 9 overlapping fragments (**Figure 9**) that range in size from 15,979 bp to 28,795 bp in length (**Table 7**). These fragments are designed so that they share at least 1.0 kbp of overlapping sequence homology with each adjacent fragment to provide sites where homologous recombination can drive the assembly of full-length genomes (**Table 7**). These overlaps should be sufficient to support accurate and efficient recombination between the co-transfected fragments.

[0239] In order to successfully synthesize and subclone these large fragments, each *Bsa*I and *Aar*I site in VACV_ACAM2000 fragments 1 to 7 are silently mutated. As with the creation of scHPXV, the *Bsa*I restriction sites in the two ITR-encoding fragments are not mutated, in case there are DNA sequence features that were important for efficient DNA replication and concatamer resolution.

[0240] For the initial reactivation, the thymidine kinase in VACV (ACAM2000) is replaced with the yfp/gpt cassette to help recover newly reactivated VACV particles.

Table 7: The VACV ACAM2000 genome fragments used in this study. The size, location within the VACV ACAM2000 genome [GenBank Accession AY313847], and overlap with adjacent fragments is described.

Fragment Name	**Size (bp)**	**Location (bp)**	**Overlap with adjacent fragment (bp)**
GA_LITR (A2000) (SEQ ID NO: 50)	18,073	1 – 18,073	-- Frag_1 (2287)
GA_Frag_1 (A2000) (SEQ ID NO: 51)	24,895	15,787 – 40,681	LITR (2287) Frag_2 (1342)
GA_Frag_2 (A2000) (SEQ ID NO: 52)	23,297	39,340 – 62,636	Frag_1 (1342) Frag_3 (2453)
GA_Frag_3 (A2000) (SEQ ID NO: 53)	24,971	60,184 – 85,154	Frag_2 (2453) Frag_4 (1604)
GA_Frag_4 (A2000) (SEQ ID NO: 54)	26,575	83,551 – 110,125	Frag_3 (1604) Frag_5 (1896)
GA_Frag_5 (A2000) (SEQ ID NO: 55)	24,635	108,230 – 132,864	Frag_4 (1896) Frag_6 (2129)
GA_Frag_6 (A2000) (SEQ ID NO: 56)	25,934	130,736 – 156,669	Frag_5 (2129) Frag_7 (2183)
GA_Frag_7 (A2000) (SEQ ID NO: 57)	28,801	154,487 – 183, 287	Frag_6 (2183) RITR (1403)
GA_RITR (A2000) (SEQ ID NO: 58)	17,350	181,885 – 199,234	Frag_7 (1403) --

Ligation of the S and F forms of the terminal loops (from VACV strain ACAM2000) onto the left and right ends of the VACV ITRs

[0241] To prepare the VACV ITR fragments to be transfected into SFV-infected cells, the terminal hairpin loops are ligated to the left and right ITR fragments using the same methods used to attach VACV hairpins to the HPXV telomeres described. Briefly, through DNA synthesis a 5' overhang comprised of three nucleotides is left at the end of each hairpin (5'-ACA; as described in Examples 1 and 2). Meanwhile, the plasmid clones encoding the left and right VACV ITR fragments are designed to encode a *Sap*I recognition site located immediately adjacent to the first nucleotide encoding the start of the VACV genome. Digesting the ITR clone with *Sap*I creates a three base overhang (5'-TGT), complementary to the 5'-ACA overhang in the terminal hairpin loop structure. The left or right ITR fragments is mixed with a ~20-fold molar excess of the terminal loops and ligated. This produces a hairpin-terminated copy of each ITR.

Leporipoxvirus-catalyzed recombination and reactivation of scACAM2000

[0242] Following digestion and DNA clean up of the VACV genomic DNA fragments, they are transfected into SFV-infected BGMK cells. As described previously, the SFV helper virus will catalyze the recombination between fragments sharing flanking homologous sequences, resulting in the creation of full-length VACV genomes that can be packaged and released from the cell. It is unlikely that hybrid viruses will be produced in this assay. After 4 days, the BGMK cells are harvested and reactivated VACV virus particles are released by freeze-thaw, followed by plating on the susceptible BSC-40 cells. The reactivated VACV (ACAM2000) plaques are the only viruses to form plaques on BSC-40 cells. Plaques are picked to produce clonal virus stocks followed by isolation of genomic DNA to be sequenced by nextGen Illumina sequencing to confirm the integrity of the recovered viruses and identify whether scACAM2000 is successfully reactivated.

Example 12. Safety and immunogenicity of scHPXV in a small animal model

Materials and Methods

[0243] *Murine intranasal model of scHPXV infection*. Six-to-eight week old BALB/c mice are purchased from Charles River Laboratories. Groups of five mice are intranasally infected (or mock infected) with 5×10^3 PFU VACV (strain WR), 1×10^7 VACV (Dryvax DPP15), or 1×10^5 PFU, 1×10^6 PFU, or 1×10^7 PFU of scHPXV YFP-gpt::095 (described in Examples 1-6) or scHPXV (wt) (described in Examples 7 and 8) in 10µl of PBS. The mice are weighed daily for 28 days, and the following clinical signs are scored: ruffled fur, difficulty breathing, reduced mobility, and pox lesions. Mice that lose 25% of the initial weight are euthanized. These vaccinated mice are subsequently challenged intranasally with 1×10^6 PFU VACV (strain WR), weighed and monitored daily for clinical signs of disease as described above for 13 days. Mice that lose 25% of the initial weight are euthanized as per protocols approved by the local animal care and use committee.

Results

scHPXV strains do not cause weight loss in an intranasal murine model of poxvirus infection

[0244] The toxicity effects of scHPXV YFP-gpt::095 or scHPXV (wt) are examined in an intranasal murine model of poxvirus infection. Mice are inoculated intranasally with the indicated dose(s) of scHPXV (wt) and their weights are monitored over a 28-day period. No weight loss is apparent in mice inoculated with any of the doses of scHPXV YFP-gpt::095 or scHPXV (wt) over the 28-day period (**Figure 10**). This is in contrast to animals inoculated with either Dryvax DPP15 (10^7 PFU) or VACV WR (5×10^3 PFU), who lose an average of 15% and 10% of their initial weight, respectively. These data suggest that even at the highest dose of scHPXV (wt) and scHPXV YFP-gpt::095 tested (10^7 PFU), no adverse effects are observed. With a known smallpox vaccine strain, DPP15,

however, transient weight loss is detected in mice by ~7 days post inoculation, although these mice return to their initial weight by ~ 10 days post inoculation.

scHPXV YFP-gpt::095 (10^6 & 10^7 PFU) and scHPXV (wt) (10^5, 10^6, 10^7 PFU) confer immune protection against a lethal VACV WR challenge in BALB/c mice

[0245] Following the 28-day immunization of BALB/c mice with the indicated strains of scHPXV YFP-gpt::095, scHPXV (wt), DPP15, and VACV WR (**Figure 10**), mice are subsequently challenged with a lethal intranasal dose of VACV WR (10^6 PFU) to assess the protective efficacy of scHPXV (wt) or scHPXV YFP-gpt::095 in mice. All of the mice initially treated with PBS succumb to infection whereas the animals initially exposed to 10^7 PFU of Dryvax (DPP15) or 5 x 10^3 PFU VACV WR show no weight loss (**Figure 11A**) and no signs of illness (**Figure 11B**). Animals vaccinated with the two lower doses of the scHPXV YFP-gpt::095 show weight loss (**Figure 11A**) and signs of severe illness based on clinical scores (**Figure 11B**) and two animals in the lowest scHPXV YFP-gpt::095 doses also are observed to succumb to infection (**Figure 12**). The remaining animals in these low-dose groups of scHPXV YFP-gpt::095 are observed ultimately to recover from the infection, but their weights remain lower than the average weights in the rest of the groups. Animals previously exposed to 10^5 to 10^7 PFU of scHPXV (wt) show only minor transient weight loss in the first few days following poxvirus challenge (**Figure 11A**), and no clinical signs of illness (**Figure 11B**). These data show that scHPXV can infect and immunize mice against a lethal VACV challenge and can do so without causing disease during the initial immunization step.

WHAT IS CLAIMED IS:

1. A synthetic chimeric poxvirus (scPV) that is replicated and reactivated from DNA derived from synthetic DNA, the viral genome of said virus differing from a wild type genome of said virus in that it is characterized by one or more modifications, the modifications being derived from a group comprising chemically-synthesized DNA, cDNA or genomic DNA.

2. The scPV of claim 1, wherein the synthetic DNA is selected from one or more of: chemically synthesized DNA, PCR amplified DNA, engineered DNA and polynucleotides comprising nucleoside analogs.

3. The scPV of claim 1, wherein the synthetic DNA is chemically synthesized DNA.

4. The scPV of any one of claims 1 to 3, wherein the one or more modifications comprise one or more deletions, insertions, substitutions, or a combination thereof.

5. The scPV of any one of claims 1 to 4, wherein the one or more modifications comprise one or more modifications to introduce one or more unique restriction sites.

6. The scPV of any one of claims 1 to 5, wherein the viral genome comprises heterologous terminal hairpin loops.

7. The scPV of any one of claims 1 to 6, wherein the viral genome comprises terminal hairpin loops derived from vaccinia virus.

8. The scPV of claim 7, wherein the left and right terminal hairpin loops a) comprise the slow form and the fast form of the vaccinia virus terminal hairpin loop, respectively, b) comprise the fast form and the slow form of the vaccinia virus terminal hairpin loop, respectively, c) both comprise the slow form of the vaccinia virus terminal hairpin loop, or d) both comprise the fast form of the vaccinia virus terminal loop.

9. The scPV of any one of claims 1 to 8, wherein the virus is replicated and reactivated from overlapping chemically-synthesized DNA fragments that correspond to substantially all of the viral genome of the scPV.

10. The scPV of claim 9, wherein the virus is replicated and reactivated from 1-14 overlapping fragments.

11. The scPV of claim 9, wherein the virus is replicated and reactivated from 8-12 overlapping fragments.

12. The scPV of claim 9, wherein the virus is replicated and reactivated from 10 overlapping fragments.

13. The scPV of any one of claims 1 to 12, wherein the virus is reactivated using leporipox virus-catalyzed recombination and reactivation.

14. The scPV of claim 13, wherein the leporipox virus is selected from the group consisting of: Shope fibroma virus (SFV), hare fibroma virus, rabbit fibroma virus, squirrel fibroma virus, and myxoma virus.

15. A synthetic chimeric orthopox virus (scOPV) that is replicated and reactivated from DNA derived from synthetic DNA, the viral genome of said virus differing from a wild type genome of said virus in that it is characterized by one or more modifications, the modifications being derived from a group comprising chemically-synthesized DNA, cDNA or genomic DNA.

16. The scOPV of claim 15, wherein the synthetic DNA is selected from one or more of: chemically synthesized DNA, PCR amplified DNA, engineered DNA and polynucleotides comprising nucleoside analogs.

17. The scOPV of claim 15, wherein the synthetic DNA is chemically synthesized DNA.

18. The scOPV of any one of claims 15 to 17, wherein the viral genome of the scOPV is based on an OPV selected from the group consisting of: camelpox (CMLV) virus, cowpox virus (CPXV), ectromelia virus (ECTV), horsepox virus

(HPXV), monkeypox virus (MPXV), vaccinia virus (VACV), variola virus (VARV), rabbitpox virus (RPXV), raccoon poxvirus, skunkpox virus, Taterapox virus, Uasin Gishu disease virus, and volepox virus.

19. The scOPV of any one of claims 15 to 18, wherein the viral genome of the scOPV is based on a VACV.

20. The scOPV of claim 19, wherein the viral genome of the scOPV is based on the genome of VACV strain ACAM2000 and differs from the ACAM2000 genome in that it is characterized by one or more modifications.

21. The scOPV of claim 19, wherein the viral genome of the scOPV is based on the genome of VACV strain IOC and differs from the IOC genome in that it is characterized by one or more modifications.

22. The scOPV of claim 19, wherein the viral genome of the scOPV is based on the genome of VACV strain MVA and differs from the MVA genome in that it is characterized by one or more modifications.

23. The scOPV of claim 19, wherein the viral genome of the scOPV is based on the genome of VACV strain MVA-BN and differs from the MVA-BN genome in that it is characterized by one or more modifications

24. The scOPV of claim 19, wherein the wild type VACV genome is the genome of a strain selected from the group consisting of: Western Reserve, Clone 3, Tian Tian, Tian Tian clone TT9, Tian Tian clone TP3, NYCBH, Wyeth, Copenhagen, Lister 107, Lister-LO, IHD-W, LC16m18, Lederle, Tashkent clone TKT3, Tashkent clone TKT4, USSR, Evans, Praha, LIVP, Ikeda, EM-63, Malbran, Duke, 3737, CV-1, Connaught Laboratories, Serro 2, CM-01, Dryvax clone DPP13, Dryvax clone DPP15, Dryvax clone DPP20, Dryvax clone DPP17, Dryvax clone DPP21, and chorioallantois vaccinia virus Ankara.

25. The scOPV of any one of claims 15 to 24, wherein the one or more modifications comprise one or more deletions, insertions, substitutions, or a combination thereof.

26. The scOPV of any one of claims 15 to 25, wherein the one or more modifications comprise one or more modifications to introduce one or more unique restriction sites.

27. The scOPV of any one of claims 15 to 26, wherein the one or more modifications comprise one or more modifications to eliminate one or more restriction sites.

28. The scOPV of claim 27, wherein the one or more modifications comprise one or more modifications to eliminate one or more *AarI* restriction sites.

29. The scOPV of claim 28, wherein the one or more modifications comprise one or more modifications to eliminate all *AarI* restriction sites.

30. The scOPV of any one of claims 15 to 29, wherein the one or more modifications comprise one or more modifications to eliminate one or more *BsaI* restriction sites.

31. The scOPV of any one of claims 15 to 30, wherein the viral genome comprises heterologous terminal hairpin loops.

32. The scOPV of any one of claims 15 to 30, wherein the viral genome comprises terminal hairpin loops derived from vaccinia virus.

33. The scOPV of claim 32, wherein the left and right terminal hairpin loops a) comprise the slow form and the fast form of the vaccinia virus terminal hairpin loop, respectively, b) comprise the fast form and the slow form of the vaccinia virus terminal hairpin loop, respectively, c) both comprise the slow form of the vaccinia virus terminal hairpin loop, or d) both comprise the fast form of the vaccinia virus terminal loop.

34. The scOPV of claim 33, wherein the slow form comprises a nucleotide sequence that is at least 85% identical to the nucleotide sequence of SEQ ID NO: 11 and the fast form comprises a nucleotide sequence that is at least 85% identical to the nucleotide sequence of SEQ ID NO: 12.

35. The scOPV of claim 33, wherein the slow form comprises a nucleotide sequence that is at least 90% identical to the sequence of SEQ ID NO: 11 and the fast form comprises a nucleotide sequence that is at least 90% identical to the nucleotide sequence of SEQ ID NO: 12.

36. The scOPV of claim 33, wherein the slow form comprises a nucleotide sequence that is at least 95% identical to the nucleotide sequence of SEQ ID NO: 11 and the fast form comprises a nucleotide sequence that is at least 95% identical to the nucleotide sequence of SEQ ID NO: 12.

37. The scOPV of claim 33, wherein the slow form consists of the nucleotide sequence of SEQ ID NO: 11 and the fast form consists of the nucleotide sequence of SEQ ID NO: 12.

38. The scOPV of any one of claims 15 to 37, wherein the virus is replicated and reactivated from overlapping chemically-synthesized DNA fragments that correspond to substantially all of the viral genome of the OPV.

39. The scOPV of claim 38, wherein the virus is replicated and reactivated from 1-14 overlapping fragments.

40. The scOPV of claim 38, wherein the virus is replicated and reactivated from 8-12 overlapping fragments.

41. The scOPV of claim 38, wherein the virus is replicated and reactivated from 10 overlapping fragments.

42. The scOPV of any one of claims 15 to 41, wherein the virus is reactivated using leporipox virus-catalyzed recombination and reactivation.

