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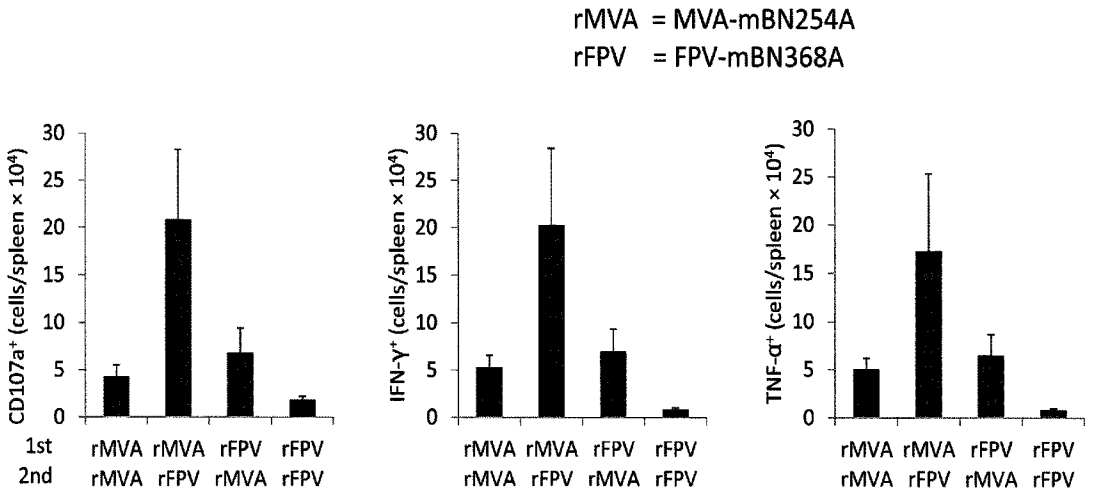
(54) **Titre : VIRUS CONTRE LES FILOVIRUS A BASE DU VIRUS DE LA VACCINE ANKARA MODIFIE RECOMBINANT (MVA)**
(54) **Title: RECOMBINANT MODIFIED VACCINIA VIRUS ANKARA (MVA) FILOVIRUS VACCINE**



(57) **Abrégé/Abstract:**
The present invention relates to an improved filovirus vaccine comprising a recombinant modified vaccinia virus Ankara-based (MVA-based) vaccine against filovirus infection and to related products, methods and uses. Specifically, the present invention relates to genetically engineered (recombinant) MVA and FPV vectors comprising at least one heterologous nucleotide sequence encoding an antigenic determinant of a Marburg virus (MARV) or Ebola virus glycoprotein. Specifically, the invention relates to recombinant MVA comprising Ebola virus glycoprotein and virion protein 40. The invention also relates to products, methods and uses thereof as well as prime/boost regimens of MVA and genetically engineered (recombinant) FPV, e.g., suitable to induce a protective immune response in a subject.

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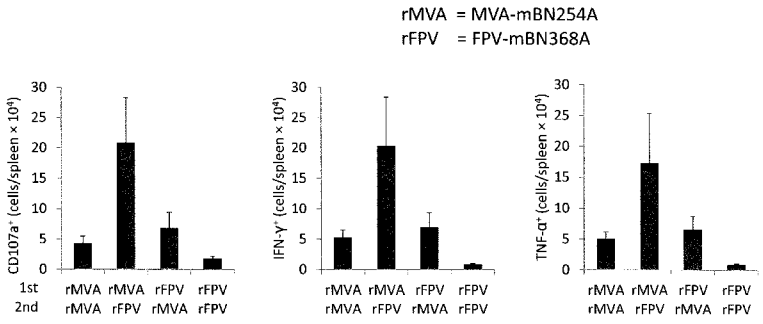
Figure 8B



(57) Abstract: The present invention relates to an improved filovirus vaccine comprising a recombinant modified vaccinia virus Ankara-based (MVA-based) vaccine against filovirus infection and to related products, methods and uses. Specifically, the present invention relates to genetically engineered (recombinant) MVA and FPV vectors comprising at least one heterologous nucleotide sequence encoding an antigenic determinant of a Marburg virus (MARV) or Ebola virus glycoprotein. Specifically, the invention relates to recombinant MVA comprising Ebola virus glycoprotein and virion protein 40. The invention also relates to products, methods and uses thereof as well as prime/boost regimens of MVA and genetically engineered (recombinant) FPV, e.g., suitable to induce a protective immune response in a subject.

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RECOMBINANT MODIFIED VACCINIA VIRUS ANKARA (MVA) FILOVIRUS VACCINE

FIELD OF THE INVENTION

The present invention relates to an improved filovirus vaccine comprising a recombinant modified vaccinia virus Ankara-based (MVA-based) vaccine against filovirus disease and to related products, methods and uses. Specifically, the present invention relates to genetically engineered (recombinant) MVA vectors comprising a heterologous nucleotide sequence encoding an antigenic determinant of a filovirus protein. The present invention also relates to vaccination methods, in particular homologous and heterologous prime-boost vaccination regimes employing two viral vector compositions. More particularly, the invention relates to a recombinant MVA for use in a homologous prime-boost vaccination regime and/or a recombinant MVA and a recombinant fowlpox virus (FPV) for use in a heterologous prime-boost vaccination regime. The invention also relates to products, methods and uses thereof, *e.g.*, suitable to induce a protective immune response in a subject.

BACKGROUND OF THE INVENTION

Filoviruses are enveloped, non-segmented, negative-strand RNA viruses of the virus family *Filoviridae*. Two members of this virus family have been identified to date: Marburg virus (MARV) and Ebola virus (EBOV). Filoviruses are extremely virulent, easily transmissible from person-to-person, and extraordinarily lethal, causing severe hemorrhagic fever in humans and non-human primates. Filovirus infections have a fatality rate in humans ranging from 23% to as high as 90%. Despite their transmissibility and lethality, however, no approved therapy or preventive vaccine is available.

During outbreaks, isolation of patients and use of protective clothing and disinfection procedures (together called viral hemorrhagic fever (VHF) isolation precautions or barrier nursing) has been sufficient to interrupt further transmission of Marburg or Ebola viruses, and thus to control and end the outbreak. Because there is no known effective treatment for the hemorrhagic fevers caused by filoviruses, transmission prevention through application of VHF isolation precautions is currently the only available means to control filovirus outbreaks.

The first filovirus was recognized in 1967 after a number of laboratory workers in Germany and Yugoslavia, who had been handling tissues from African green monkeys, developed severe hemorrhagic fever. A total of 31 cases and seven deaths were associated with these outbreaks. The virus was named Marburg virus (MARV) after Marburg, Germany, the site of one of the outbreaks. After the initial outbreaks the virus disappeared and did not reemerge until 1975, when a traveler, most likely exposed in Zimbabwe, became ill in Johannesburg, South Africa; the traveler's traveling companion and a nurse were also infected. A few sporadic cases of Marburg hemorrhagic fever (MHF) have been identified since that time, but the disease remains relatively rare.

The second filovirus, Ebola virus (EBOV), was first identified in 1976 when two outbreaks of Ebola hemorrhagic fever (EHF) occurred in northern Zaire (now the Democratic Republic of Congo) and southern Sudan. The outbreaks involved viruses which eventually proved to be two different species of Ebola virus, which were named after the nations in which they were discovered. Both viruses proved to be highly lethal, with 90% of the cases in Zaire and 50% of the cases in Sudan resulting in death. Since 1976, Ebola virus has appeared sporadically in Africa, with a few small- to medium-sized outbreaks confirmed between 1976 and 1979, and again in Gabon between 1994 and 1996. Larger epidemics of Ebola HF occurred in Kikwit, Zaire in 1995 and in Gulu, Uganda in 2000.

It appears that filoviruses are transmitted to humans from ongoing life cycles in one or more non-human animals. Despite numerous attempts to locate the natural reservoir or reservoirs of Ebola and Marburg viruses, however, their origins remain mysterious. Consequently, it also remains unclear just how the virus is transmitted from its natural reservoir(s) to humans. Once a human has been infected, however, further infections occur by person-to-person transmission. Specifically, transmission involves close personal contact between an infected individual or their body fluids and another person. During recorded outbreaks of hemorrhagic fever caused by filovirus infection, people who cared for (*i.e.*, fed, washed, medicated) or worked very closely with infected individuals were especially at risk of becoming infected themselves. Nosocomial (hospital) transmission through contact with infected body fluids (*i.e.*, via reuse of unsterilized syringes, needles, or other medical equipment contaminated with these fluids) has also been an important factor in the spread of disease. Minimizing close contact between uninfected and infected patients usually reduces the number of new filovirus infections in humans during an outbreak. Although filoviruses have

displayed some capability of infection through small-particle aerosols in the laboratory, airborne spread among humans has not been clearly demonstrated.

Five strains of Ebola virus have been identified so far, and are named after their site of first appearance: Bundibugyo (BEBOV), Ivory Coast (EBOV-CdI, also called Tai Forest virus or TAFV), Reston (EBOV-Reston), Sudan (SEBOV), and Zaire (ZEBOV); the Zaire, Sudan, and Bundibugyo strains are commonly involved in morbidity and death in humans. Ebola-Reston is the only known filovirus that does not cause severe disease in humans, although it can be fatal in monkeys. Several strains of Marburg virus have been identified so far, with the Musoke strain having the highest lethality rate. *See* Figure 1.