43. The scOPV of claim 42, wherein the leporipox virus is selected from the group consisting of: Shope fibroma virus (SFV), hare fibroma virus, rabbit fibroma virus, squirrel fibroma virus, and myxoma virus.

44. A synthetic chimeric horsepox virus (scHPXV) that is replicated and reactivated from synthetic DNA, the viral genome differing from a wild type

genome of HPXV in that it is characterized by one or more modifications, the modifications being derived from a group comprising chemically-synthesized DNA, cDNA or genomic DNA.

45. The scHPXV of claim 44, wherein the synthetic DNA is selected from one or more of: chemically synthesized DNA, PCR amplified DNA, engineered DNA and polynucleotides comprising nucleoside analogs.

46. The scHPXV of claim 44, wherein the synthetic DNA is chemically synthesized DNA.

47. The scHPXV of any one of claims 44 to 46, wherein the viral genome is based on the genome of HPXV strain MNR-76 and differs from the MNR-76 genome in that it is characterized by one or more modifications.

48. The scHPXV of any one of claims 44 to 47, wherein the one or more modifications comprise one or more deletions, insertions, substitutions, or a combination thereof.

49. The scHPXV of any one of claims 44 to 48, wherein the one or more modifications comprise one or more modifications to introduce one or more unique restriction sites.

50. The scHPXV of claim 49, wherein the one or more modifications are present in *HPXV044* or *HPXV095*.

51. The scHPXV of any one of claims 44 to 50, wherein the one or more modifications comprise one or more mutations listed in Table 3.

52. The scHPXV of any one of claims 44 to 51, wherein the one or more modifications comprise one or more modifications to eliminate one or more restriction sites.

53. The scHPXV of claim 52, wherein the one or more modifications comprise one or more modifications to eliminate one or more *AarI* restriction sites.

54. The scHPXV of claim 53, wherein the one or more modifications comprise one or more modifications to eliminate all *AarI* restriction sites.

55. The scHPXV of any one of claims 44 to 54, wherein the one or more modifications comprise one or more modifications to eliminate one or more *BsaI* restriction sites.

56. The scHPXV of any one of claims 44 to 55, wherein the one or more modifications comprise one or more mutations listed in Table 2.

57. The scHPXV of any one of claims 44 to 56, wherein the viral genome comprises heterologous terminal hairpin loops.

58. The scHPXV of claim 57, wherein the viral genome comprises terminal hairpin loops derived from vaccinia virus.

59. The scHPXV of claim 58, wherein the left and right terminal hairpin loops a) comprise the slow form and the fast form of the vaccinia virus terminal hairpin loop, respectively, b) comprise the fast form and the slow form of the vaccinia virus terminal hairpin loop, respectively, c) both comprise the slow form of the vaccinia virus terminal hairpin loop, or d) both comprise the fast form of the vaccinia virus terminal loop.

60. The scHPXV of claim 59, wherein the slow form comprises a nucleotide sequence that is at least 85% identical to the sequence of SEQ ID NO: 11 and the fast form comprises a nucleotide sequence that is at least 85% identical to the nucleotide sequence of SEQ ID NO: 12.

61. The scHPXV of claim 59, wherein the slow form comprises a nucleotide sequence that is at least 90% identical to the sequence of SEQ ID NO: 11 and the fast form comprises a nucleotide sequence that is at least 90% identical to the nucleotide sequence of SEQ ID NO: 12.

62. The scHPXV of claim 59, wherein the slow form comprises a nucleotide sequence that is at least 95% identical to the sequence of SEQ ID NO: 11 and the

fast form comprises a nucleotide sequence that is at least 95% identical to the nucleotide sequence of SEQ ID NO: 12.

63. The scHPXV of claim 59, wherein the slow form consists of the nucleotide sequence of SEQ ID NO: 11 and the fast form consists of the nucleotide sequence of SEQ ID NO: 12.

64. The scHPXV of claim 57, wherein the viral genome comprises terminal hairpin loops derived from camelpox virus, cowpox virus, ectromelia virus, monkeypox virus, variola virus, rabbitpox virus, raccoon poxvirus, skunkpox virus, Taterapox virus, Uasin Gishu disease virus, or volepox virus.

65. The scHPXV of any one of claims 44 to 64, wherein the virus is replicated and assembled from overlapping chemically-synthesized DNA fragments that correspond to substantially all of the viral genome of HPXV.

66. The scHPXV of claim 65, wherein the virus is replicated and reactivated from 1-14 overlapping fragments.

67. The scHPXV of claim 65, wherein the virus is replicated and reactivated from 8-12 overlapping fragments.

68. The scHPXV of claim 65, wherein the virus is replicated and reactivated from 10 overlapping fragments.

69. The scHPXV of any one of claims 44 to 68, wherein the virus is reactivated using leporipox virus-catalyzed recombination and reactivation.

70. The scHPXV of claim 69, wherein the leporipox virus is selected from the group consisting of: Shope fibroma virus (SFV), hare fibroma virus, rabbit fibroma virus, squirrel fibroma virus, and myxoma virus.

71. A method of producing a synthetic chimeric poxvirus (scPV) comprising the steps of:
(i) chemically synthesizing overlapping DNA fragments that correspond to substantially all of the viral genome of the poxvirus;

(ii) transfecting the overlapping DNA fragments into helper virus-infected cells;
(iii) culturing said cells to produce a mixture of helper virus and synthetic chimeric poxviral particles in said cells; and
(iv) plating the mixture on host cells specific to the scPV to recover the scPV.

72. The method of claim 71, wherein the helper virus is a leporipox virus.

73. The method of claim 72, wherein the leporipox virus is selected from the group consisting of: Shope fibroma virus (SFV), hare fibroma virus, rabbit fibroma virus, squirrel fibroma virus, and myxoma virus.

74. The method of claim 73, wherein the leporipox virus is SFV.

75. The method of claim 71, wherein the helper virus is fowlpox virus.

76. The method of claim 71, wherein the helper virus is a psoralen-inactivated helper virus.

77. The method of any one of claims 71 to 74, wherein the helper virus-infected cells are BGMK cells.

78. The method of any one of claims 71 to 74, wherein step (i) further comprises chemically synthesizing terminal hairpin loops from a poxvirus and ligating them onto the fragments comprising the left and right termini of the viral genome.

79. A method of producing a synthetic chimeric orthopox virus (scOPV) comprising the steps of:
(i) chemically synthesizing overlapping DNA fragments that correspond to substantially all of the viral genome of the OPV;
(ii) transfecting the overlapping DNA fragments into helper virus-infected cells;
(iii) culturing said cells to produce a mixture of helper virus and scOPV particles in said cells; and
(iv) plating the mixture on OPV-specific host cells to recover the scOPV.

80. The method of claim 79, wherein the helper virus is a leporipox virus.

81. The method of claim 80, wherein the leporipox virus is selected from the group consisting of: Shope fibroma virus (SFV), hare fibroma virus, rabbit fibroma virus, squirrel fibroma virus, and myxoma virus.

82. The method of claim 81, wherein the leporipox virus is SFV.

83. The method of claim 79, wherein the helper virus is fowlpox virus.

84. The method of claim 79, wherein the helper virus is a psoralen-inactivated helper virus.

85. The method of any one of claims 79 to 82, wherein the helper virus-infected cells are BGMK cells.

86. The method of any one of claims 79 to 85, wherein the OPV-specific host cells are BSC-40 cells.

87. The method of any one of claims 79 to 86, wherein the OPV is selected from the group consisting of: camelpox virus, cowpox virus, ectromelia virus, horsepox virus, monkeypox virus, vaccinia virus, variola virus, rabbitpox virus, raccoon poxvirus, skunkpox virus, Taterapox virus, Uasin Gishu disease virus, volepox virus.

88. The method of any one of claims 79 to 87, wherein step (i) further comprises chemically synthesizing terminal hairpin loops from an OPV and ligating them onto the fragments comprising the left and right termini of the viral genome.

89. A method of producing a synthetic chimeric horsepox virus (scHPXV) comprising the steps of:
(i) chemically synthesizing overlapping DNA fragments that correspond to substantially all of the HPXV genome;
(ii) transfecting the overlapping DNA fragments into helper virus-infected cells;
(iii) culturing said cells to produce a mixture of helper virus and scHPXV particles in said cells; and
(iv) plating the mixture on HPXV-specific host cells to recover the scHPXV.

90. The method of claim 89, wherein the helper virus is a leporipox virus.

91. The method of claim 90, wherein the leporipox virus is selected from the group consisting of: Shope fibroma virus (SFV), hare fibroma virus, rabbit fibroma virus, squirrel fibroma virus, and myxoma virus.

92. The method of claim 91, wherein the leporipox virus is SFV.

93. The method of claim 89, wherein the helper virus is fowlpox virus.

94. The method of claim 89, wherein the helper virus is a psoralen-inactivated helper virus.

95. The method of any one of claims 89 to 92, wherein the helper virus-infected cells are BGMK cells.

96. The method of any one of claims 89 to 95, wherein the HPXV-specific host cells are BSC-40 cells.

97. The method of any one of claims 89 to 96, wherein step (i) further comprises chemically synthesizing terminal hairpin loops from an OPV and ligating them onto the fragments comprising the left and right termini of the HPXV genome.

98. The method of any one of claims 89 to 97, wherein the overlapping DNA fragments comprise:
i) nucleotide sequences that are at least 85% identical to the sequences of SEQ ID NOs: 1–10;
ii) nucleotide sequences that are at least 90% identical to the sequences of SEQ ID NOs: 1–10;
(iii) nucleotide sequences that are at least 95% identical to the sequences of SEQ ID NOs: 1–10; or
(iv) nucleotide sequences that consist of the sequences of SEQ ID NOs: 1–10.

99. A method of producing a synthetic chimeric horsepox virus (scHPXV) comprising:

(i) chemically synthesizing overlapping DNA fragments that correspond to substantially all of the HPXV genome;

(ii) transfecting the overlapping DNA fragments into Shope fibroma virus (SFV)-infected cells;

(iii) culturing said cells to produce a mixture of SFV and scHPXV particles in said cells; and

(iv) plating the mixture on HPXV-specific host cells to recover the scHPXV.

100. The method of claim 99, wherein the SFV-infected cells are BGMK cells.

101. The method of claim 99 or 100, wherein the HPXV-specific host cells are BSC-40 cells.

102. A synthetic chimeric poxvirus (scPV) generated by the method of any one of claims 71 to 78.

103. A synthetic chimeric orthopox virus (scOPV) generated by the method of any one of claims 79 to 88.

104. A synthetic chimeric horsepox virus (scHPXV) generated by the method of any one of claims 89 to 101.

105. A composition comprising a pharmaceutically acceptable carrier and the scPV of any one of claims 1 to 14 and 102.

106. A composition comprising a pharmaceutically acceptable carrier and the scOPV of any one of claims 15 to 43 and 103.

107. A method of triggering or boosting an immune response against variola virus, comprising administering to a subject in need thereof a composition comprising the scOPV of any one of claims 15 to 43 and 103.

108. A method of triggering or boosting an immune response against vaccinia virus, comprising administering to a subject in need thereof a composition comprising the scOPV of any one of claims 15 to 43 and 103.

109. A method of triggering or boosting an immune response against monkeypox virus, comprising administering to a subject in need thereof a composition comprising the scOPV of any one of claims 15 to 43 and 103.

110. A method of immunizing a human subject to protect said subject from variola virus infection, comprising administering to said subject a composition comprising the scOPV of any one of claims 15 to 43 and 103.

111. A method of treating a variola virus infection, comprising administering to a subject in need thereof a composition comprising the scOPV of any one of claims 15 to 43 and 103.

112. A composition comprising a pharmaceutically acceptable carrier and the scHPXV of any one of claims 44 to 70 and 104.

113. A method of triggering or boosting an immune response against variola virus, comprising administering to a subject in need thereof a composition comprising the scHPXV of any one of claims 44 to 70 and 104.

114. A method of triggering or boosting an immune response against vaccinia virus, comprising administering to a subject in need thereof a composition comprising the scHPXV of any one of claims 44 to 70 and 104.

115. A method of triggering or boosting an immune response against monkeypox virus, comprising administering to a subject in need thereof a composition comprising the scHPXV of any one of claims 44 to 70 and 104.

116. A method of immunizing a human subject to protect said subject from variola virus infection, comprising administering to said subject a composition comprising the scHPXV of any one of claims 44 to 70 and 104.

117. A method of treating a variola virus infection, comprising administering to a subject in need thereof a composition comprising the scHPXV of any one of claims 44 to 70 and 104.

118. A kit comprising a composition comprising the scPV of any one of claims 1 to 13 and 102.

119. A kit comprising a composition comprising the OPV of any one of claims 15 to 43 and 103 and instructions for the use thereof.

120. A kit comprising a composition comprising the scHPXV of any one of claims 44 to 70 and 104 and instructions for the use thereof.

121. The method of any one of claims 107-111 or 113-117, wherein the composition is administered in a poxvirus treatment facility.

122. The method of any one of claims 107-111, 113-117 or 121, wherein the composition is administered by a specialist in smallpox adverse events.

123. The method of claim 122, wherein the smallpox adverse events are selected from: eczema vaccinatum, progressive vaccinia, postvaccinal encephalitis, myocarditis, and dilated cardiomyopathy.

124. A poxvirus treatment facility wherein subjects in need of immunization or treatment with a composition comprising the virus of any one of claims 1-70 and 102-106 or the composition of any one of claims 105, 106, or 112 may be immunized or treated in an environment such that they are sequestered from other subjects not intended to be immunized or treated.

125. The poxvirus treatment facility of claim 124 being characterized by specialists in smallpox adverse events to administer the immunization or treatment.

126. The poxvirus treatment facility of claim 124 or 125, wherein the smallpox adverse events are selected from: eczema vaccinatum, progressive vaccinia, postvaccinal encephalitis, myocarditis, and dilated cardiomyopathy.

127. The poxvirus treatment facility of any one of claims 124-126, wherein the subjects in need of immunization or treatment are immunized or treated according to the methods of any one of claims 107-111 or 113-117.

Figure 1A

Unmodified HPXV genome (strain MNR-76; GI DQ792504)

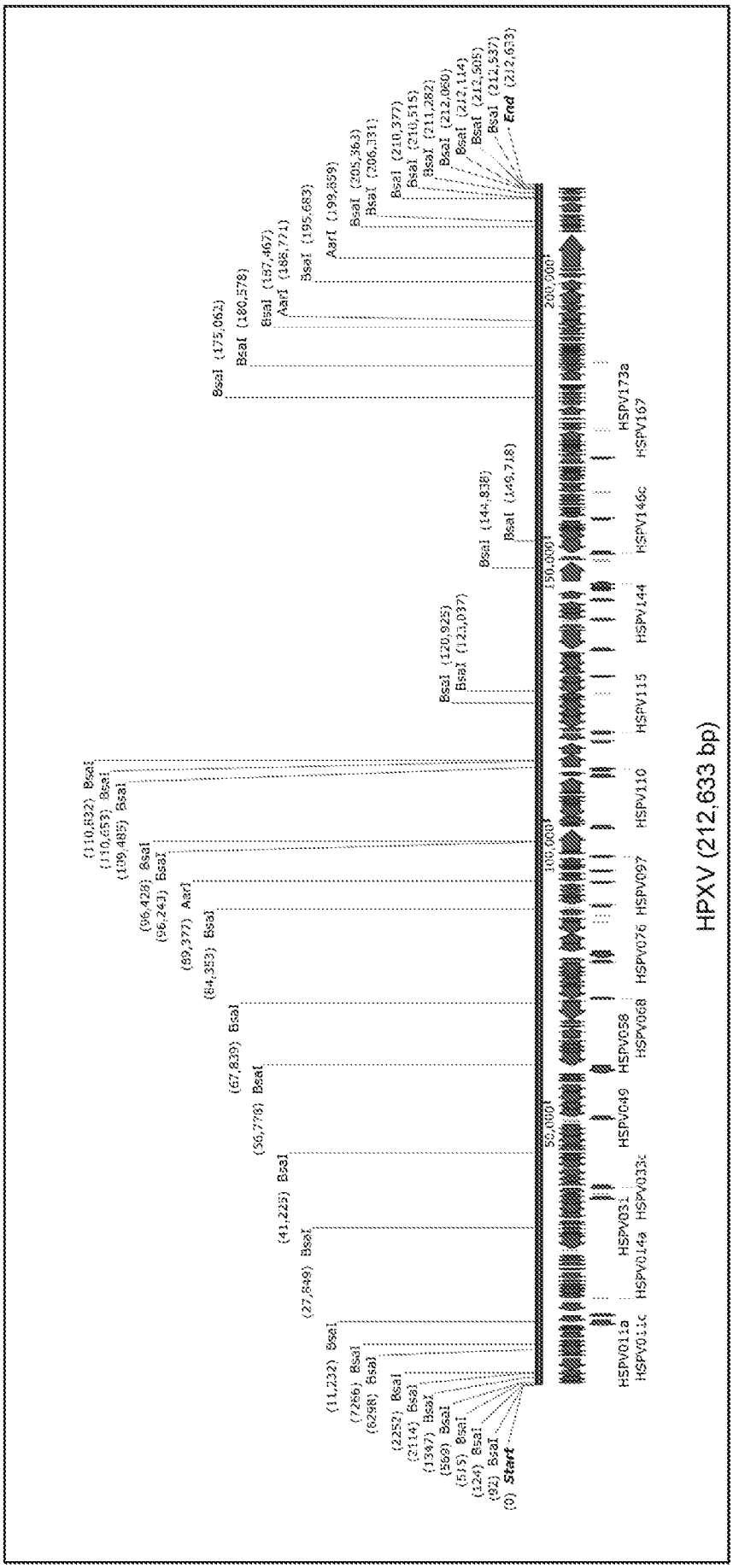


Figure 1B

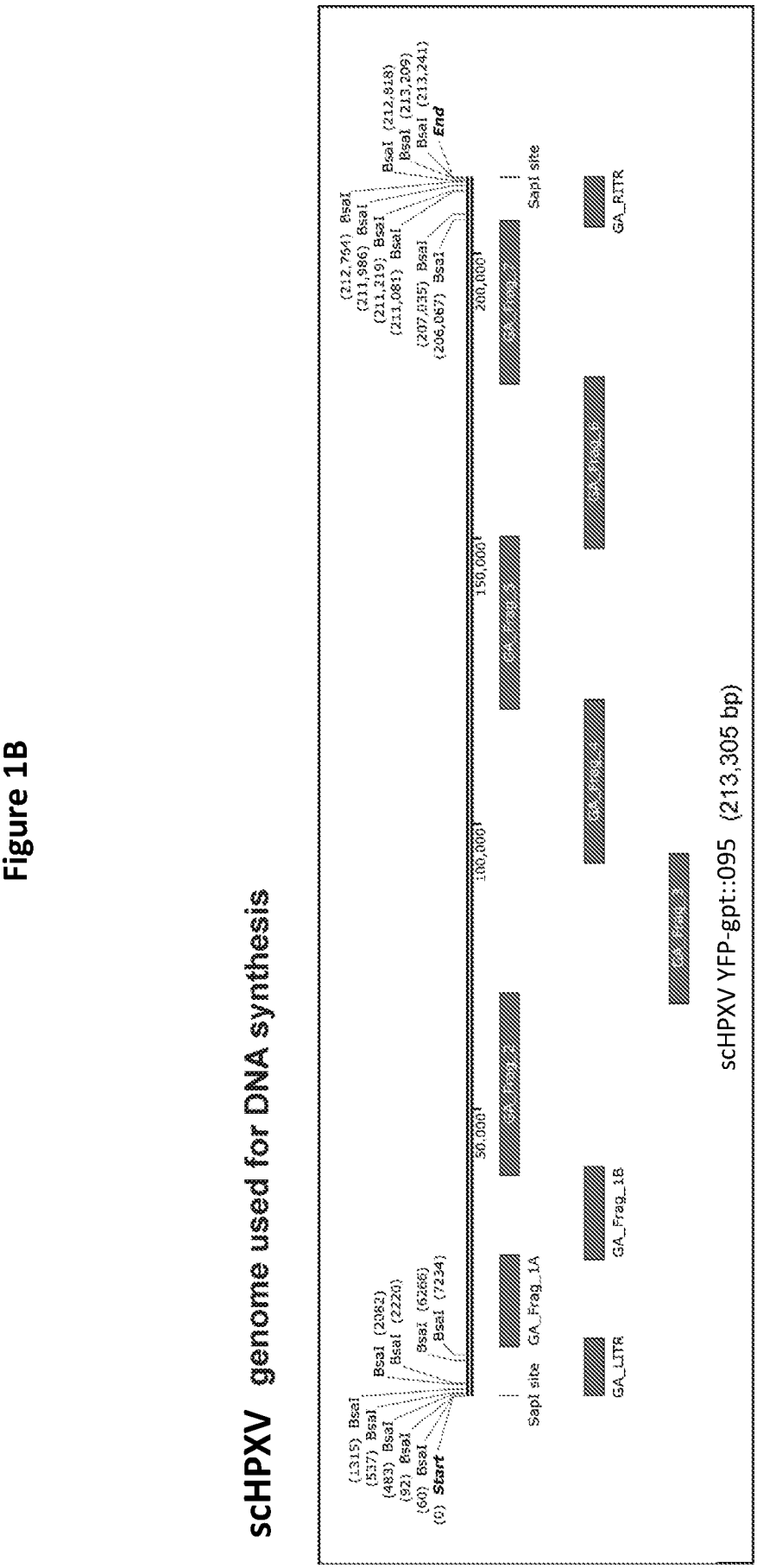

scHPXV genome used for DNA synthesis
(1315) BsaI
(537) BsaI
(483) BsaI
(92) BsaI
(60) BsaI
(6) Start
BsaI (2082)
BsaI (2220)
BsaI (6266)
BsaI (7234)
SapI site
GA_Frag_1A
GA_LTR
GA_Frag_1B
50,000
100,000
150,000
200,000
SapI site
GA_RITR
(212,754) BsaI
(211,986) BsaI
(211,219) BsaI
(211,081) BsaI
(207,835) BsaI
(206,067) BsaI
BsaI (212,818)
BsaI (213,209)
BsaI (213,241)
End
scHPXV YFP-gpt::095 (213,305 bp)

Figure 2

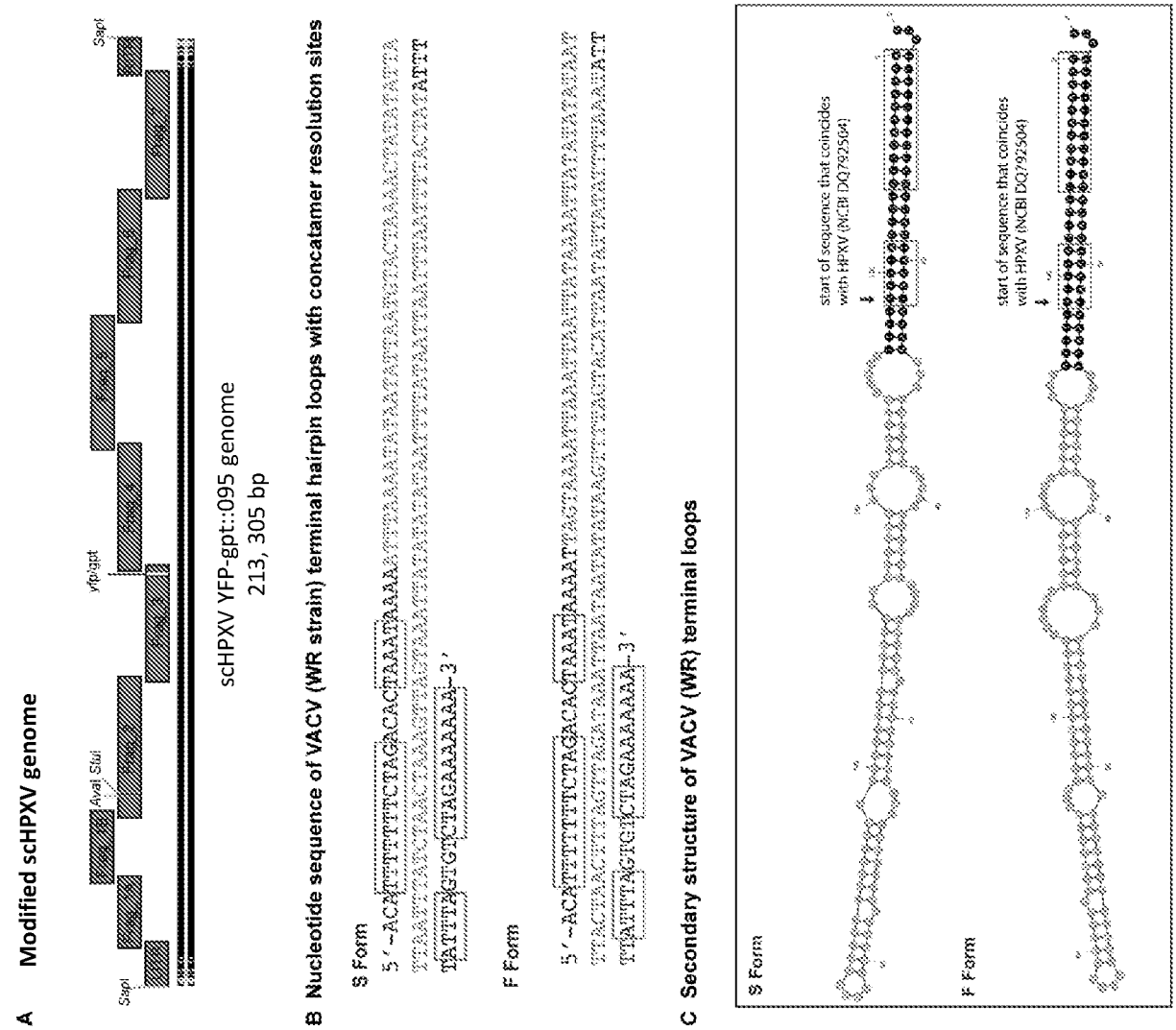

A Modified scHPXV genome
SapI
AvaI
StuI
yfp/gpt
SapI
scHPXV YFP-gpt::095 genome
213, 305 bp
B Nucleotide sequence of VACV (WR strain) terminal hairpin loops with concatamer resolution sites
S Form
5'-ACATTTTTTTCTAGACACTAAATAAAATTTAAAAATATAATTAATTAAATTACTAAAACTATAGTTA
TTAATTTATCTAACTTAAAGTTAATTAATATAATTTATAATTTAATTTAATTTTACTTTATT
TATTTAGTGTCTAGAAAAAAA-3'
F Form
5'-ACATTTTTTTCTAGACACTAAATAAAATTAGTAAATTAAATTTAATTATAAATTATAAATATAAT
TTACTAACTTTAGTTAGTATAAATTAATAATAAAGTTTTACTACATTAATTATATTTAAAATT
TTATTTAGTGTCTAGAAAAAAA-3'
C Secondary structure of VACV (WR) terminal loops
S Form
start of sequence that coincides
with HPXV (NCBI DQ792564)
F Form
start of sequence that coincides
with HPXV (NCBI DQ792504)

Figure 3A

Schematic diagram of scHPXV YFP-gpt::095 left and right ITR digested with *PvuII*

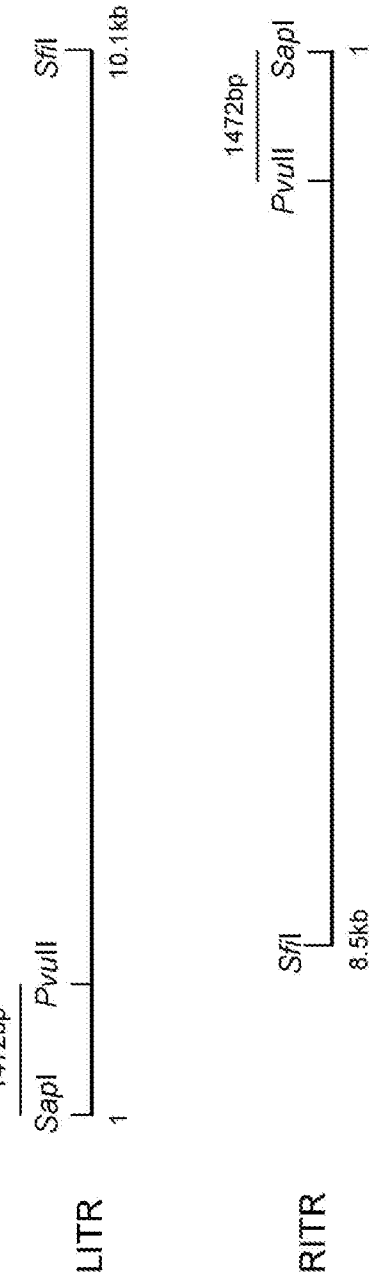


Figure 3B

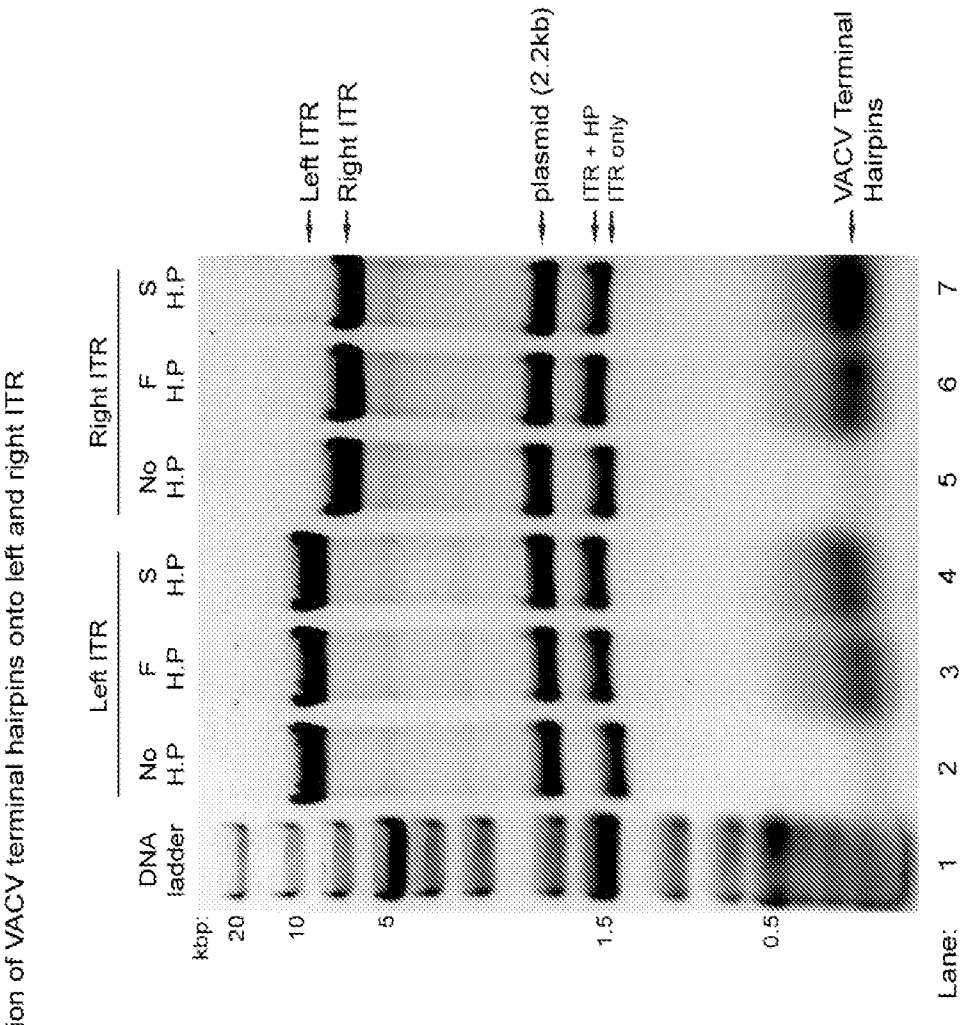

Ligation of VACV terminal hairpins onto left and right ITR
Left ITR
Right ITR
DNA ladder
No H.P
F H.P
S H.P
No H.P
F H.P
S H.P
kbp:
20
10
5
1.5
0.5
Left ITR
Right ITR
plasmid (2.2kb)
ITR + HP
ITR only
VACV Terminal Hairpins
Lane:
1
2
3
4
5
6
7