Structurally, filovirus virions may appear in several shapes, including long, sometimes branched filaments, as well as shorter filaments shaped like a "6", the letter "U", or a circle. Viral filaments can measure up to 14 micrometers (μm) in length, have a uniform diameter of 80 nanometers (nm), and are enveloped in a lipid membrane. Each virion contains one single-stranded, negative-sense RNA molecule approximately 19 kilobase pairs (kb) in length, which contains seven sequentially arranged genes in the order of nucleoprotein (NP), virion protein 35 (VP35), virion protein 40 (VP40), envelope glycoprotein (GP), virion protein 30 (VP30), virion protein 24 (VP24), and RNA-directed RNA polymerase protein (L). Upon entry into the host cell cytoplasm, the RNA is transcribed to generate polyadenylated, subgenomic mRNA species encoding the proteins. Transcription and translation lead to the synthesis of seven structural polypeptides, with presumed identical functions for each of the different filoviruses. Four proteins (NP, VP30, VP35 and L) are associated with the viral genomic RNA in the nucleocapsid complex. The three remaining structural proteins are membrane-associated; GP is a type I transmembrane protein, while VP24 and VP40 are probably located on the inner side of the membrane. The envelope glycoprotein (GP) appears in the viral envelope as a homotrimer (also referred to as a 'peplomer') comprising three copies of a heterodimer. The heterodimer contains two fragments of the full-length GP precursor (referred to as 'GP0') known as 'GP1' and 'GP2' produced by furin cleavage. GP1 and GP2 are linked by a disulfide bond. A non-structural, secreted glycoprotein (sGP) is expressed by EBOV, but not MARV (H. Feldmann & M.P. Kiley, *Curr. Top. Microbiol. Immunol.* 235:1-21 (1999)). New viral particles are created by budding from the surface of host cells (*see below*).

The filovirus life cycle begins with virion attachment to specific cell-surface receptors, followed by fusion of the virion envelope with cellular membranes and release of the virus nucleocapsid into the cytosol. The viral RNA-directed RNA polymerase (RNAP,

also known as the 'L' protein) partially uncoats the nucleocapsid and transcribes the genes into positive-stranded mRNAs, which are then translated into structural and nonstructural proteins. *See* Figure 2. The RNAP binds to a single promoter located at the 3' end of the genome. Transcription either terminates after a gene or continues to the next gene downstream, meaning that genes close to the 3' end of the genome are transcribed in the greatest abundance, while those towards the 5' end of the genome are least likely to be transcribed. Gene order is therefore a simple but effective form of transcriptional regulation. The most abundant protein produced is the nucleoprotein (NP), cellular concentration of which determines when the RNAP switches from gene transcription to genome replication. Replication results in full-length, positive-stranded anti-genomes that are in turn transcribed into negative-stranded virus progeny genome copies. Newly synthesized structural proteins and genomes self-assemble and accumulate near the inside of the cell membrane. Virus particles are enveloped as they bud from the infected host cell, producing mature infectious virions.

Prior vaccine development

Many strategies have been evaluated during attempts to develop a safe, immunogenic vaccine capable of inducing protective immunity against infection by one or more filovirus species, with decidedly mixed results. An overview is summarized in Marzi and Feldmann (A. Marzi and H. Feldmann *Expert Rev. Vaccines* 13(4):521-531 (2014)). For instance, while a trivalent DNA vaccine comprising a mixture of three DNA plasmids, one expressing the envelope glycoprotein from ZEBOV, a second expressing the envelope glycoprotein from SEBOV, and a third expressing the nucleoprotein from ZEBOV was safe, immunogenic, and able to induce an antibody response against at least one of the three antigens in humans. CD8+ T-cell responses were detected in fewer than 1/3 of the vaccinated population (J.E. Martin et al., *Clin. Vaccine Immunol.* 13(11):1267-1277 (2006)). Similarly, a complex, pentavalent adenovirus-based 'pan-filovirus' vaccine comprising a mixture of four different recombinant adenoviruses expressing envelope glycoproteins from ZEBOV, SEBOV, Marburg-Ci67 (strain Ratayczak), Marburg-Musoke, and Marburg-Ravn, as well as nucleoproteins from ZEBOV and Marburg-Musoke, protected non-human primates from ZEBOV or MARV challenge and induced antibody responses to both types of virus, although it remains unclear whether the vaccine induced any CD8+ T-cell response (D.L. Swenson et al., *Clin. Vaccine Immunol.* 15(3):460-467 (2008)).

Intranasal administration of a recombinant paramyxovirus - human parainfluenza virus, serotype 3 (HPIV3) - expressing either the envelope glycoprotein or both the envelope glycoprotein and nucleoprotein from ZEBOV protected guinea pigs from subsequent

challenge with EBOV. Rodent models are frequently poorly predictive of results in primates, with a number of previous EBOV vaccine candidates that were effective in rodents failing completely in non-human primates (A. Bukreyev et al., *J. Virol.* 80(5):2267-2279 (2006)). Intranasal administration of a recombinant HPIV3 expressing either the envelope glycoprotein or both the envelope glycoprotein and nucleoprotein from ZEBOV in rhesus monkeys showed that any construct expressing the envelope glycoprotein was moderately immunogenic and protected more than 80% of the animals against disease after post-vaccination challenge with ZEBOV (A. Bukreyev et al., *J. Virol.* 81(12):6379-6388 (2007)). Finally, a recombinant vesicular stomatitis virus (VSV) in which the VSV glycoprotein was replaced by the ZEBOV envelope glycoprotein protected 50% of guinea pigs, 100% of mice following treatment as late as 24 hours after an otherwise uniformly lethal infection. Four out of eight rhesus macaques (50%) were protected when treated 20 to 30 min after exposure providing a post-exposure treatment option for Ebola virus infection (H. Feldmann, *PLoS Pathogens* 3(1):54-61 (2007)).

Geisbert et al. evaluated the effects of vaccine strategies that had protected mice or guinea pigs from lethal EBOV infection in nonhuman primates. They used RNA replicon particles derived from an attenuated strain of Venezuelan equine virus (VEEV) expressing EBOV glycoprotein and nucleoprotein, recombinant Vaccinia virus (VACV) expressing EBOV glycoprotein, liposomes containing lipid A and inactivated EBOV, and a concentrated, inactivated whole-virion preparation. They found that none of these strategies successfully protected nonhuman primates from robust challenge with EBOV (T.H Geisbert et al., *Emerging Infectious Diseases* 8(3):503-507 (2002)).

Others have used Virus Like Particles (VLPs) expressed in mammalian, bacterial, plant or insect cells as non-replicating subunit vaccines (D.L. Swenson et al., *Vaccine* 23:3033-3042 (2005); K. L. Warfield et al., *JID* 196(2):430-437 (2007), N. Kushnir et al., *Vaccine* 31(1):58-83 (2012), K. L. Warfield ET AL., *PLOS ONE* 10(3):e0118881 (2015), K. L. Warfield and M.J. Aman *JID* 204:1053-1059 (2011), V.M. Wahl-Jensen et al., *J Virol.* 79(16):10442-10450 (2005), WO 2003/039477, WO 2006/046963, WO 2006/073422, WO 2004/042001, US 8,900,595, US 7,211,378) to induce antibody responses. However, filovirus VLPs require a cost-intensive and challenging production process and need to be stored at ambient temperature over time.

Thus, after expending considerable time and effort, a few promising vaccine candidates have emerged at preclinical stages, but at present no approved preventive vaccine is available. Given the transmissibility and lethality of filovirus infection, there is a pressing need for an effective vaccine.

BRIEF SUMMARY OF THE INVENTION

It is discovered in the present invention that various prime-boost combinations of replication deficient and replication incompetent vectors generate effective immune protection against filovirus infection.

Accordingly, one general aspect of the present invention relates to a combination vaccine comprising:

a) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding an antigenic protein of at least one filovirus subtype, together with a pharmaceutically acceptable carrier; and

b) a second composition comprising an immunologically effective amount of a fowlpox vector comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein one of the compositions is a priming composition and the other composition is a boosting composition.

In an additional aspect, the present invention relates to a combination vaccine comprising:

(a) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding an antigenic protein of at least two filovirus subtypes, together with a pharmaceutically acceptable carrier; and

(b) a second composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein one of the compositions is a priming composition and the other composition is a boosting composition.

In an additional aspect, the present invention relates to a kit comprising:

(a) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding an antigenic protein of at least one filovirus subtypes, together with a pharmaceutically acceptable carrier; and

(b) a second composition comprising an immunologically effective amount of a fowlpox vector comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein one of the compositions is a priming composition and the other composition is a boosting composition.

In an additional aspect, the present invention relates to a kit comprising:

(a) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding antigenic proteins of at least two filovirus subtypes, together with a pharmaceutically acceptable carrier; and

(b) a second composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein one of the compositions is a priming composition and the other composition is a boosting composition.

In an additional aspect, the present invention relates to a recombinant Modified Vaccinia Virus (MVA) vector comprising a nucleotide sequence encoding two or more antigenic determinants of a filovirus protein for use in the treatment and/or prevention of a filovirus-caused disease. In yet another aspect, the invention relates to a recombinant MVA vector comprising a nucleotide sequence encoding an antigenic protein of a filovirus glycoprotein and encoding a filovirus virion protein 40 (VP40) for use in the treatment and/or prevention of a filovirus-caused disease. In another embodiment, the invention relates to a recombinant MVA vector comprising a nucleotide sequence selected from the group consisting of a) SEQ ID NO:5, SEQ ID NO:19 and SEQ ID NO:30, b) SEQ ID NO:5, SEQ ID NO:19, SEQ ID NO:28 and SEQ ID NO:30 and c) SEQ ID NO:19 and SEQ ID NO:33. In a certain aspect, the invention relates to a composition comprising said recombinant MVA vector, a vaccine comprising said recombinant MVA vector, a pharmaceutical comprising said recombinant MVA vector and a pharmaceutical carrier, diluent and/or additive, and a cell comprising said recombinant MVA vector. In a certain aspect, the invention relates to said recombinant MVA vector for use as a medicament or vaccine for treating and/or preventing a filovirus-caused disease in a subject and a method for affecting an immune response in a subject comprising administering to the subject said

recombinant MVA vector. In an additional aspect, the present invention relates to a kit comprising said recombinant MVA vector in a first vial or container for a first administration (priming) and in a second vial or container for a second administration (boosting).

The present invention also relates to a recombinant FPV vector comprising a nucleotide sequence encoding at least one antigenic determinant of a filovirus protein (e.g. any of the filovirus proteins as mentioned *supra* or *infra,* preferably an filovirus envelope glycoprotein) under the control of the FPV-40K promoter having SEQ ID NO:26. In an additional aspect, the invention relates to a recombinant fowlpox virus (FPV) vector comprising a nucleotide sequence encoding one, two or more antigenic determinants of a filovirus protein for use in the treatment and/or prevention of a filovirus-caused disease. In a certain aspect, the invention relates to a composition comprising said recombinant FPV vector, a vaccine comprising said recombinant FPV vector, a pharmaceutical comprising said recombinant FPV vector and a pharmaceutical carrier, diluent and/or additive and a cell comprising said recombinant FPV vector. In a certain aspect, the invention relates to said recombinant FPV vector for use as a medicament or vaccine for treating and/or preventing a filovirus-caused disease in a subject and a method for affecting an immune response in a subject comprising administering to the subject said recombinant FPV vector.

In an additional aspect, the present invention relates to a combination vaccine comprising:

(a) an immunologically effective amount of a MVA vector comprising a nucleic acid encoding antigenic proteins of at least two filovirus subtypes, together with a pharmaceutically acceptable carrier; and

(b) an immunologically effective amount of a fowlpox vector comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein one of the vectors is a priming vaccine and the other vector is a boosting vaccine.

In an additional aspect, the present invention relates to a combination vaccine comprising:

(a) an immunologically effective amount of a MVA vector comprising a nucleic acid encoding antigenic proteins of at least two filovirus subtypes, together with a pharmaceutically acceptable carrier; and

(b) an immunologically effective amount of one or more additional MVA vectors comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein one of the MVA vectors is a priming vaccine and the other MVA vectors is a boosting vaccine.

In an additional aspect, the present invention relates to a combination vaccine comprising:

(a) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding at least one antigenic determinant of a filovirus protein; and

(b) a second composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding at least one antigenic determinant of a filovirus protein;

OR

(c) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding at least one antigenic determinant of a filovirus protein; and

(d) a second composition comprising an immunologically effective amount of an FPV vector comprising a nucleic acid encoding at least one antigenic determinant of a filovirus protein;

wherein one of the compositions is a priming composition and the other composition is a boosting composition.

In an additional aspect, the present invention relates to a method of inducing an immune response against a filovirus in a subject, the method comprising administering to the subject:

(a) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding an antigenic protein of at least one filovirus subtype, together with a pharmaceutically acceptable carrier; and

(b) a second composition comprising an immunologically effective amount of a fowlpox vector comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein one of the compositions is a priming composition and the other composition is a boosting composition.

In an additional aspect, the present invention relates to a method of inducing an immune response against a filovirus in a subject, the method comprising administering to the subject:

(a) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding antigenic proteins of at least two filovirus subtypes, together with a pharmaceutically acceptable carrier; and

(b) a second composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein one of the compositions is a priming composition and the other composition is a boosting composition.

The invention also covers a method of generating a recombinant MVA vector for use in the treatment and/or prevention of a filovirus-caused disease comprising the steps of:

(a) infecting a host cell with a MVA virus,

(b) transfecting the infected cell with a recombinant vector comprising at least one nucleotide sequence encoding an antigenic determinant of any of the filovirus proteins of any of the embodiments of the invention, said nucleic acid sequence further comprising a genomic MVA virus sequence capable of directing the integration of the at least one nucleotide sequence into the MVA virus genome, and

(c) identifying, isolating and optionally purifying the generated recombinant MVA virus.

In another embodiment, the order of step a) and b) of the method of generating a recombinant MVA vector of any of the above embodiments can be changed such that step b) is the first step and a) the second.

The invention also covers a method of generating a recombinant FPV vector for use in the treatment and/or prevention of a filovirus-caused disease comprising the steps of:

(a) infecting a host cell with an FPV virus,

(b) transfecting the infected cell with a recombinant vector comprising at least one nucleotide sequence encoding an antigenic determinant of any of the

filovirus proteins of any of the embodiments of the invention, said nucleic acid sequence further comprising a genomic FPV virus sequence capable of directing the integration of the at least one nucleotide sequence into the FPV virus genome, and

(c) identifying, isolating and optionally purifying the generated recombinant FPV virus.

In another embodiment, the order of step a) and b) of the method of generating a recombinant FPV vector of any of the above embodiments can be changed such that step b) is the first step and a) the second.

In an additional aspect, the present invention relates to a method of inducing an immune response against a filovirus in a subject comprising administering to the subject:

(a) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding at least one antigenic determinant of a filovirus protein; and

(b) a second composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding at least one antigenic determinant of a filovirus protein;

OR

(c) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding at least one antigenic determinant of a filovirus protein; and

(d) a second composition comprising an immunologically effective amount of an FPV vector comprising a nucleic acid encoding at least one antigenic determinant of a filovirus protein

wherein one of the compositions is a priming composition and the other composition is a boosting composition.

In an additional aspect, the present invention relates to a method of providing protective immunity or a protective immune response in a subject, the method comprising administering to the subject:

(a) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding an antigenic protein of at

least one filovirus subtype, together with a pharmaceutically acceptable carrier; and

(b) a second composition comprising an immunologically effective amount of a fowlpox vector comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein one of the compositions is a priming composition and the other composition is a boosting composition.

In an additional aspect, the present invention relates to a method of providing protective immunity or a protective immune response in a subject comprising administering to the subject:

(a) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding antigenic proteins of at least two filovirus subtypes, together with a pharmaceutically acceptable carrier; and

(b) a second composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein one of the compositions is a priming composition and the other composition is a boosting composition.

In an additional aspect, the present inventions relates to a method for production of filovirus-like particles in a subject comprising administering to the subject:

(a) an immunologically effective amount of a MVA vector comprising a nucleic acid encoding antigenic proteins of at least one filovirus glycoprotein and a filovirus virion protein 40 (VP40), together with a pharmaceutically acceptable carrier; and

(b) an immunologically effective amount of a fowlpox vector or a MVA vector comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein one of the vectors is a priming vaccine and the other vector is a boosting vaccine.

In an additional aspect, the present inventions relates to a method for production of filovirus-like particles in a subject comprising administering to the subject:

(a) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding antigenic proteins of at least one filovirus glycoprotein and a filovirus virion protein 40 (VP40), together with a pharmaceutically acceptable carrier; and

(b) a second composition comprising an immunologically effective amount of a fowlpox vector or a MVA vector comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein one of the compositions is a priming composition and the other composition is a boosting composition.

In an additional aspect, the invention relates to a method of inducing an enhanced immune response against a filovirus in a subject, the method comprising production of filovirus-like particles in the subject by administering to the subject:

(a) an immunologically effective amount of a MVA vector comprising a nucleic acid encoding antigenic proteins of at least one filovirus glycoprotein and a filovirus virion protein 40 (VP40), together with a pharmaceutically acceptable carrier; and

(b) an immunologically effective amount of a fowlpox vector or a MVA vector comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein one of the vectors is a priming vaccine and the other vector is a boosting vaccine.

In an additional aspect, the invention relates to a method of inducing an enhanced immune response against a filovirus in a subject, the method comprising production of filovirus-like particles in the subject by administering to the subject:

(a) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding antigenic proteins of at least one filovirus glycoprotein and a filovirus virion protein 40 (VP40), together with a pharmaceutically acceptable carrier; and

(b) a second composition comprising an immunologically effective amount of a fowlpox vector or a MVA vector comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein one of the compositions is a priming composition and the other composition is a boosting composition.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings illustrate several embodiments of the invention and together with the description, serve to explain the principles of the invention.

Figure 1 shows a phylogenetic tree depicting the relationships between various identified filovirus strains. The tree was constructed using coding regions of envelope glycoprotein (GP) genes and the maximum parsimony method. Both the Ravn and Ratayczak strains of Marburg virus had a 23% fatality rate, while the Musoke and Angola strains had fatality rates ranging from 50% to 88%. The Sudan strain had a 41-65% fatality rate, and the Zaire strain had a 57-90% fatality rate. Both the Cote d'Ivoire and Reston strains have not yet caused disease in man, though Reston has caused disease in pigs.

Figure 2 shows the structure and genetic organization of the filovirus genome.

Figure 3A shows the structure and genetic organization of MVA-mBN252B. **Figure 3B** shows the structure and genetic organization of MVA-mBN226B. **Figure 3C** shows the structure and genetic organization of MVA-mBN254A including the selection marker. **Figure 3D** shows the structure and genetic organization of MVA-mBN368A including the selection marker.

Figure 4A shows the structure and genetic organization of plasmid pBNX186. Flank 1 (F1 IGR 88/89) and flank 2 (F2 IGR 88/89) are sequences of MVA-BN surrounding IGR 88/89. F1 IGR 88/89 and F2 IGR 88/89 are used for insertion of the expression cassette and the selection cassette (NPT II and eGFP) into MVA-BN in a homologous recombination event. The *E. coli* drug selection gene Neomycin Phosphotransferase (NPT II) and an enhanced Green Fluorescent Protein (eGFP) were connected via an internal ribosomal entry site (IRES) and inserted under the control of a strong synthetic poxvirus promoter (PrS) in order to allow selection for recombinant viruses. F2 and F2-repeat sequences of IGR 88/89 flank the selection cassette enabling the removal of the selection cassette via homologous recombination in the absence of selective pressure.