Figure 4A

PCR analysis of *BsaI* sites in VACV and scHPXV YFP-gpt::095 (plaque pick 1 and 3)

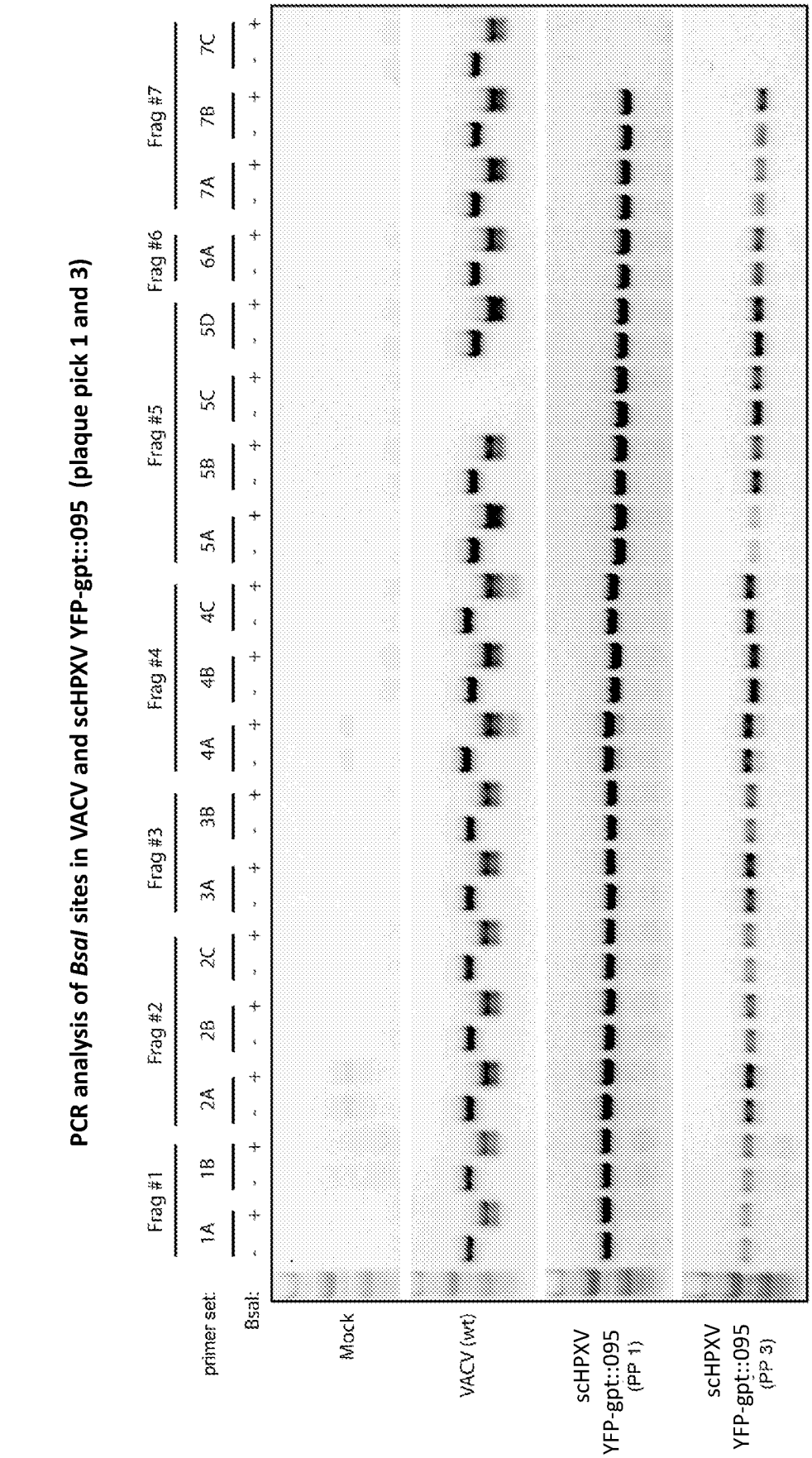


Figure 4B

PFGE of scHPXV YFP-gpt::095 following *BsaI* and *HindIII* digest

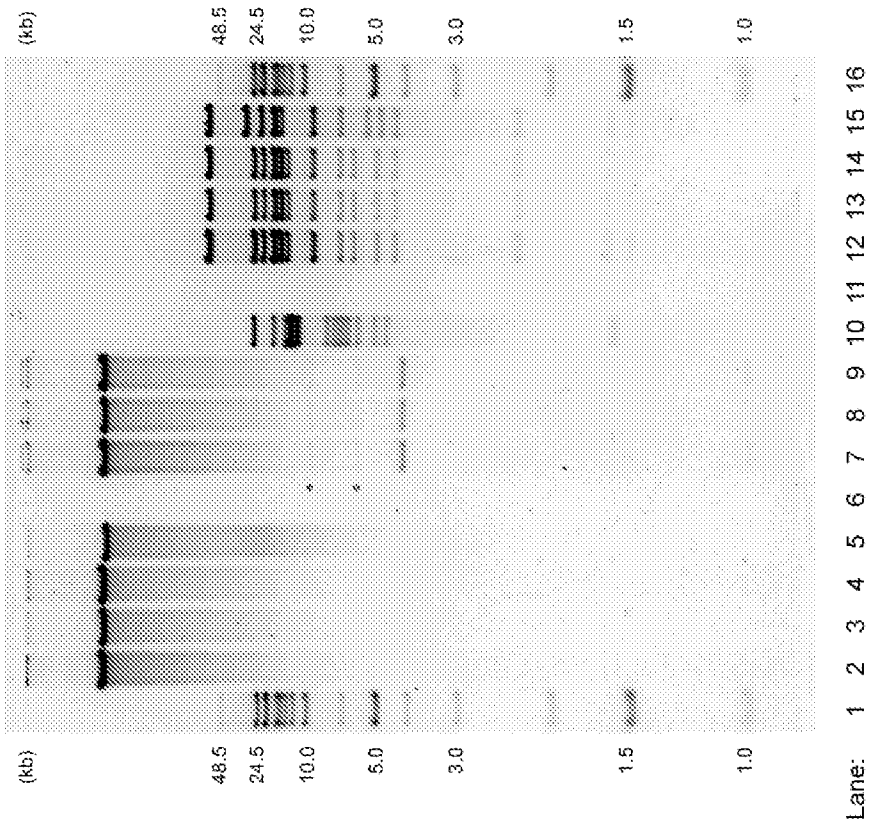


Figure 4C

Agarose electrophoresis of scHPXV YFP-gpt::095 following *BsaI* and *HindIII* digest

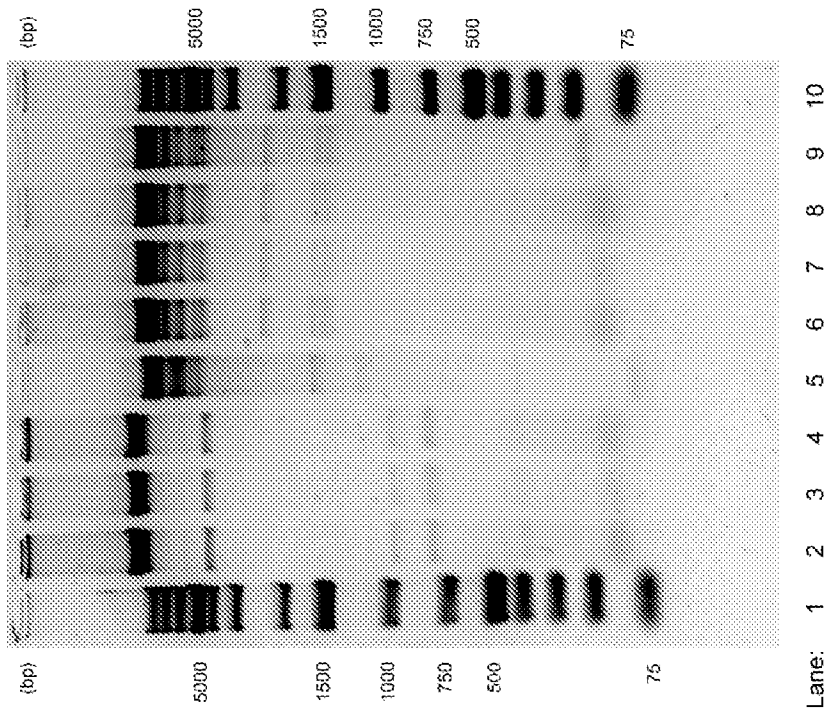


Figure 5A


scHPXV
YFP-gpt::095
(Cs on end)
Consensus
Coverage
SapI
Terminal Loop
Concatamer Resolution site
VACV WR terminal hairpin loop

Figure 5B

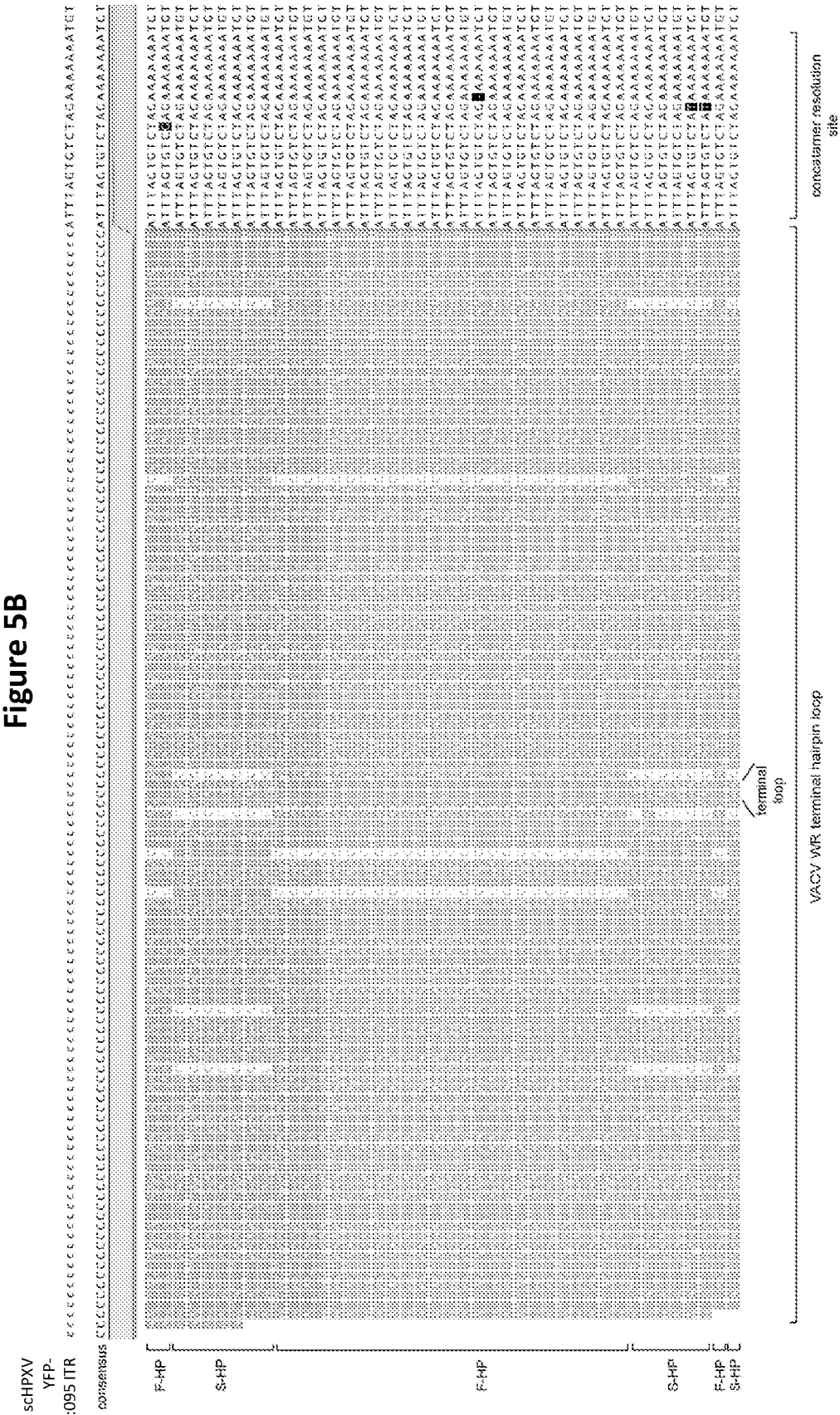

scHPXV YFP-gpt::095 ITR
consensus
F-HP
S-HP
F-HP
S-HP
F-HP
S-HP
terminal loop
VACV WR terminal hairpin loop
concatamer resolution site