Figure 4B shows the structure and genetic organization of plasmid pBNX197. Flank 1 (F1 IGR 148/149) and flank 2 (F2 IGR 148/149) are sequences of MVA-BN surrounding IGR 148/149. F1 IGR 148/149 and F2 IGR 148/149 are used for insertion of the expression cassette and the selection cassette (GPT and RFP) into MVA-BN in a

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homologous recombination event. The *E. coli* Guanine-Xanthine-Phosphoribosyl-Transferase drug selection gene (GPT) and a Red Fluorescence Protein gene (RFP) were inserted as a fusion gene under the control of a strong synthetic poxvirus promoter (PrS) in order to allow selection for recombinant viruses. LoxP sequences flank the selection cassette enabling the Cre recombinase-mediated removal of the selection cassette. **Figure 4C** shows the structure and genetic organization of plasmid pBN274, which expresses Cre recombinase. **Figure 4D** shows the structure and genetic organization of plasmid pBNX221. Flank 1 (F1 IGR BamHI J FowlPox) and flank 2 (F2 IGR BamHIJ FowlPox) are sequences of FPV surrounding the insertion site BamHI J. F1 IGR BamHI J FowlPox and F2 IGR BamHI J FowlPox are used for insertion of the expression cassette and the selection cassette (GPT and RFP) into FPV in a homologous recombination event. The *E. coli* Guanine-Xanthine-Phosphoribosyl-Transferase drug selection gene (GPT) and a Red Fluorescence Protein gene (RFP) were inserted as a fusion gene under the control of a strong synthetic poxvirus promoter (PrS) in order to allow selection for recombinant viruses. LoxP sequences flank the selection cassette enabling the Cre recombinase-mediated removal of the selection cassette. **Figure 4E** shows the structure and genetic organization of plasmid pBNX214. Flank 1 (F1 IGR 148/149) and flank 2 (F2 IGR 148/149) are sequences of MVA-BN surrounding IGR 148/149. F1 IGR 148/149 and F2 IGR 148/149 are used for insertion of the expression cassette and the selection cassette (GPT and RFP) into MVA-BN in a homologous recombination event. pBNX214 already includes the PrS5E promoter for the expression of transgenes. The *E. coli* Guanine-Xanthine-Phosphoribosyl-Transferase drug selection gene (GPT) and a Red Fluorescence Protein gene (RFP) were inserted as a fusion gene under the control of a strong synthetic poxvirus promoter (PrS) in order to allow selection for recombinant viruses. LoxP sequences flank the selection cassette enabling the Cre recombinase-mediated removal of the selection cassette.

Figure 5A shows the structure and genetic organization of plasmid pBN433. The GP-MARV-Musoke was inserted under control of the promoter PrS into the BspEI/NheI site of pBNX197. In addition the plasmid also contains MVA-BN DNA sequences flanking the IGR 148/149 of the MVA-BN genome and the loxP-flanked selection cassette. The loxP sites allow the later elimination of the selection cassette by Cre recombinase-mediated recombination. **Figure 5B** shows the structure and genetic organization of plasmid pBN384. The glycoprotein genes of Ebola virus Zaire-Mayinga (GP-ZEBOV-Mayinga) and Marburg virus Musoke (GP-MARV-Musoke) were inserted under control of the promoters Pr7.5 and PrS into the MluI/NheI sites of pBNX197. In addition, the

plasmid also contains MVA-BN DNA sequences flanking the IGR 148/149 of the MVA-BN genome and the loxP-flanked selection cassette. The loxP sites allow the later elimination of the selection cassette by CRE recombinase-mediated recombination. **Figure 5C** shows the structure and genetic organization of plasmid pBN385. The glycoprotein gene of Ebola virus Sudan (GP-SEBOV) and the Nucleoprotein of Ebola virus Ivory Coast (NP-EBOV-CdI) were inserted under control of the synthetic promoters PrS and PrLE1 into the MluI/NheI sites of pBNX186. In addition, the plasmid also contains MVA-BN DNA sequences flanking the IGR 148/149 of the MVA-BN genome and a selection cassette flanked by F2 and F2rpt in order to allow the later elimination of the selection cassette via homologous recombination in the absence of selective pressure. **Figure 5D** shows the structure and genetic organization of plasmid pBN436. The glycoprotein gene of Ebola virus Zaire-Mayinga (GP-ZEBOV-Mayinga) was inserted into the BspEI/NotI sites of pBNX214 under control of the PrS5E promoter. In addition, the plasmid also contains MVA-BN DNA sequences flanking the IGR 148/149 of the MVA-BN genome and the loxP-flanked selection cassette. The loxP sites allow the later elimination of the selection cassette by Cre recombinase-mediated recombination. **Figure 5E** shows the structure and genetic organization of plasmid pBN555. The glycoprotein gene of Ebola virus Zaire-Mayinga (GP-ZEBOV-Mayinga) under control of the FPV-40K promoter was inserted into the MluI/NotI sites of pBNX221. In addition, the plasmid also contains FPV DNA sequences flanking the Insertion site BamHI J of the FPV genome and the loxP-flanked selection cassette. The loxP sites allow the later elimination of the selection cassette by Cre recombinase-mediated recombination.

Figure 6 shows the levels of antibodies against GP in cynomolgus macaques following vaccination with MVA-BN-Filo (MVA-mBN226B) as measured by ELISA. Animals were vaccinated twice four weeks apart with MVA-BN-Filo (on Day -42 and Day -14), and blood was drawn at intervals for analysis via ELISA: prior to vaccination (Day -42, red curve (1)), after the first but prior to the second vaccination (Day -14, green curve (2)), and after the second vaccination (Day -5, orange curve (3)). The graph on the left shows Marburg GP specific antibodies in serum, the graph in the middle shows Ebola Zaire GP specific antibodies in serum, and the graph on the right shows Ebola Sudan GP specific antibodies in serum. Hyperimmune serum from cynomolgus macaques immunized with either Marburg Angola GP (left graph), Ebola Zaire GP (middle graph), or Ebola Sudan GP (right graph) was used as positive control in each ELISA.

Figure 7 shows the results of vaccination with MVA-BN-Filo (MVA-mBN226B) following challenge with MARV-Musoke. **Figure 7A** shows that vaccination with MVA-BN-Filo

protected 100% of animals from challenge with MARV-Musoke. **Figure 7B** shows clinical scores post-challenge; vaccinated animals challenged with MARV-Musoke showed no symptoms or histological changes associated with hemorrhagic fever and harbored no virus in liver, spleen, adrenal glands, lymph nodes, or lungs.

Figure 8 shows the antibody and CD8 T cell response after heterologous MVA/FPV immunization. H-2K^{k+} B6CBA F1 mice were immunized s.c. with 5 × 10^7 $TCID_{50}$ MVA-ZEBOV-GP (MVA; MVA-mBN354A, see **Figure 3C**) or FPV-ZEBOV-GP (FPV; FPVmBN368A, see **Figure 3D**) on day 0 and 21. **A)** Mice were bled on day 21 and 41 for antibody analysis. Shown is the mean concentration of ZEBOV-GP-specific antibodies +/- SEM. **B)** On day 41, mice were sacrificed and spleens were analyzed flow-cytometrically after re-stimulation with $GP_{577-584}$ peptide. Shown is the absolute number of CD107a$^+$, IFN-γ^+ and TNF-α^+ CD8 T cells per spleen x 10^4 +/- SEM. rMVA = recombinant MVA-ZEBOV-GP (MVA-mBN254); rFPV = recombinant FPV-ZEBOV-GP (FPV-mBN368).

Figure 9 shows the ZEBOV-GP specific CD8 T cell response after immunization (s.c) of mice with MVA/FPV. Shown is the absolute number of CD107a$^+$, IFN-γ^+ and TNF-α^+ CD8 T cells per spleen x 10^4 +/- SEM. 1: MVA-mBN254/FPV-mBN368; 2: MVA-mBN226/FPV-mBN368, 3: MVA-mBN255/FPV-mBN368.

Figure 10 shows ZEBOV-GP specific antibodies of cynomolgus macaques which received prime-boost vaccinations on Study Day 0 and 28 with MVA-BN-ZEBOV/GP (MVA-mBN254) at a dose of 5x10^8 $TCID_{50}$ (n=3), with MVA-BN-ZEBOV/GP-VP40 (MVA-mBN255) at a dose of 5x10^8 $TCID_{50}$ (n=3) according to Example 6. Results are presented as the geometric mean concentration (ng/ml) together with the standard error of the mean (SEM).

Figure 11 shows neutralizing antibody responses of cynomolgus macaques which received three vaccinations on Study Day 0, 28 and with MVA-BN-ZEBOV/GP at a dose of 5x10^8 $TCID_{50}$ (n=2), or with MVA-BN-ZEBOV/GP-VP40 (5x10^8 $TCID_{50}$, n=2). Additional animals (n=2) received TBS as negative control on Study Day 0 and 56. Sera were analyzed by ZEBOV-GP-specific pseudo virion neutralizing assay. Results are presented as individual antibody titer neutralizing 80% of ZEBOV-GP expressing VSV.

Figure 12 A) and **B)** shows the formation of filovirus-like particles in HeLa cells infected with MVA-BN-ZEBOV/GP-VP40 (MVA-mBN255). **A, B)** Transmission electron microscopy (TEM) analysis of MVA-BN-ZEBOV/GP-VP40 (VLP) and MVA wt infected HeLa cells. HeLa cells were infected with MVA-BN-ZEBOV/GP-VP40 (**A**) or BAC-

derived MVA-wt (**B**) at an MOI of 10 and thin sections were generated and processed for TEM. Arrow: Transverse section of VLP generated by MVA-BN-ZEBOV/GP-VP40. **C)** Shows an immunoblot analysis of (co-)expression of GP and VP40 in Hela cells. **D)** Shows an immunoblot of immunoprecipitates from the supernatants of HeLa cells (aliquots of the same supernatants as shown in **C)** infected with MVA-BN-ZEBOV/GP-VP40 at an MOI of 10 for 2 days. VP40 and GP can only be co-precipitated if present in intact VLPs but not after disruption of VLPs with Triton™-X-100 (1%). 166: MVA-mBN166, 254: MVA-mBN254, 255: MVA-mBN255.

Figure 13 shows the structure of certain recombinant MVA/FPV constructs.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have found that a vaccine comprising a recombinant modified vaccinia virus Ankara (MVA) comprising a heterologous nucleotide sequence encoding an antigenic determinant of a Marburg virus (MARV) glycoprotein (GP) provides a filoviral vaccine capable of inducing both cellular and humoral responses sufficient to confer protective immunity to Marburg virus, as well as to smallpox. The insertion of additional heterologous nucleotide sequences encoding an antigenic determinant of an Ebola virus Zaire (ZEBOV) glycoprotein (GP), Ebola Virus Sudan (SEBOV) glycoprotein (GP), and/or an EBOV nucleoprotein (NP) into the recombinant MVA produces a multivalent vaccine capable of inducing immune responses to both MARV and EBOV, and even to multiple strains of MARV and/or EBOV, such as, for example Sudan Ebola virus (SEBOV) and Zaire Ebola virus (ZEBOV), the two types associated with the lethal forms of Ebola hemorrhagic fever. Thus, a recombinant MVA vector comprising a nucleotide sequence encoding an antigenic determinant of an EBOV GP reveals very good immune responses against Ebola strains. Moreover, the excellent safety profile of MVA and its derivatives (*e.g.*, MVA-BN), as well as their ability to accommodate multiple heterologous nucleotide sequences enables the production of a safe single component multivalent pan-filovirus vaccine, in contrast to a number of multi-component vaccines in early stages of development (see below).

Given the fact that prior art attempts to generate an immune response against filoviruses, in particular in non-human primates against MARV and EBOV, failed, the present invention came as a surprise. It could not have been expected from what is taught and what was achieved in the prior art that a MVA-based vaccine would generate an immune response that confers protection in non-human primates against filovirus infection, in particular against MARV. Of course, from the data generated by the present inventors and their observations, it is more than reasonable and plausible

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to conclude that the MVA-based vaccine would also induce an immune response in humans. Indeed, the FDA accepts non-human primate models as proof that a vaccine which confers protection in these non-human primates is likewise suitable in humans.

The present inventors have also found that a vaccination regime comprising a recombinant modified vaccinia virus Ankara (MVA) comprising a heterologous nucleotide sequence encoding an antigenic determinant of EBOV, such as, for example Sudan Ebola virus (SEBOV) and/or Zaire Ebola virus (ZEBOV) in combination with a recombinant modified FPV comprising a heterologous nucleotide sequence encoding an antigenic determinant of EBOV, for example Sudan Ebola virus (SEBOV) and/or Zaire Ebola virus (ZEBOV) provides a filoviral vaccine capable of inducing both cellular and humoral responses sufficient to confer protective immunity.

In the study underlying the present invention it has also been found that the use of a MVA vector comprising a nucleic acid encoding an antigenic protein of at least one filovirus subtype, in particular a filovirus glycoprotein, and a fowlpox vector comprising at least one nucleic acid encoding an antigenic protein of a first filovirus glycoprotein as a heterologous prime and boost generates a protective immune response against a filovirus immunogen by induction of a high level of antibody response and an up to 5-fold higher cytotoxic CD8 T cell response, in particular wherein the MVA vector was used as at least one prime composition and the fowlpox as a boost composition.

The recombinant MVA and/or FPV may be either monovalent, *i.e.*, comprising only one heterologous sequence encoding an antigenic determinant of EBOV, or multivalent, *i.e.,* comprising at least two heterologous sequences encoding antigenic determinants of EBOV.

The invention thus provides vaccines or vaccine combinations for use in generating an immune response that confers dual protection or cross protection against infections by at least two filovirus subtypes in particular Marburg virus and/or Ebola virus subtypes and vaccines or vaccine combinations which can be used for manufacturing of a vaccine against at least two filovirus subtypes in particular Marburg virus and/or Ebola virus subtypes. Thus, vaccines for cross-protection against filoviruses such as Ebola Zaire-Mayinga and Zaire-Kikwit and/or Marburg-Musoke and Marburg-Angola could be provided. It is now also discovered for the first time, that immunization with a MVA vector expressing certain antigens such as the VP40 protein of ZEBOV together with other heterologous nucleotide sequences encoding for at least one surface glycoprotein of a filovirus, in particular of ZEBOV, can generate filovirus-like particles *e.g.*, Ebola virus-like particles containing the filovirus glycoprotein on their surface. This was unexpected since it had been reported that transport of filoviral GP to the cell

surface was largely inhibited by MVA (Sänger et al. *J. Virol. Meth.* 81, 29-35 (2001)). However, since filovirus particle budding occurs at the cell surface (Noda et al., *PLoS Pathog.* 2(9):e99 (2006)) efficient GP surface transport is required for formation of GP-containing filovirus-VLP. In the study underlying the present invention the recombinant MVA expressing filovirus virion protein 40 (VP40) and a glycoprotein *e.g.*, GP-ZEBOV-Mayinga capable of producing VLPs induced an enhanced immune response with various prime-boost combinations and protected non-human primates against filovirus infection. The studies performed could also show that a homologous prime-boost based solely on recombinant MVA expressing a filovirus glycoprotein and a filovirus virion protein 40 (VP40) protein protected against a filovirus infection in non-human primates.

It has further been found that the use of a MVA vector comprising a nucleic acid encoding an antigenic glycoprotein of at least one filovirus subtype, in particular a glycoprotein of a Marburg virus and/or Ebola virus, and a nucleic acid encoding an antigenic protein of a virion protein 40 (VP40) as a heterologous prime boost with a fowlpox vector comprising at least one nucleic acid encoding an antigenic protein of a first filovirus glycoprotein generates an enhanced CD8 T cell response. In was further found that the use of a MVA vector comprising a nucleic acid encoding an antigenic glycoprotein of at least one filovirus subtype, in particular a glycoprotein of an Ebola virus and a nucleic acid encoding an antigenic protein of a virion protein 40 (VP40) induced a higher neutralizing antibody response in non-human primates *e.g.,* already after priming which was further improved after boosting and thus generates an immune response against one or more filovirus infections, in particular Zaire-Mayinga and Zaire-Kikwit. It has also been shown that immunization with a MVA vector expressing certain antigens such as the filovirus virion protein 40 (VP40) together with a filovirus glycoprotein can produce VPLs that express a filovirus envelope glycoprotein lining the entire surface of the VLPs which resemble intact filovirus virions. In this way, incorporation of a nucleic acid encoding for a filovirus VP40 protein into the MVA vector was shown to enhance the immune response of the viral vector expressing the antigenic protein or proteins, in particular the MVA vector.

Reference will now be made in detail to exemplary embodiments of the invention, examples of which are illustrated in the accompanying drawings.

Recombinant MVA virus

In one aspect, the present invention provides a recombinant modified vaccinia virus Ankara (MVA) comprising a nucleotide sequence encoding an antigenic determinant of a filovirus glycoprotein (GP), in particular an envelope glycoprotein. In another aspect, the present invention provides a recombinant MVA vector comprising a heterologous nucleotide sequence encoding an antigenic determinant of a filovirus glycoprotein, in particular an envelope glycoprotein, and a heterologous nucleotide sequence encoding an antigenic determinant of a further filovirus protein. MVA has been generated by more than 570 serial passages on chicken embryo fibroblasts of the dermal vaccinia strain Ankara (Chorioallantois vaccinia virus Ankara virus, CVA; for review see Mayr et al. (1975), *Infection* 3: 6-14) that was maintained in the Vaccination Institute, Ankara, Turkey for many years and used as the basis for vaccination of humans. However, due to the often severe post-vaccination complications associated with vaccinia viruses, there were several attempts to generate a more attenuated, safer smallpox vaccine.

During the period of 1960 to 1974, Prof. Anton Mayr succeeded in attenuating CVA by over 570 continuous passages in CEF cells (Mayr et al. (1975)). It was shown in a variety of animal models that the resulting MVA was avirulent (Mayr, A. & Danner, K. (1978), *Dev. Biol. Stand.* 41:225-234). As part of the early development of MVA as a pre-smallpox vaccine, there were clinical trials using MVA-517 in combination with Lister Elstree (Stickl (1974), *Prev. Med.* 3:97-101; Stickl and Hochstein-Mintzel (1971), *Munch. Med. Wochenschr.* 113:1149-1153) in subjects at risk for adverse reactions from vaccinia. In 1976, MVA derived from MVA-571 seed stock (corresponding to the 571st passage) was registered in Germany as the primer vaccine in a two-stage parenteral smallpox vaccination program. Subsequently, MVA-572 was used in approximately 120,000 Caucasian individuals, the majority children between 1 and 3 years of age, with no reported severe side effects, even though many of the subjects were among the population with high risk of complications associated with vaccinia (Mayr et al. (1978), *Zentralbl. Bacteriol.* (B) 167:375-390). MVA-572 was deposited at the European Collection of Animal Cell Cultures as ECACC V94012707.

As a result of the passaging used to attenuate MVA, there are a number of different strains or isolates, depending on the number of passages conducted in CEF cells. For example, MVA-572 was used in a small dose as a pre-vaccine in Germany during the smallpox eradication program, and MVA-575 was extensively used as a veterinary vaccine. MVA as well as MVA-BN lacks approximately 13% (26.6 kb from six regions) of the genome compared with ancestral CVA virus. The deletions affect a number of virulence and host range genes, as well as the gene for Type A inclusion bodies. MVA-

575 was deposited on December 7, 2000, at the European Collection of Animal Cell Cultures (ECACC) under Accession No. V00120707. The attenuated CVA-virus MVA (Modified Vaccinia Virus Ankara) was obtained by serial propagation (more than 570 passages) of the CVA on primary chicken embryo fibroblasts.

Even though Mayr et al. demonstrated during the 1970s that MVA is highly attenuated and avirulent in humans and mammals, certain investigators have reported that MVA is not fully attenuated in mammalian and human cell lines since residual replication might occur in these cells (Blanchard et al. (1998), *J. Gen. Virol.* 79:1159-1167; Carroll & Moss (1997), *Virology* 238:198-211; U.S. Patent No. 5,185,146; Ambrosini et al. (1999), *J. Neurosci. Res.* 55: 569). It is assumed that the results reported in these publications have been obtained with various known strains of MVA, since the viruses used essentially differ in their properties, particularly in their growth behaviour in various cell lines. Such residual replication is undesirable for various reasons, including safety concerns in connection with use in humans.

Strains of MVA having enhanced safety profiles for the development of safer products, such as vaccines or pharmaceuticals, have been developed by Bavarian Nordic: MVA was further passaged by Bavarian Nordic and is designated MVA-BN. A representative and preferred sample of MVA-BN was deposited on August 30, 2000 at the European Collection of Cell Cultures (ECACC) under Accession No. V00083008. MVA-BN is further described in WO 02/42480 (US 2003/0206926) and WO 03/048184 (US 2006/0159699).