Figure 6A

Overall Illumina read coverage in scHPXV YFP-gpt::095 region 96,050 to 96,500

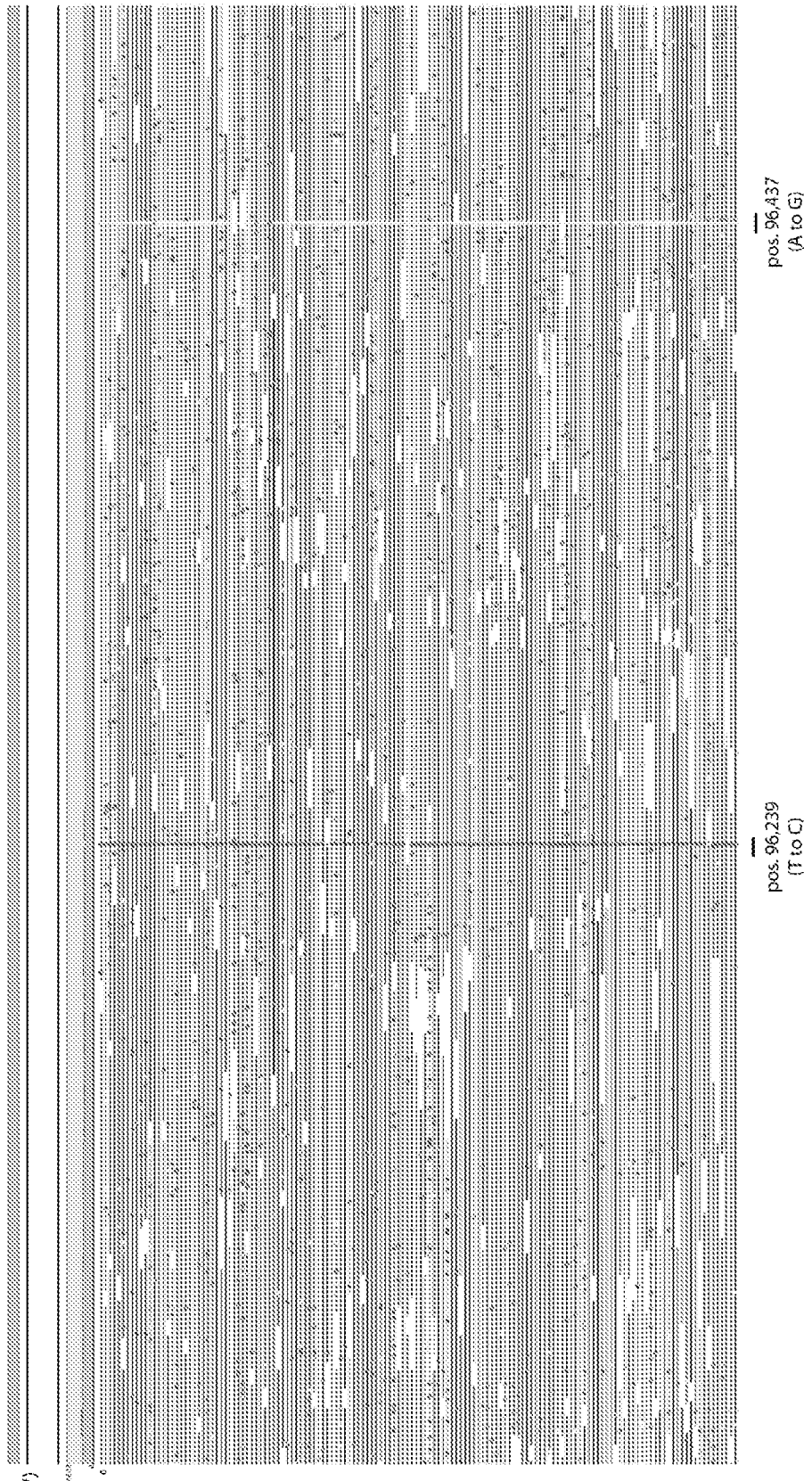


Figure 6B

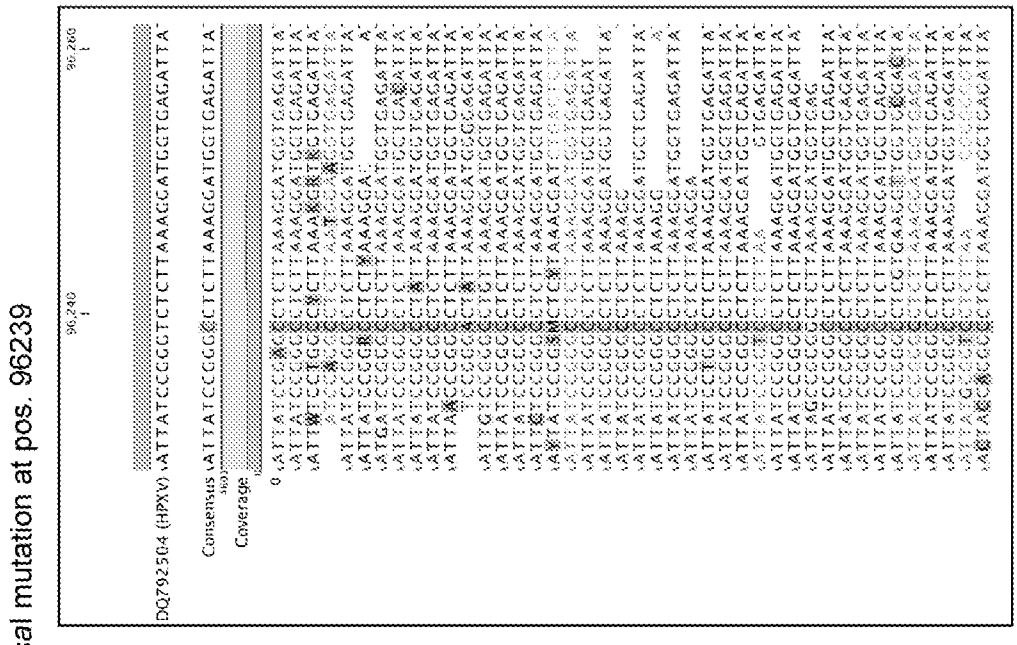

BsaI mutation at pos. 96239
96,240
1
96,260
DQ792504 (HPXV)
Consensus
Coverage

Figure 6C

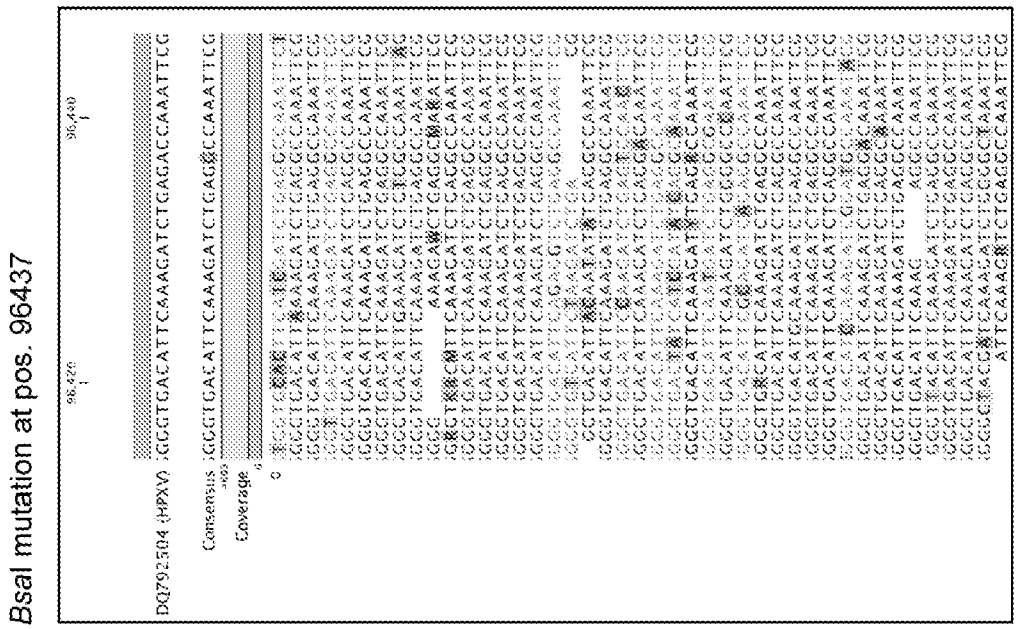

BsaI mutation at pos. 96437
96,420
96,440
DQ792504 (HPXV)
Consensus
Coverage

Figure 7A

Nucleotide changes in HPXV044 to create unique restriction sites in scHPXV YFP-gpt::095

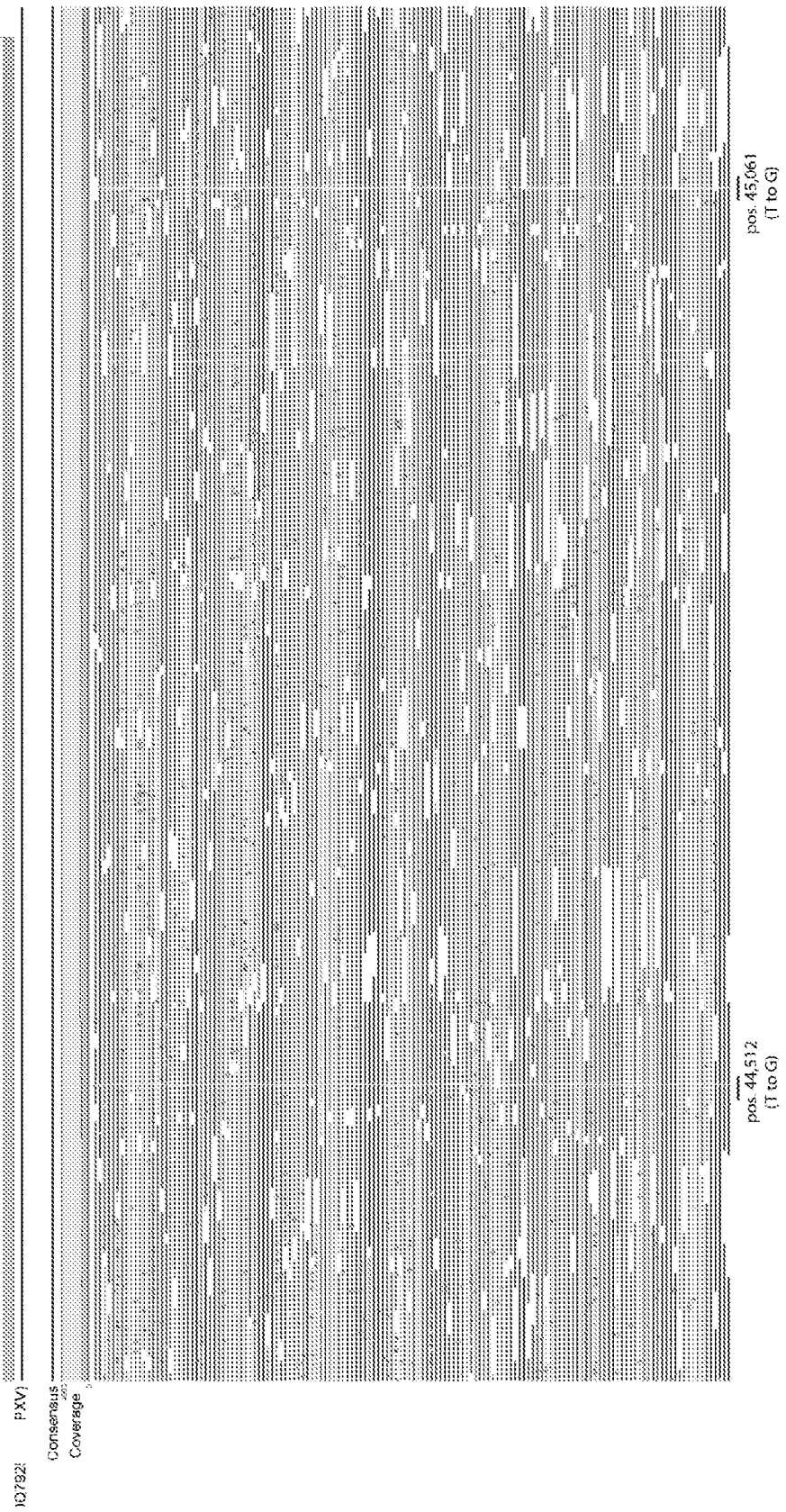


Figure 7B

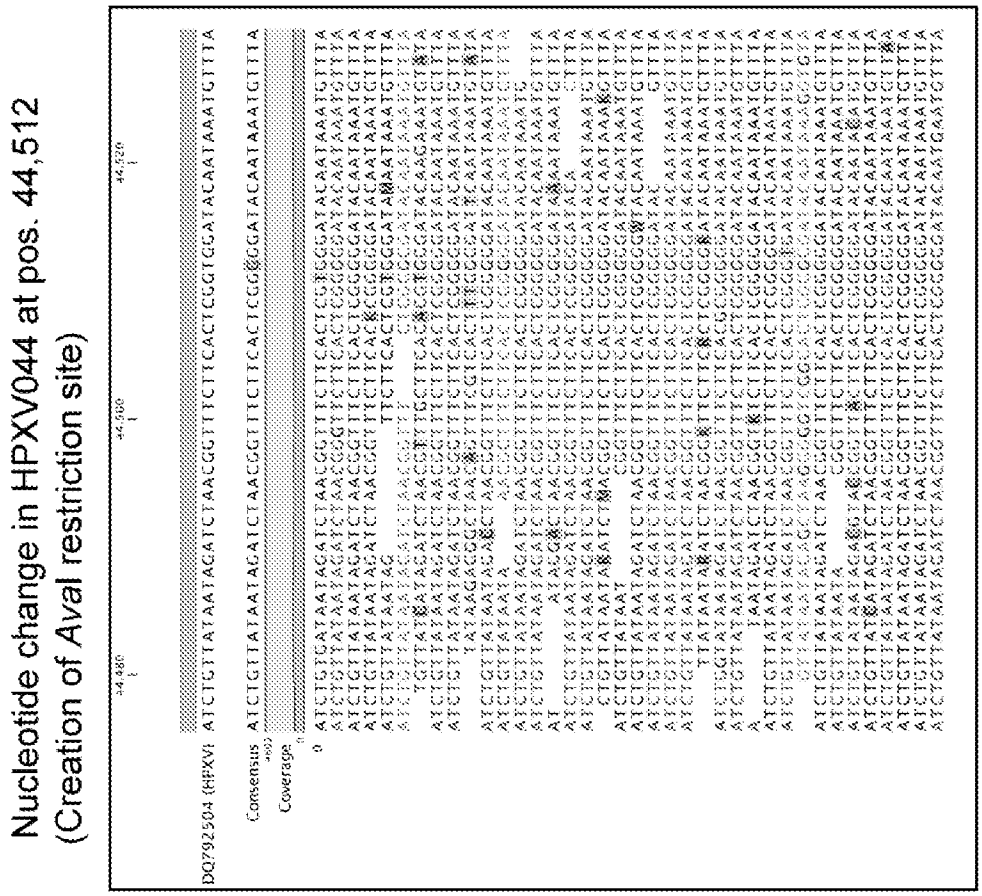

Nucleotide change in HPXV044 at pos. 44,512
(Creation of AvaI restriction site)
44,488
44,500
44,512
DQ792504 (HPXV1
Consensus
Coverage

Figure 7C

Nucleotide change in HPXV044 at pos. 45,061
(Creation of *StuI* restriction site)