MVA-BN can attach to and enter human cells where virally-encoded genes are expressed very efficiently. MVA-BN is strongly adapted to primary chicken embryo fibroblast (CEF) cells and does not replicate in human cells. In human cells, viral genes are expressed, and no infectious virus is produced. MVA-BN is classified as Biosafety Level 1 organism according to the Centers for Disease Control and Prevention in the United States. Preparations of MVA-BN and derivatives have been administered to many types of animals, and to more than 2000 human subjects, including immune-deficient individuals. All vaccinations have proven to be generally safe and well tolerated. Despite its high attenuation and reduced virulence, in preclinical studies MVA-BN has been shown to elicit both humoral and cellular immune responses to vaccinia and to heterologous gene products encoded by genes cloned into the MVA genome (E. Harrer et al. (2005), *Antivir. Ther.* 10(2):285-300; A. Cosma et al. (2003), *Vaccine* 22(1):21-9; M. Di Nicola et al. (2003), *Hum. Gene Ther.* 14(14):1347-1360; M. Di Nicola et al. (2004), *Clin. Cancer Res.*, 10(16):5381-5390).

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"Derivatives" or "variants" of MVA refer to viruses exhibiting essentially the same replication characteristics as MVA as described herein, but exhibiting differences in one or more parts of their genomes. MVA-BN as well as a derivative or variant of MVA-BN fails to reproductively replicate in vivo in humans and mice, even in severely immune suppressed mice. More specifically, MVA-BN or a derivative or variant of MVA-BN has preferably also the capability of reproductive replication in chicken embryo fibroblasts (CEF), but no capability of reproductive replication in the human keratinocyte cell line HaCaT (Boukamp et al (1988), *J. Cell Biol.* 106:761-771), the human bone osteosarcoma cell line 143B (ECACC Deposit No. 91112502), the human embryo kidney cell line 293 (ECACC Deposit No. 85120602), and the human cervix adenocarcinoma cell line HeLa (ATCC Deposit No. CCL-2). Additionally, a derivative or variant of MVA-BN has a virus amplification ratio at least two fold less, more preferably three-fold less than MVA-575 in Hela cells and HaCaT cell lines. Tests and assay for these properties of MVA variants are described in WO 02/42480 (US 2003/0206926) and WO 03/048184 (US 2006/0159699).

The term "not capable of reproductive replication" or "no capability of reproductive replication" is, for example, described in WO 02/42480, which also teaches how to obtain MVA having the desired properties as mentioned above. The term applies to a virus that has a virus amplification ratio at 4 days after infection of less than 1 using the assays described in WO 02/42480 or in U.S. Patent No. 6,761,893.

The term "fails to reproductively replicate" refers to a virus that has a virus amplification ratio at 4 days after infection of less than 1. Assays described in WO 02/42480 or in U.S. Patent No. 6,761,893 are applicable for the determination of the virus amplification ratio.

The amplification or replication of a virus is normally expressed as the ratio of virus produced from an infected cell (output) to the amount originally used to infect the cell in the first place (input) referred to as the "amplification ratio". An amplification ratio of "1" defines an amplification status where the amount of virus produced from the infected cells is the same as the amount initially used to infect the cells, meaning that the infected cells are permissive for virus infection and reproduction. In contrast, an amplification ratio of less than 1, *i.e.*, a decrease in output compared to the input level, indicates a lack of reproductive replication and therefore attenuation of the virus.

The advantages of MVA-based vaccine include their safety profile as well as availability for large scale vaccine production. Preclinical tests have revealed that MVA-BN demonstrates superior attenuation and efficacy compared to other MVA strains (WO 02/42480). An additional property of MVA-BN strains is the ability to induce

substantially the same level of immunity in vaccinia virus prime/vaccinia virus boost regimes when compared to DNA-prime/vaccinia virus boost regimes.

The recombinant MVA-BN viruses, the most preferred embodiment herein, are considered to be safe because of their distinct replication deficiency in mammalian cells and their well-established avirulence. Furthermore, in addition to its efficacy, the feasibility of industrial scale manufacturing can be beneficial. Additionally, MVA-based vaccines can deliver multiple heterologous antigens and allow for simultaneous induction of humoral and cellular immunity.

In a preferred embodiment, the recombinant MVA vector of any of the embodiments used for generating the recombinant virus is a MVA-BN virus or a derivative having the capability of reproductive replication in vitro in chicken embryo fibroblasts (CEF) cells, but no capability of reproductive replication in the human keratinocyte cell line HaCat, the human bone osteosarcoma cell line 143B, the human embryo kidney cell line 293, and the human cervix adenocarcinoma cell line HeLa.

In another embodiment, the recombinant MVA vector of any of the embodiments used for generating the recombinant virus is MVA-BN as deposited at the European Collection of Animal Cell cultures (ECACC) under accession number V00083008.

MVA vectors useful for the present invention can be prepared using methods known in the art, such as those described in WO 02/042480 and WO 02/24224.

In another aspect, a MVA viral strain suitable for generating the recombinant virus may be strain MVA-572, MVA-575 or any similarly attenuated MVA strain. Also suitable may be a mutant MVA, such as the deleted chorioallantois vaccinia virus Ankara (dCVA). A dCVA comprises del I, del II, del III, del IV, del V, and del VI deletion sites of the MVA genome. The sites are particularly useful for the insertion of multiple heterologous sequences. The dCVA can reproductively replicate (with an amplification ratio of greater than 10) in a human cell line (such as human 293, 143B, and MRC-5 cell lines), which then enable the optimization by further mutation useful for a virus-based vaccination strategy (see WO 2011/092029).

Recombinant FPV

In one aspect, the present invention provides a recombinant FPV comprising a nucleotide sequence encoding an antigenic determinant of a filovirus glycoprotein (GP), in particular an envelope glycoprotein. In another aspect, the present invention provides a recombinant FPV comprising a heterologous nucleotide sequence encoding an antigenic determinant of a filovirus glycoprotein, in particular an envelope

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glycoprotein, and a heterologous nucleotide sequence encoding an antigenic determinant of a further filovirus protein.

An FPV according to the invention is a prototype species within the genus of the Avipoxvirus. Numerous FPV strains are described and are available for example from CEVA Laboratories, Cynamid Webster, Fort Dodge, Intercontinental Laboratories, Intervet (NOBILIS VARIOLE), Merial (DIFTOSEC CT strain), Schering-Plough, Select Laboratories, Solvay, Syntro-Zeon and Vineland Laboratories. FP1 is a Duvette strain modified to be used as a vaccine in one day old chickens. The strain is a commercial fowlpox virus vaccine strain designated 0 DCEP 25/CEP67/2309 October, 1980 and is available from Institute Merieux, Inc. FP5 is a commercial fowlpox virus vaccine strain of chicken embryo origin available from American Scientific Laboratories (Division of Schering Corp.) Madison, Wisconsin, United States Veterinary License No. 165, serial No. 30321. Various attenuated strains of fowlpox virus are known such as FPV M (mild vaccine strain) and FPV S (standard vaccine strain) obtainable from Cyanamid Websters PtY, Ltd Australia. The US Department of Agriculture (USDA) challenge strain has been further described by C.L. Afonso et al., *J. Virol.* 74(8):3815-3831 (2000), 74(8):3815-3831 (2000). FP9 is a fowlpox strain used for vaccine purposes obtained in the late 1980s by Tomeley, Binns, Boursnell and Brown at the IAH Houghton Laboratories (St Ives, UK). It was derived from plaque purification of a virus that had been passaged 438 times in chicken embryo fibroblasts (CEF) culture from HP1 (A. Mayr & K. Malicki (1966), *Zentralbl Veterinarmed* (B) 13:1–13, Skinner et al. (2005), *Expert Res. Vaccines* 4(1):63-76). Other attenuated strains are POXVAC-TC as such described in S. Jenkins et al. (1991), *Aids Research and Human Retroviruses* 7(12):991:998. Deposited strains encompass for example fowlpox virus ATCC® VR-229 (typical fowlpox scabs from combs of chickens in New Jersey prior to 1928) and fowlpox virus ATCC® VR-250 (chicken, Kentucky, 1950).

In another aspect, a FPV viral strain suitable for generating the recombinant virus can be any strain mentioned *supra* or any similar FPV strain. In another aspect, the FPV is selected from the group of FP1, FP5, FP9, FPV M, FPV S, ATCC® VR-229, ATCC® VR-250, the USDA strain and POXVAC-TC. In yet another embodiment, the FPV of any of the embodiments is an attenuated FPV.

An advantage of FPV is that the virus causes disease only in avian species, but is able to enter and express transgenes in mammalian cells, while being immunologically non-cross-reactive with vaccinia virus and can thus escape pre-existing immunity in smallpox-experienced humans.

Recombinant FPV vectors suitable for generating the recombinant FPV can be constructed by well-established methods. Live attenuated fowlpox viruses may be produced by multiple passage of the virus in avian cells. The preparation of the FPV vectors is described, for example in Michael J.P. Lawman and Patricia D. Lawman (*eds.*) *Cancer Vaccines*: Method and Protocols, Methods in Molecular Biology, vol. 1139, Chapter 32 Paul M. Howley, Kerrilyn R. Diener and John D. Hayball p.407-427. The generation of recombinant FPV useful for virus-based vaccination strategy has also been described in EP 0 284 416 B1, WO 88/02022, WO 89/03429, WO 89/03879, WO89/07644, WO 89/12684, WO 90/02191, WO 91/02072, WO 89/03879 and WO 94/019014. The genome sequence and genome organization has been described by Afonso et al. and Laidlaw and Skinner (C.L. Afonso et al. (2000), *J. Virol.* 74(8):3815-3831, S.M. Laidlaw and M.A. Skinner (2004), *Journal of General Virology* 85:305-322). An exemplary genome sequence of FPV can be found in GenBank Accession No. AF198100.1.