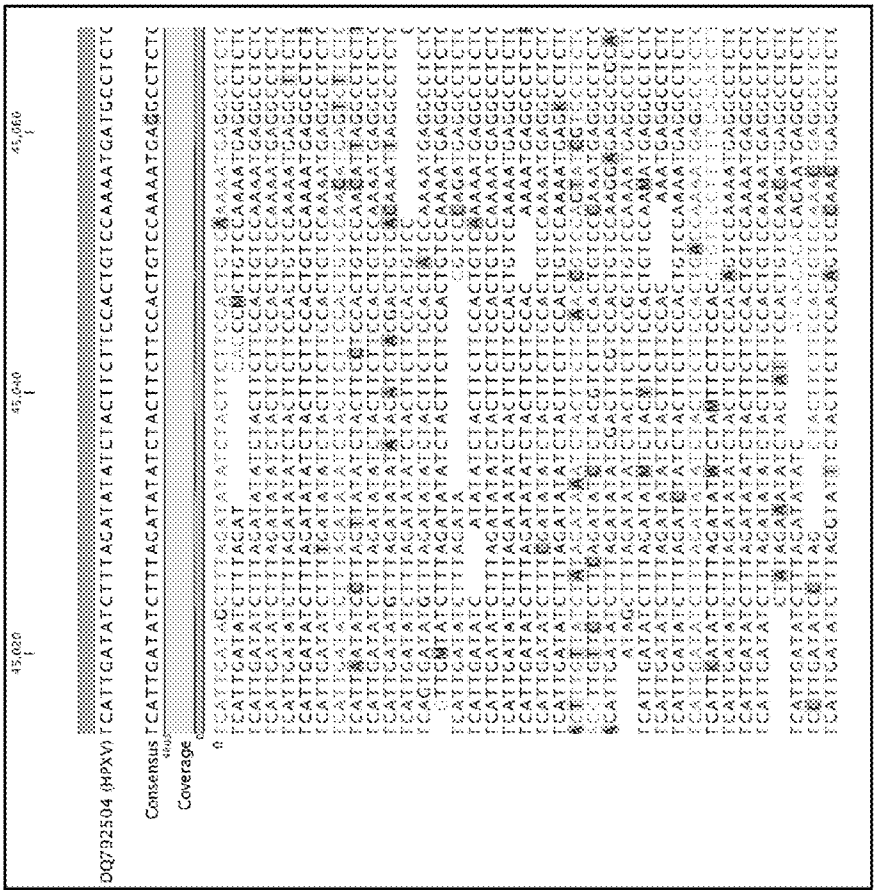


Figure 8A

Growth of scHPXV YFP-gpt::095 in cell lines

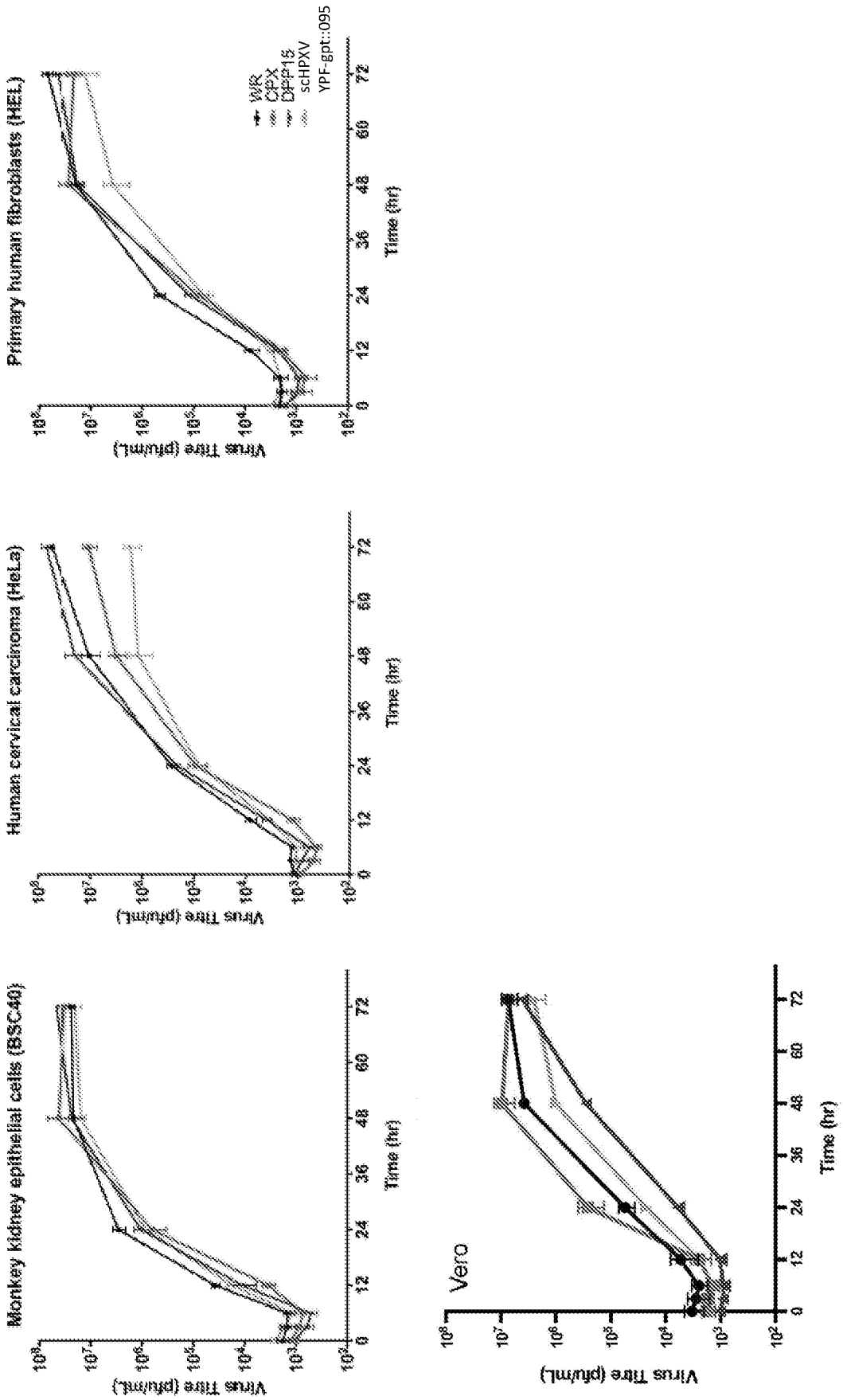


Figure 8B

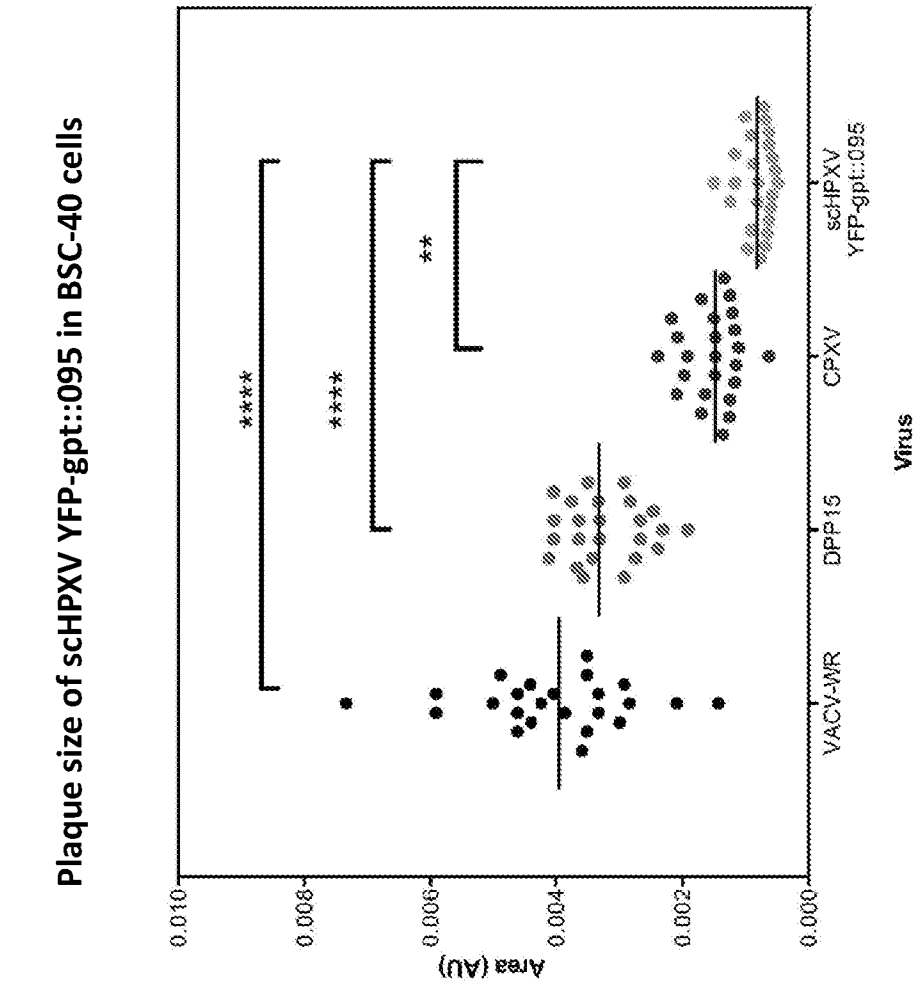

Plaque size of scHPXV YFP-gpt::095 in BSC-40 cells
Area (AU)
0.010
0.008
0.006
0.004
0.002
0.000
****
****
**
VACV-WR
DPP15
CPXV
scHPXV YFP-gpt::095
Virus

Figure 8C

Plaque morphology of scHPXV YFP-gpt::095 in BSC-40 cells

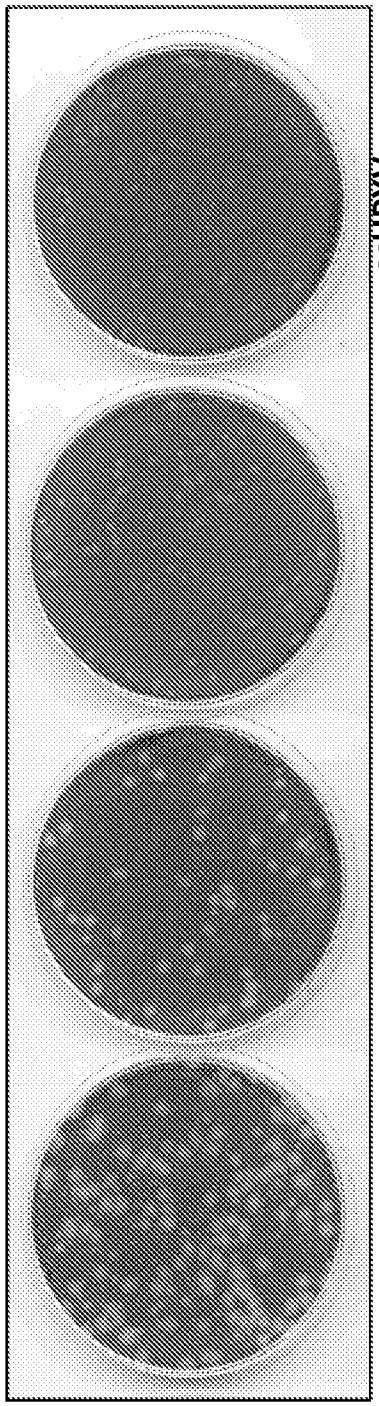


Figure 9

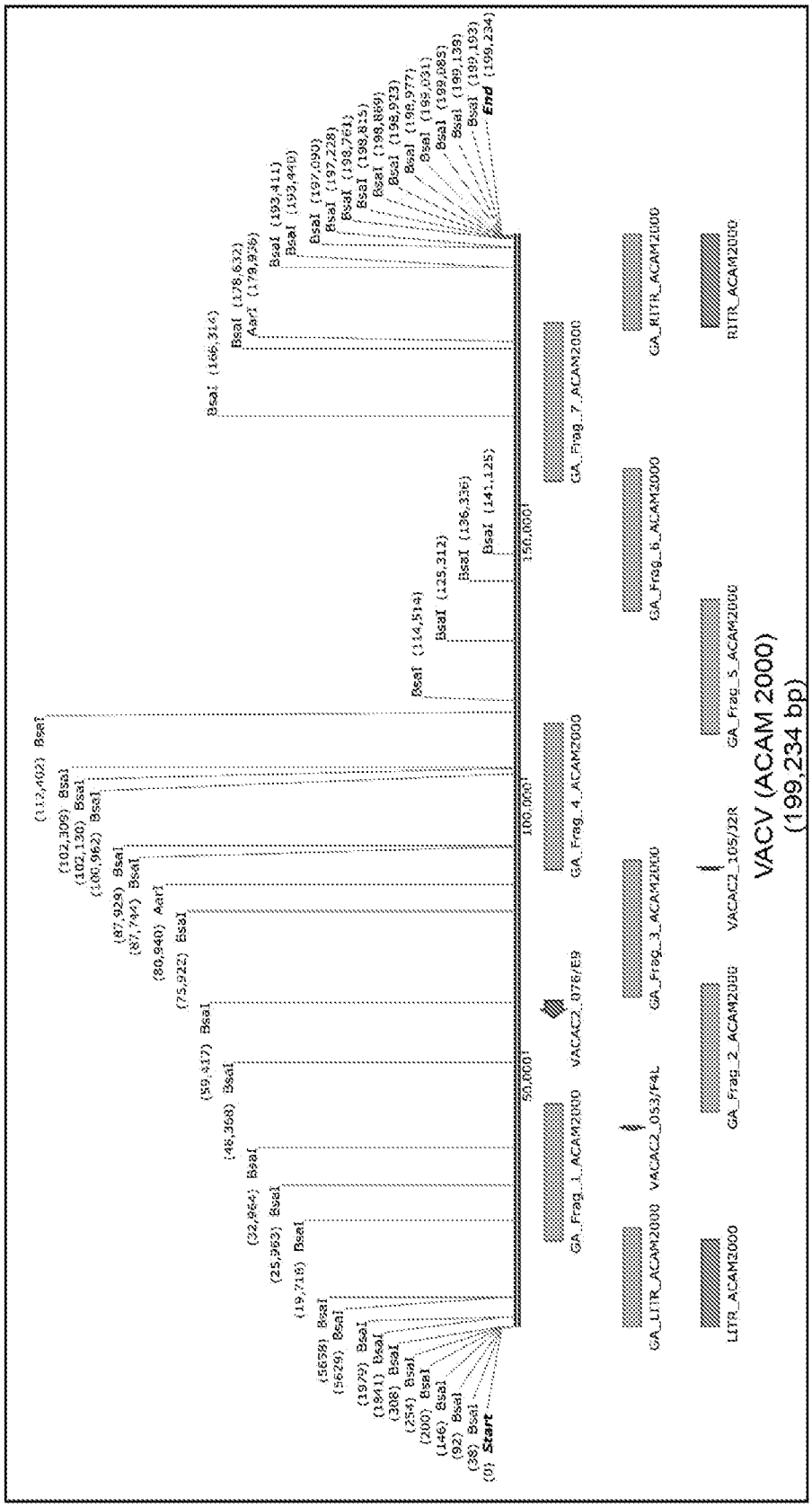

VACV (ACAM 2000)
(199,234 bp)
Start
End (199,234)
50,000
100,000
150,000
GA_Frag_1_ACAM2000
VACAC2_076/E8
GA_Frag_4_ACAM2000
GA_Frag_7_ACAM2000
GA_LITR_ACAM2000
VACAC2_053/F4L
GA_Frag_3_ACAM2000
GA_Frag_6_ACAM2000
GA_RITR_ACAM2000
LITR_ACAM2000
GA_Frag_2_ACAM2000
VACAC2_165/J2R
GA_Frag_5_ACAM2000
RITR_ACAM2000

Figure 10

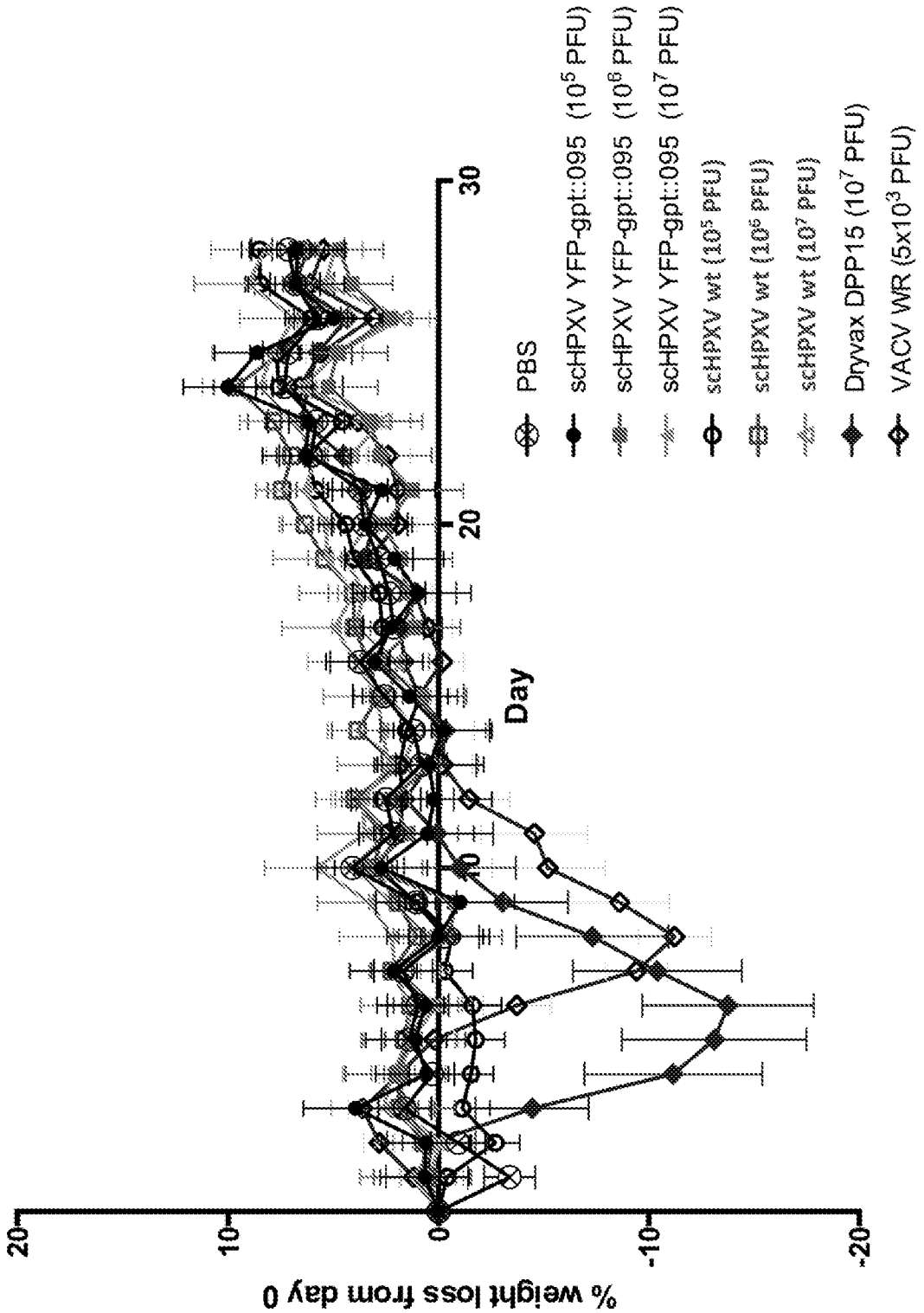

% weight loss from day 0
20
10
0
-10
-20
0
10
20
30
Day
PBS
scHPXV YFP-gpt::095 (10^5 PFU)
scHPXV YFP-gpt::095 (10^6 PFU)
scHPXV YFP-gpt::095 (10^7 PFU)
scHPXV wt (10^5 PFU)
scHPXV wt (10^6 PFU)
scHPXV wt (10^7 PFU)
Dryvax DPP15 (10^7 PFU)
VACV WR (5x10^3 PFU)

Figure 11A

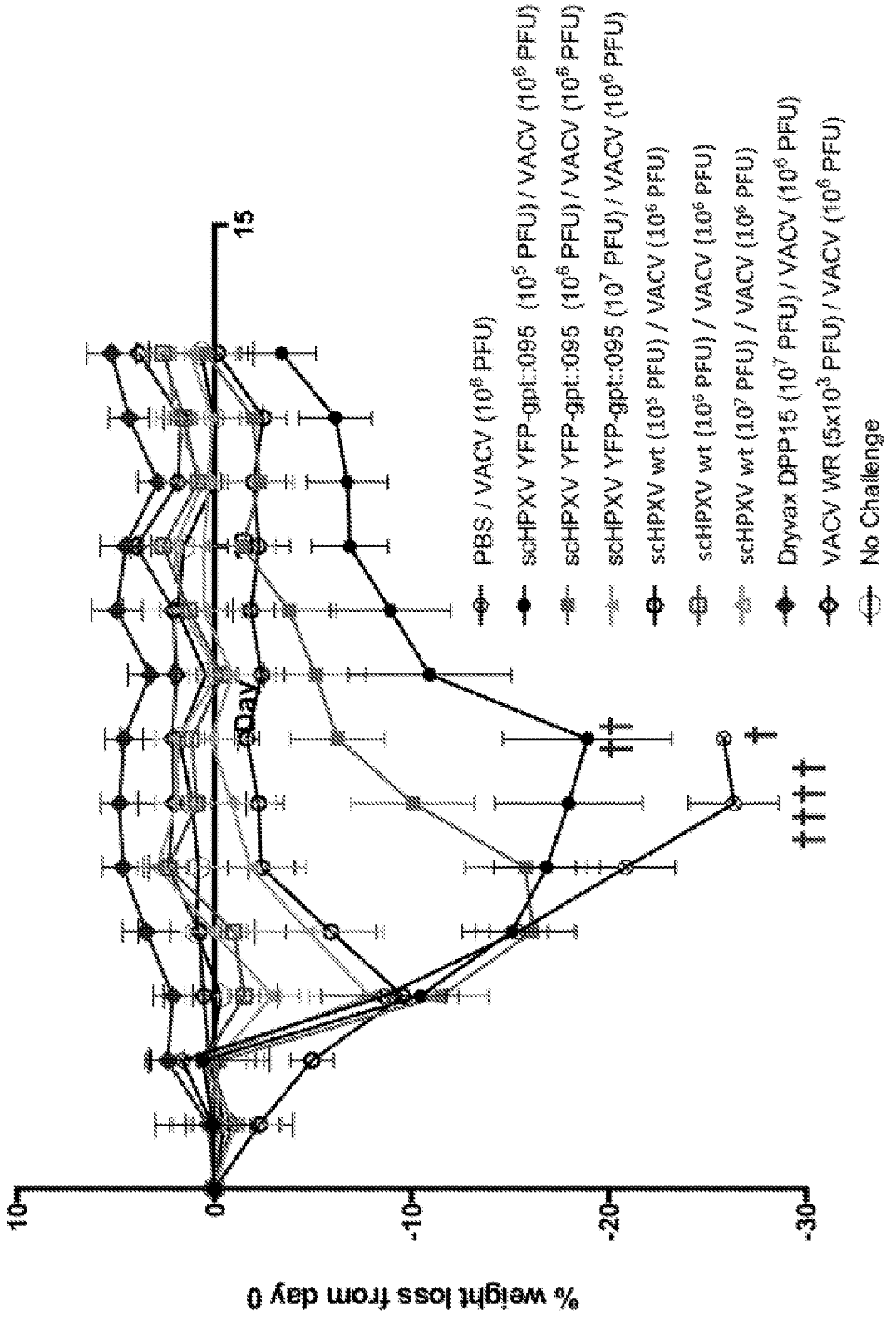

% weight loss from day 0
10
0
-10
-20
-30
0
5
10
15
Day
PBS / VACV (10^6 PFU)
scHPXV YFP-gpt::095 (10^5 PFU) / VACV (10^6 PFU)
scHPXV YFP-gpt::095 (10^6 PFU) / VACV (10^6 PFU)
scHPXV YFP-gpt::095 (10^7 PFU) / VACV (10^6 PFU)
scHPXV wt (10^5 PFU) / VACV (10^6 PFU)
scHPXV wt (10^6 PFU) / VACV (10^6 PFU)
scHPXV wt (10^7 PFU) / VACV (10^6 PFU)
Dryvax DPP15 (10^7 PFU) / VACV (10^6 PFU)
VACV WR (5x10^3 PFU) / VACV (10^6 PFU)
No Challenge
++
+
++++

Figure 11B

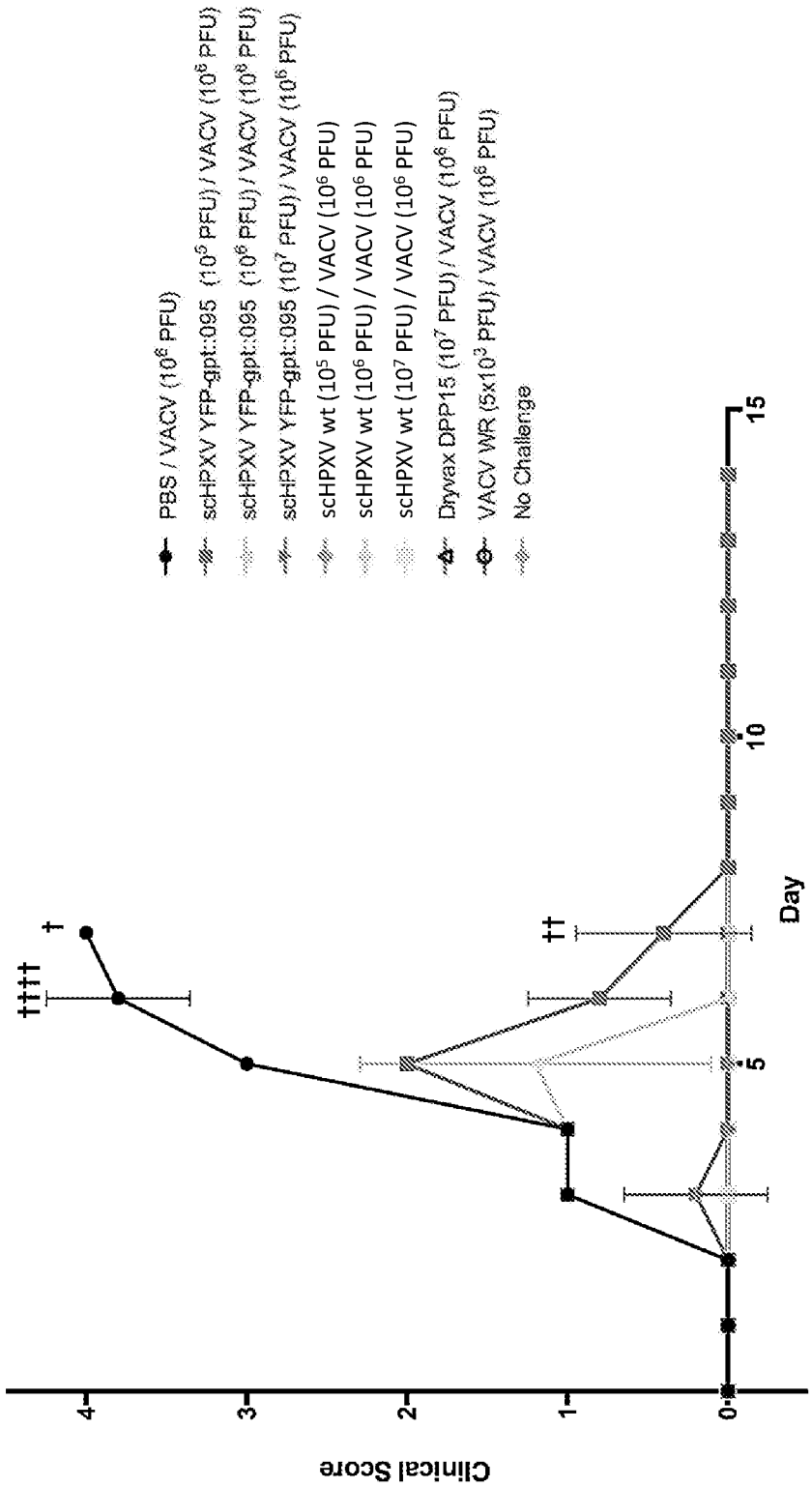

PBS / VACV (10^6 PFU)
scHPXV YFP-gpt::095 (10^5 PFU) / VACV (10^6 PFU)
scHPXV YFP-gpt::095 (10^6 PFU) / VACV (10^6 PFU)
scHPXV YFP-gpt::095 (10^7 PFU) / VACV (10^6 PFU)
scHPXV wt (10^5 PFU) / VACV (10^6 PFU)
scHPXV wt (10^6 PFU) / VACV (10^6 PFU)
scHPXV wt (10^7 PFU) / VACV (10^6 PFU)
Dryvax DPP15 (10^7 PFU) / VACV (10^6 PFU)
VACV WR (5x10^3 PFU) / VACV (10^6 PFU)
No Challenge
Clinical Score
Day
0
1
2
3
4
5
10
15
†
††
††††

Figure 12

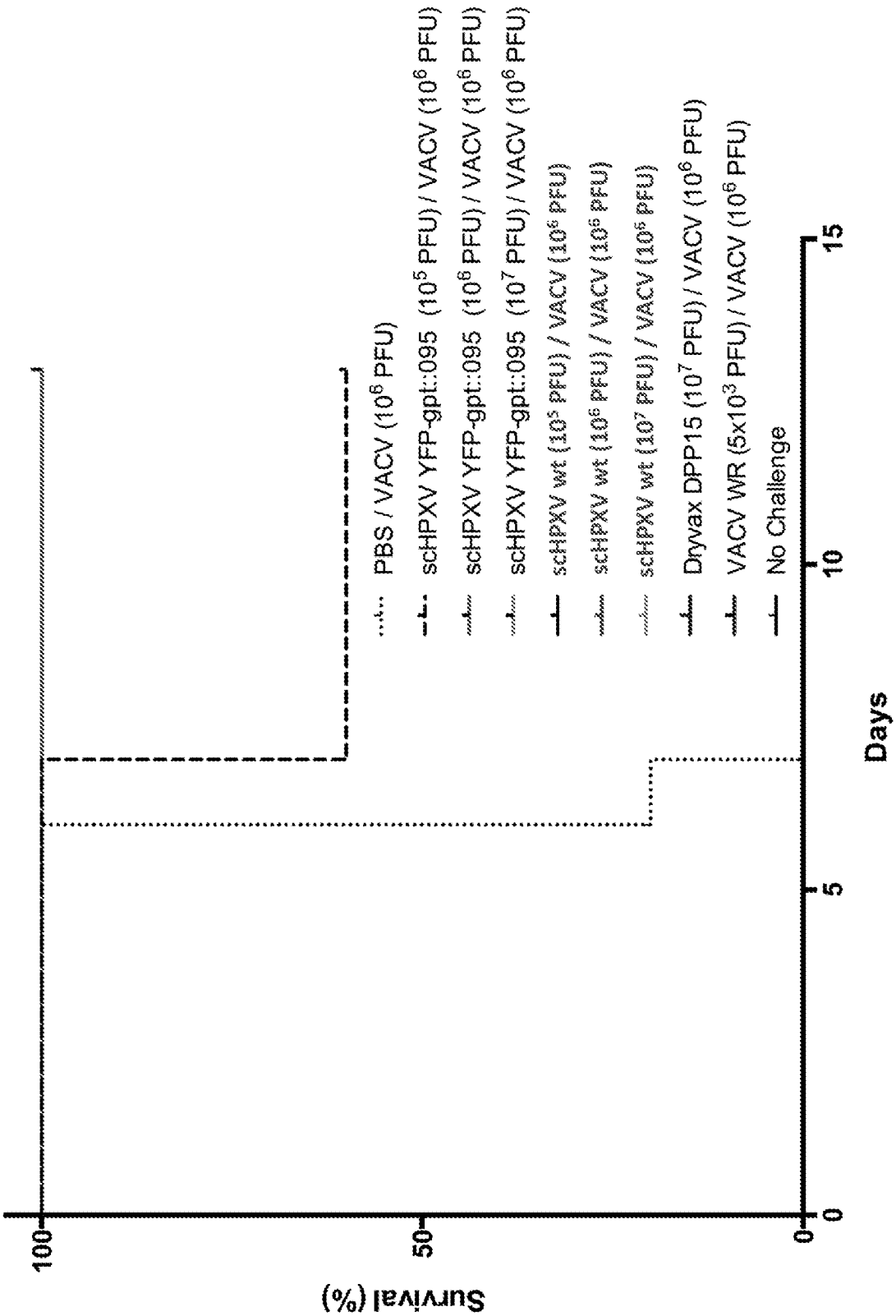

Survival (%)
100
50
0
0
5
10
15
Days
PBS / VACV (10^6 PFU)
scHPXV YFP-gpt::095 (10^5 PFU) / VACV (10^6 PFU)
scHPXV YFP-gpt::095 (10^6 PFU) / VACV (10^6 PFU)
scHPXV YFP-gpt::095 (10^7 PFU) / VACV (10^6 PFU)
scHPXV wt (10^5 PFU) / VACV (10^6 PFU)
scHPXV wt (10^6 PFU) / VACV (10^6 PFU)
scHPXV wt (10^7 PFU) / VACV (10^6 PFU)
Dryvax DPP15 (10^7 PFU) / VACV (10^6 PFU)
VACV WR (5x10^3 PFU) / VACV (10^6 PFU)
No Challenge