Antigenic Determinants

The term "antigenic determinant" refers to any molecule that stimulates a host's immune system to make an antigen-specific immune response, whether a cellular response or a humoral antibody response. Antigenic determinants may include proteins, polypeptides, antigenic protein fragments, antigens, and epitopes which still elicit an immune response in a host and form part of an antigen, homologues or variants of proteins, polypeptides, and antigenic protein fragments, antigens and epitopes including, for example, glycosylated proteins, polypeptides, antigenic protein fragments, antigens and epitopes, and nucleotide sequences encoding such molecules. Thus, proteins, polypeptides, antigenic protein fragments, antigens and epitopes are not limited to particular native nucleotide or amino acid sequences but encompass sequences identical to the native sequence as well as modifications to the native sequence, such as deletions, additions, insertions and substitutions.

The term "epitope" refers to a site on an antigen to which B- and/or T-cells respond, either alone or in conjunction with another protein such as, for example, a major histocompatibility complex ("MHC") protein or a T-cell receptor. Epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by secondary and/or tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents, while epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 5, 6, 7, 8, 9, 10 or more amino acids - but generally less than 20 amino acids - in a unique spatial conformation. Methods of determining spatial

conformation of epitopes include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, *e.g.*, "Epitope Mapping Protocols" in *Methods in Molecular Biology*, Vol. 66, Glenn E. Morris, Ed (1996).

Preferably, a homologue or variant has at least about 50%, at least about 60% or 65%, at least about 70% or 75%, at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, or 89%, more typically, at least about 90%, 91%, 92%, 93%, or 94% and even more typically at least about 95%, 96%, 97%, 98% or 99%, most typically, at least about 99% identity with the referenced protein, polypeptide, antigenic protein fragment, antigen and epitope at the level of nucleotide or amino acid sequence.

Techniques for determining sequence identity between nucleic acids and amino acids are known in the art. Two or more sequences can be compared by determining their "percent identity." The percent identity of two sequences, whether nucleic acid or amino acid sequences, is the number of exact matches between two aligned sequences divided by the length of the shorter sequences and multiplied by 100.

"Percent (%) amino acid sequence identity" with respect to proteins, polypeptides, antigenic protein fragments, antigens and epitopes described herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference sequence (*i.e.*, the protein, polypeptide, antigenic protein fragment, antigen or epitope from which it is derived), 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 example, using publically available computer software such as BLAST, ALIGN, or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximum alignment over the full-length of the sequences being compared.

The same applies to "percent (%) nucleotide sequence identity", *mutatis mutandis*.

For example, an appropriate alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman, (1981), *Advances in Applied Mathematics* 2:482-489. This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff, Atlas of Protein Sequences and Structure, M. O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA, and normalized by Gribskov (1986), *Nucl. Acids Res.* 14(6):6745-6763. An exemplary implementation of this algorithm to determine

percent identity of a sequence is provided by the Genetics Computer Group (Madison, Wis.) in the "BestFit" utility application. The default parameters for this method are described in the Wisconsin Sequence Analysis Package Program Manual, Version 8 (1995) (available from Genetics Computer Group, Madison, Wis.). A preferred method of establishing percent identity in the context of the present invention is to use the MPSRCH package of programs copyrighted by the University of Edinburgh, developed by John F. Collins and Shane S. Sturrok, and distributed by IntelliGenetics, Inc. (Mountain View, California). From this suite of packages the Smith-Waterman algorithm can be employed where default parameters are used for the scoring table (for example, gap open penalty of 12, gap extension penalty of one, and a gap of six). From the data generated the "Match" value reflects "sequence identity." Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code=standard; filter=none; strand=both; cutoff=60; expect=10; Matrix=BLOSUM62; Descriptions=50 sequences; sort by=HIGH SCORE; Databases=non-redundant, GenBank+EMBL+DDBJ+PDB+ GenBank CDS translations+Swiss protein+Spupdate+PIR. Details of these programs can be found at the following internet address: http://www.ncbi.nlm.gov/cgi-bin/BLAST.

In some embodiments, the heterologous nucleic acid encodes antigenic domains or antigenic protein fragments rather than the entire antigenic protein. These fragments can be of any length sufficient to be antigenic or immunogenic. Fragments can be at least 8 amino acids long, preferably 10-20 amino acids, but can be longer, such as, *e.g.*, at least 50, 100, 200, 500, 600, 800, 1000, 1200, 1600, 2000 amino acids long, or any length in between.

In some embodiments, at least one nucleic acid fragment encoding an antigenic protein fragment or immunogenic polypeptide thereof is inserted into the viral vector of the invention. In another embodiment, about 2-6 different nucleic acids encoding different antigenic proteins are inserted into one or more of the viral vectors. In some embodiments, multiple immunogenic fragments or subunits of various proteins can be used. For example, several different epitopes from different sites of a single protein or from different proteins of the same strain, or from a protein orthologue from different strains can be expressed from the vectors.

Definitions

It must be noted that, as used herein, the singular forms "a", "an", and "the", include plural references unless the context clearly indicates otherwise. Thus, for example,

reference to "an antigenic determinant" includes one or more antigenic determinants and reference to "the method" includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

Unless otherwise indicated, the term "at least" preceding a series of elements is to be understood to refer to every element in the series. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the present invention.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integer or step. When used herein the term "comprising" can be substituted with the term "containing" or "including" or sometimes when used herein with the term "having". Any of the aforementioned terms (comprising, containing, including, having), whenever used herein in the context of an aspect or embodiment of the present invention may be substituted with the term "consisting of", though less preferred.

When used herein "consisting of" excludes any element, step, or ingredient not specified in the claim element. When used herein, "consisting essentially of" does not exclude materials or steps that do not materially affect the basic and novel characteristics of the claim.

As used herein, the conjunctive term "and/or" between multiple recited elements is understood as encompassing both individual and combined options. For instance, where two elements are conjoined by "and/or", a first option refers to the applicability of the first element without the second. A second option refers to the applicability of the second element without the first. A third option refers to the applicability of the first and second elements together. Any one of these options is understood to fall within the meaning, and therefore satisfy the requirement of the term "and/or" as used herein. Concurrent applicability of more than one of the options is also understood to fall within the meaning, and therefore satisfy the requirement of the term "and/or."

Several documents are cited throughout the text of this specification. To the extent the cited material

contradicts or is inconsistent with this specification, the specification will supersede any such material. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

The term "substantially similar" in the context of the filovirus antigenic proteins of the invention indicates that a polypeptide comprises a sequence with at least 90%, preferably at least 95% sequence identity to the reference sequence over a comparison window of 10-20 amino acids. Percentage of sequence identity is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

The term “subtype” herein can be replaced with “species”. It includes strains, isolates, clades or variants of any filovirus such as Marburg or Ebola virus. The terms “strain” “clade” or “isolate” are technical terms, well known to the practitioner, referring to the taxonomy of microorganisms. The taxonomic system classifies all so far characterised microorganisms into the hierarchic order of Families, Genera, Species, Strains (Fields Virology, ed. by Fields B. N., Lippincott-Raven Publishers, 4th edition 2001). While the criteria for the members of a Family is their phylogenetic relationship, a Genera comprises all members which share common characteristics, and a Species is defined as a polythetic class that constitutes a replicating lineage and occupies a particular ecological niche. The term “strain” or “clade” describes a microorganism, *i.e.*, virus, which shares common characteristics, like basic morphology or genome structure and organization, but varies in biological properties, like host range, tissue tropism, geographic distribution, attenuation or pathogenicity. For example there are five Ebola virus subtypes known, *i.e.,* Zaire Ebola virus, Sudan Ebola virus, Reston Ebola virus, Bundibugyo Ebola virus and Ivory Coast Ebola virus. Zaire Ebola virus strains are for example Zaire-Mayinga, Zaire-Kikwit, Zaire-Gabon (1994), Zaire-Gabon (Feb. 1996), Zaire-Gabon (Oct. 1996). There is only one Marburg virus subtype or species *i.e.,* Lake Victoria marburgvirus know so far with the strains including Marburg-Musoke and Marburg-Angola. For further strains or isolates see also Figure 1.

The term “$TCID_{50}$” is the abbreviation of "tissue culture infectious dose", that amount of a pathogenic agent that will produce pathological change in 50% of cell cultures inoculated,

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The term "$TCID_{50}$" is the abbreviation of "tissue culture infectious dose", that amount of a pathogenic agent that will produce pathological change in 50% of cell cultures inoculated, expressed as $TCID_{50}$/ml. A method for determining $TCID_{50}$ is well known to the person skilled in the art. It is for example described in *e.g.*, Example 2 of WO 03/053463.

The term "subject" as used herein is a living multi-cellular vertebrate organisms, including, for example, humans, non-human mammals and (non-human) primates. The term "subject" may be used interchangeably with the term "animal" herein.

The term "filovirus-caused disease" referred to in any of the embodiments can be any disease caused by an infection of any filovirus strain, isolate or variant thereof as mentioned herein or any combination of any filovirus strain, isolate or variant (as mentioned anywhere *supra* or *infra* and/or in any of the embodiments *supra* or *infra*) thereof.

As used herein, the term "enhanced" when used with respect to an immune response against a filovirus, such as an antibody response (*e.g.,* neutralizing antigen specific antibody response or ZEBOV-GP-specific antibody response), a cytokine response or a CD8 T cell response (*e.g.,* immunodominant CD8 T cell response), refers to an increase in the immune response in an animal administered with a homologous prime-boost combination vaccine of MVA relative to the corresponding immune response observed from the animal administered with a homologous prime-boost combination vaccine of MVA vectors, wherein the MVA vectors do not express any filovirus virion protein 40 or refers to an increase in the immune response in an animal administered with a heterologous prime-boost combination vaccine of MVA and FPV vectors according to the invention, relative to the corresponding immune response observed from the animal administered with a heterologous prime-boost combination vaccine of MVA and FPV vectors according to the invention, wherein the MVA vector does not express any filovirus virion protein 40. Preferably, "enhanced" when used with respect to an immune response, such as an antibody response *e.g.,* neutralizing antibody response, a cytokine response or a CD8 T cell response, refers to an increase in the immune response in an animal administered with a heterologous prime-boost combination vaccine of MVA as a prime and FPV vectors as boost according to the invention, relative to the corresponding immune response observed from the animal administered with a reverse prime-boost combination, wherein the FPV vector is provided as a prime and the MVA vector is provided to boost the immune response, using the same prime-boost interval.