Figure 13A

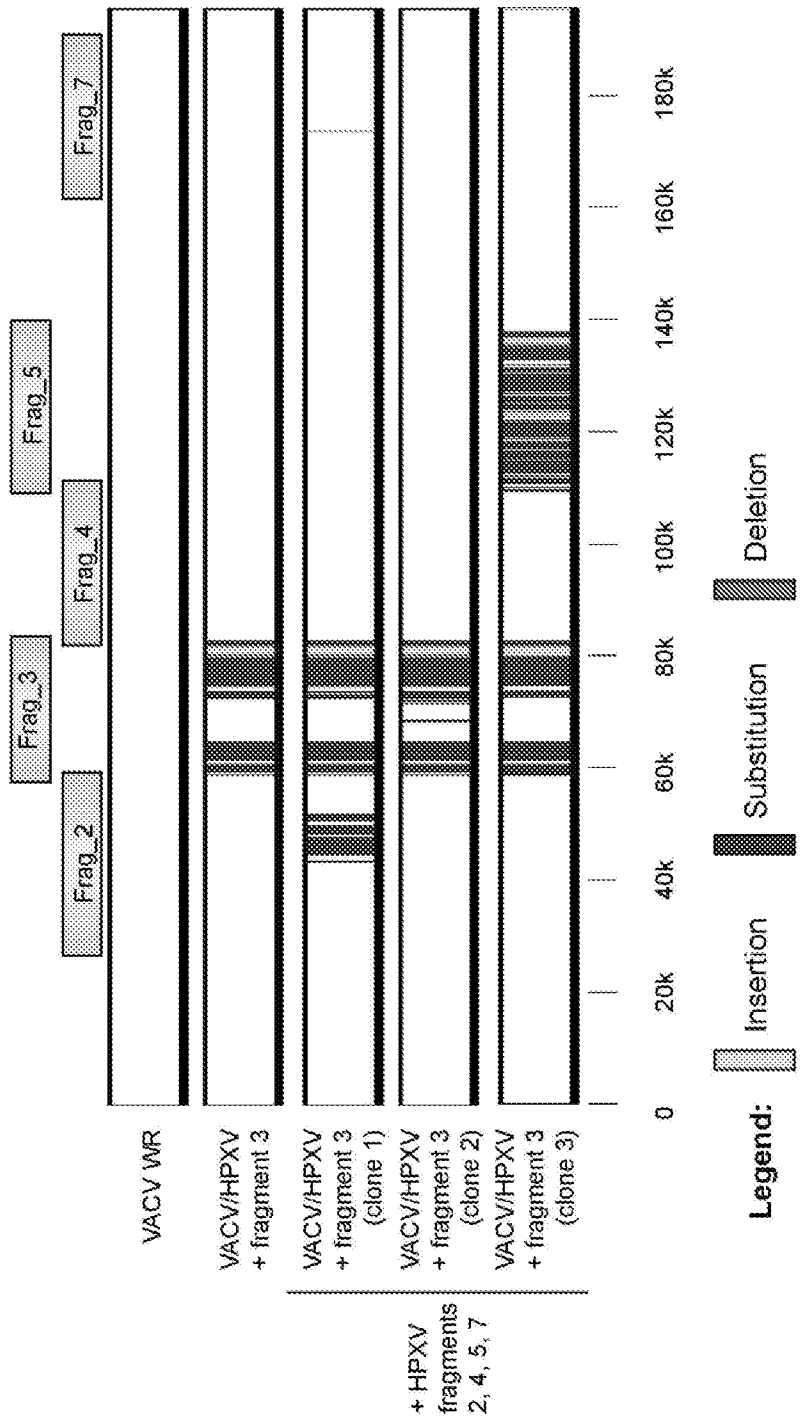

Frag_2
Frag_3
Frag_4
Frag_5
Frag_7
VACV WR
VACV/HPXV + fragment 3
VACV/HPXV + fragment 3 (clone 1)
VACV/HPXV + fragment 3 (clone 2)
VACV/HPXV + fragment 3 (clone 3)
+ HPXV fragments 2, 4, 5, 7
0
20k
40k
60k
80k
100k
120k
140k
160k
180k
Legend:
Insertion
Substitution
Deletion

Figure 13B

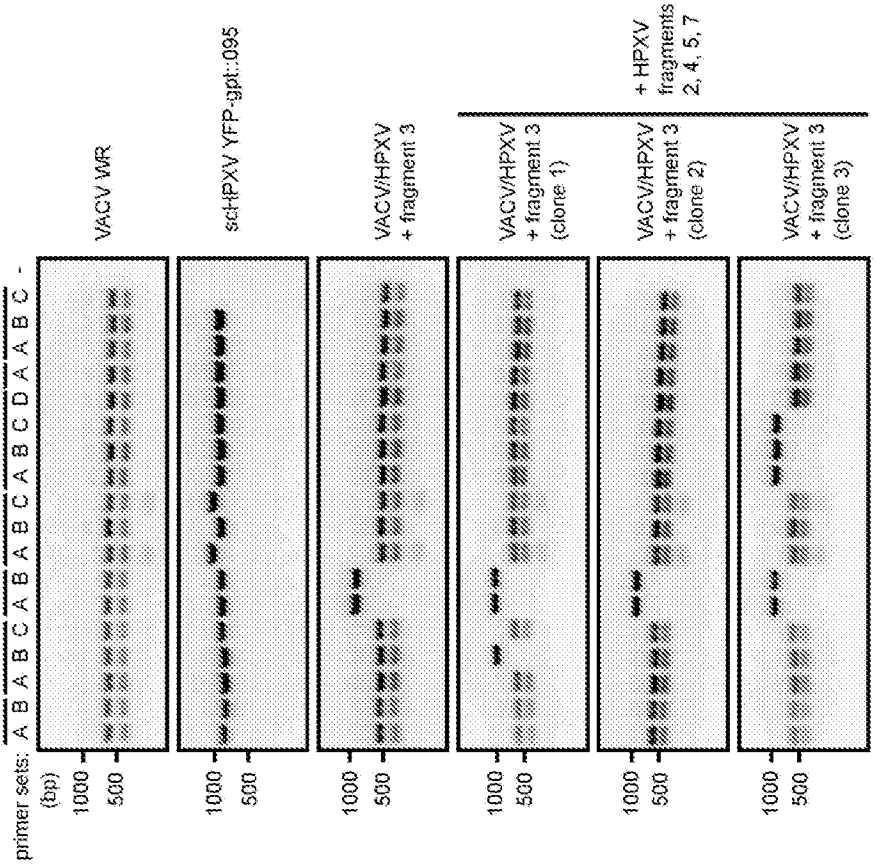

HPXV Fragments
1 2 3 4 5 6 7
A B A B C A B C A B C D A B C -
primer sets:
(bp)
1000
500
VACV WR
scHPXV YFP-gpt::095
VACV/HPXV + fragment 3
VACV/HPXV + fragment 3 (clone 1)
VACV/HPXV + fragment 3 (clone 2)
VACV/HPXV + fragment 3 (clone 3)
+ HPXV fragments 2, 4, 5, 7

Figure 14A

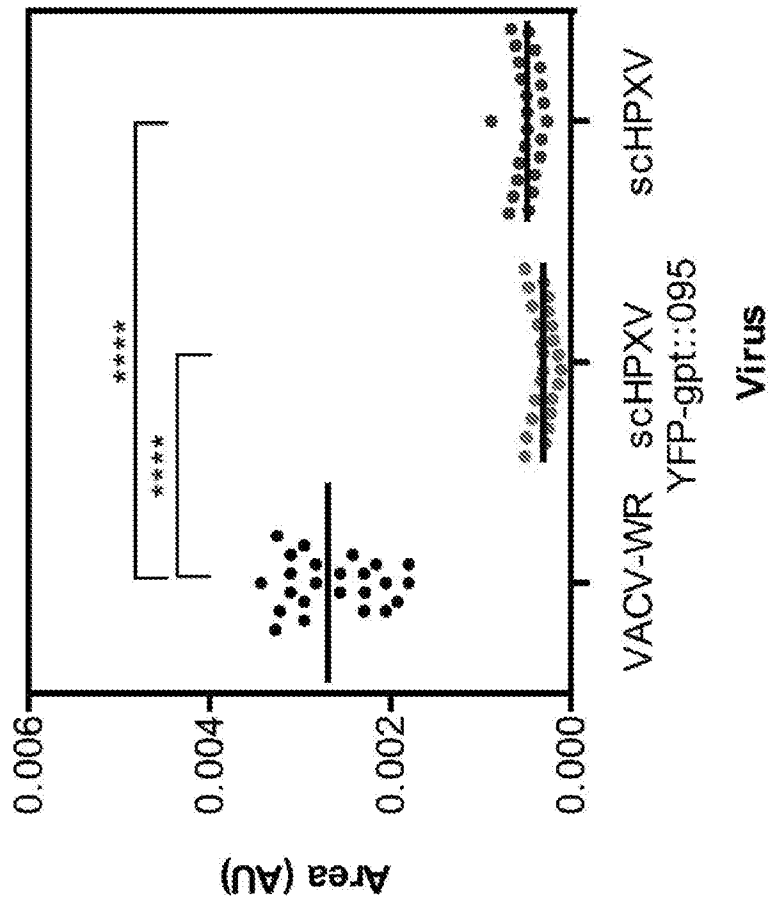

0.006
0.004
0.002
0.000
Area (AU)
****
****
*
VACV-WR
scHPXV YFP-gpt::095
scHPXV
Virus

Figure 14B

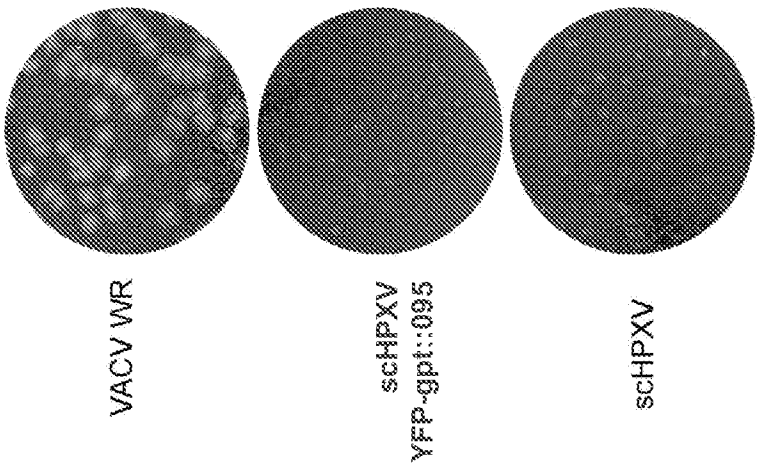
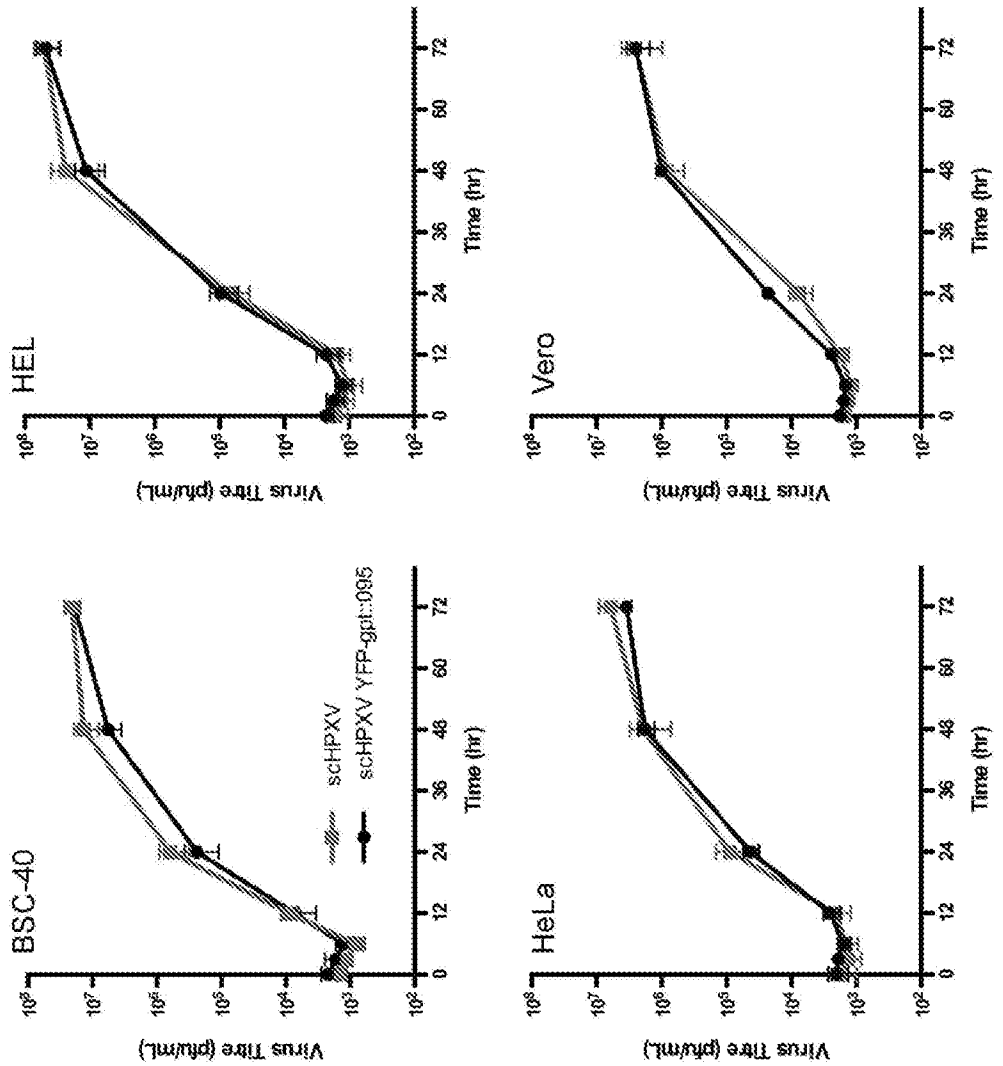


Figure 14C


BSC-40
HEL
HeLa
Vero
Virus Titre (pfu/mL)
Time (hr)
scHPXV
scHPXV YFP-gpt::095
0
12
24
36
48
60
72

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/059782

A. CLASSIFICATION OF SUBJECT MATTER

C12N 7/01 (2006.01) A61K 39/275 (2006.01)

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)

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

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

PATENW, CAPLUS, BIOSIS, EMBASE, MEDLINE: Poxvirus, orthopox, horsepox, terminal hairpin loop, chimeric, overlapping fragments and like terms; CPC: C12N2710/24011, A61K39/275.

AUSPAT, ESPACENET, PUBMED, Internal databases: Inventor names.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] 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	Date of mailing of the international search report
29 January 2018	29 January 2018
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Richard Filmer AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61262832735

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/059782

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☐ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

   No Sequence Listing was filed, although the specification describes some of the sequences referred to by their SEQ ID NOs.

   No sequences defined in the specification were the subject of a search.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/059782

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.:

because they relate to subject matter not required to be searched by this Authority, namely:

the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including

2. ☒ Claims Nos.: **34-37, 60-63**

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:

See Supplemental Box

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 additional 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 additional fees, this Authority did not invite payment of 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	International application No. PCT/US2017/059782

Supplemental Box

Continuation of **Box II**

Claims 34-37 and 60-63 make reference to SEQ ID NOs: 11 and 12, however no Sequence Listing was filed with the specification and these sequences do not appear to be defined anywhere in the specification. The scope of these claims is thus so unclear that no meaningful search or exam can be conducted.

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/US2017/059782**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2004/0014034 A1 (EVANS et al.) 22 January 2004 [0006]-[0012], [0017], [0018], [0041], [0042], [0049], [0061], [0083]-[0126], Figs. 1, 2	1-5, 7-30, 32, 33, 38-43, 71-88, 102, 103, 105-111, 118, 119, 121-127
X	YAO, X.-D. et al., "High-Frequency Genetic Recombination and Reactivation of Orthopoxviruses from DNA Fragments Transfected into Leporipoxvirus-Infected Cells", Journal of Virology, 2003, vol. 77, no. 13, pages 7281-7290 Abstract; page 7281, column 2; Materials and Methods	1-5, 7-30, 32, 33, 38-43, 71-88, 102, 103, 105-111, 118, 119, 121-127
X	YAO, X.-D. et al., "Construction of Recombinant Vaccinia Viruses Using Leporipoxvirus-Catalyzed Recombination and Reactivation of Orthopoxvirus DNA", Methods in Molecular Biology, 2004, vol. 269, pages 51-64 Materials; Methods	1-5, 7-30, 32, 33, 38-43, 71-88, 102, 103, 105-111, 118, 119, 121-127
A	LI, X.-D. et al., "Recovery of a chemically synthesized Japanese encephalitis virus reveals two critical adaptive mutations in NS2B and NS4A", Journal of General Virology, 2014, vol. 94, pages 806-815 Methods: Plasmid construction	
A	TULMAN, E. R. et al., "Genome of Horsepox Virus", Journal of Virology, 2006, vol. 80, no. 18, pages 9244-9258	
P,X	"WHO Advisory Committee on Variola Virus Research: Report of the Eighteenth Meeting", 2-3 November 2016, Geneva, Switzerland, World Health Organisation. [Retrieved from Internet 13 December 2017] <URL: http://www.who.int/csr/resources/publications/smallpox/variola-research-november-2016/en/ > Chapter 18, Pages 29-31	1-33, 38-59, 64-127
P,X	KOBLENTZ, G. D. "The De Novo Synthesis of Horsepox Virus: Implications for Biosecurity and Recommendations for Preventing the Reemergence of Smallpox". Health Security, 2017, vol. 15, no. 5, pages 620-628, Epub 24 August 2017 Abstract; "The Synthesis of Horsepox Virus"; Fig. 1	1-33, 38-59, 64-127

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/US2017/059782**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
US 2004/0014034 A1	22 January 2004	US 2004014034 A1	22 Jan 2004
		CA 2431349 A1	06 Dec 2003

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.