In the context of this invention, an "immunodominant CD8 T cell response" means the

major CD8 T cell response of a host against a recombinant antigen encoded by a MVA and/or FPV vector. Thus, an immunodominant CD8 T cell response against a recombinant antigen encoded by a homologous prime-boost of recombinant MVA or heterologous prime-boost of recombinant MVA and FPV can be generated that is greater than the CD8 T cell response against any recombinant antigen of the recombinant MVA or FPV, wherein the MVA vector does not express any filovirus virion protein 40.

The level of the CD8 T cell response can be determined by methods well known in the art such as but not limited to an ELISPOT assay (*e.g.,* interferon gamma (IFN-γ) ELISPOT. Protocols are for examples described in Current Protocols in Immunology (John Wiley & Son, Inc. (1994) (see, *e.g.*, Chapter 6, Section 19: ELISPOPT Assay to Detect Cytokine-secreting Murine and Human Cells, Supplement 10) or by Schneider, et al., *Nat. Med.* 4:397–402 (1998)) and, for example, by the techniques set forth in the examples for a specific virus of the invention. Other suitable assays comprise an ICS assay, which analyzes levels of intracellular cytokine for CD8 T cell activity. For example, the CD8 T cell response can comprise an antigen specific CD8 T cell response that is more than 50%, such as 51%, 60%, 70%, 80%, 90% or 100% of the total antigen specific T-cell responses in the animal subject. Preferably, the CD8 T cell response also represents 0.1% or more, such as 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, or more of the total cytokine responses in the animal subject. In some embodiments, after the second or third boost, the recombinant viral vectors according to the invention induce a CD8 T cell response in the host against the encoded antigen that is at least 0,5%, 1%, 5%, 10%, 15%, 20%, 25%, or 30% of the total CD8 T cell compartment.

The level of antibody responses can be determined by methods known in the art. Any suitable plaque reduction neutralization titer (PRNT) assay can be used to determine whether a polypeptide (or polynucleotide expressing such a polypeptide) induces one or more neutralizing antibodies against one or more filovirus antigens of one or more filovirus subtype. An exemplary plaque reduction neutralization titer assay for filoviruses is described in the examples. Other PRNT methods and formats are well known to those of ordinary skill in the art.

Filovirus Proteins

As used interchangeably herein, the terms “glycoprotein gene” or “GP gene” refer to the gene, or to a homologue or variant of the gene, encoding the glycoprotein, in particular the transmembrane envelope glycoprotein, in any filovirus strain or isolate, even though the exact sequence and/or genomic location of the glycoprotein gene may

differ between strains or isolates. For example, in the Maleo strain of SEBOV (SEBOV-Maleo), the glycoprotein gene (GP-SEBOV-Maleo gene) comprises nucleotides 120-1004 and 1004-2149 (endpoints included) as numbered in GenBank Accession Number U23069.1. The EBOV transcripts undergo editing during transcription such that some nucleotides are read twice. The GP-SEBOV-Maleo gene further comprises a protein coding open reading frame (ORF) spanning nucleotides 120-1004 and 1004-2149 (endpoints included) as numbered in GenBank Accession Number U23069.1. The nucleotide sequence of the GP-SEBOV-Maleo gene is set forth in SEQ ID NO:1 (GenBank Accession No. U23069.1).

As used herein, a "homologue" or "variant" preferably has at least about 50%, at least about 60% or 65%, at least about 70% or 75%, at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, or 89%, more typically, at least about 90%, 91%, 92%, 93%, or 94% and even more typically at least about 95%, 96%, 97%, 98% or 99%, most typically, at least about 99% nucleotide sequence identity with the referenced gene, protein, polypeptide, antigenic protein fragment, antigen and epitope. The term "homologue" or "variant" also encompasses deleted, truncated or otherwise mutated versions of the genes and proteins, respectively. By way of example, encompassed are, *e.g.*, soluble forms of the GP-EBOV or GP-MARV proteins lacking the signal peptide as well as the transmembrane and/or cytoplasmic domains of the full-length GP-EBOV or GP-MARV proteins.

As used interchangeably herein, the terms "glycoprotein" or "GP" refer to the glycoprotein, in particular the transmembrane envelope glycoprotein, or to a homologue or variant of the glycoprotein.

The amino acid sequence of GP-EBOV-Maleo is set forth in SEQ ID NO:2 (amino acid sequence of GenBank Accession No. U23069.1). The GP-SEBOV-Maleo protein comprises a signal peptide, an extracellular domain, a transmembrane domain, and a cytoplasmic domain (*see, e.g.*, UniProtKB/Swiss-Prot Accession No. Q66798). The signal peptide of GP-SEBOV-Maleo protein consists of amino acids 1-32 of SEQ ID NO:2; the extracellular domain of GP-SEBOV-Maleo protein consists of amino acids 33-650 of SEQ ID NO:2 or amino acids 1-650 of SEQ ID NO:2; the transmembrane domain of GP-SEBOV-Maleo protein consists of amino acids 651-671 of SEQ ID NO:2; and the cytoplasmic domain of GP-SEBOV-Maleo protein consists of amino acids 672-676 of SEQ ID NO:2.

The nucleic acid encoding the amino acid sequence of GP-ZEBOV-Mayinga is set forth in SEQ ID NO:19. The GP-ZEBOV-Mayinga comprises a protein as set forth in SEQ ID NO:20 (GenBank Accession Number ABX75367.1).

Likewise, also the terms "nucleoprotein gene" or "NP gene", as used interchangeably herein, refer to the gene, or to a homologue or variant of the gene, encoding the nucleoprotein in any filovirus strain or isolate, even though the exact sequence and/or genomic location of the nucleoprotein gene may also differ between strains or isolates. For example, in the Boniface strain of SEBOV (SEBOV-Boniface), the nucleoprotein gene (NP-SEBOV-Boniface gene) comprises nucleotides 383-2599 (endpoints included) as numbered in GenBank Accession Number AF173836.1. The NP-SEBOV-Boniface gene further comprises a protein coding open reading frame (ORF) spanning nucleotides 383-2599 (endpoints included) as numbered in GenBank Accession Number AF173836.1. The nucleotide sequence of the NP-SEBOV-Boniface gene is set forth in SEQ ID NO:3 (GenBank Accession No. AF173836.1).

The amino acid sequence of NP-EBOV-Boniface is set forth in SEQ ID NO:4 (amino acid sequence of GenBank Accession No. AF173836.1). The NP-SEBOV-Boniface protein comprises a coiled coil domain (*see, e.g.*, UniProtKB/Swiss-Prot Accession No. Q9QP77). The coiled coil domain of NP-SEBOV-Boniface protein consists of amino acids 334-363 of SEQ ID NO:4.

In certain embodiments, the nucleic acid encoding an antigenic determinant, preferably an antigenic protein, more preferably of any of the proteins as mentioned *supra* or *infra* is a full-length protein.

Recombinant MVA and FPV

Provided herein are recombinant poxviruses (*e.g.*, MVA or MVA-BN or FPV) comprising heterologous or foreign nucleic acid sequences derived from EBOV and/or MARV incorporated in a variety of insertion sites in the poxviral (*e.g.*, MVA or MVA-BN or FPV) genome. The heterologous nucleic acids can encode one or more foreign proteins and/or foreign antigens including, for example, viral antigens.

Generally, a "recombinant" MVA or FPV as described herein refers to MVAs/FPVs that are produced by standard genetic engineering methods, *i.e.*, MVAs/FPVs of the present invention are thus genetically engineered or genetically modified MVAs/FPCs. The term "recombinant MVA or FPV" thus includes MVAs/FPVs which have stably integrated recombinant nucleic acid, preferably in the form of a transcriptional unit, in their genome. A transcriptional unit may include a promoter, enhancer, terminator and/or silencer. Recombinant MVAs/FPVs of the present invention may express heterologous antigenic determinants, polypeptides or proteins (antigens) upon induction of the regulatory elements. The term "MVA/FPV" in the context of any of the

embodiments of the invention encompasses both individual and combined options for MVA, FPV or MVA and FPV.

As used herein, a "heterologous" gene, nucleic acid, antigen, or protein is understood to be a nucleic acid or amino acid sequence which is not present in the wild-type poxviral genome (*e.g.*, MVA or MVA-BN or FPV). The skilled person understands that a "heterologous gene", when present in a poxvirus such as MVA or MVA-BN or FPV, is to be incorporated into the poxviral genome in such a way that, following administration of the recombinant poxvirus to a host cell, it is expressed as the corresponding heterologous gene product, *i.e.*, as the "heterologous antigen" and/or "heterologous protein." Expression is normally achieved by operatively linking the heterologous gene to regulatory elements that allow expression in the poxvirus-infected cell. Preferably, the regulatory elements include a natural or synthetic poxviral promoter.

In one aspect, the recombinant MVA/FPV vector according to the invention comprises a heterologous nucleotide sequence encoding an antigenic determinant of a filovirus protein selected from an Ebola virus (EBOV) and/or a Marburg virus (MARV). In another embodiment, the recombinant MVA/FPV vector according to the invention comprises a heterologous nucleotide sequence encoding an antigenic determinant of one or more antigenic determinant(s) of the filovirus protein (*e.g.*, EBOV protein) which is selected from one or more EBOV subtypes selected from the group consisting of Zaire Ebola virus (ZEBOV), Sudan Ebola virus (SEBOV), Cote d'Ivoire Ebola virus (EBOV-Cdl, also called Tai Forest virus or TAFV), Reston Ebola virus (REBOV) and Bundibugyo Ebola virus (BEBOV).

According to another embodiment, the recombinant MVA/FPV vector according to the invention comprises one or more antigenic determinant(s) of a filovirus protein, preferably EBOV protein, MARV protein or full-length protein thereof, selected from the group of Zaire-Mayinga, Zaire-Kikwit, Zaire-Gabon, Cote d'Ivoire Ebola virus, Sudan-Boniface, Sudan-Maleo, Sudan-Gulu, Marburg-Ravn, Marburg-Ozolin, Marburg-Ratayczak, Marburg-Musoke, Marburg-Angola.

Preferably, the antigenic determinant of the filovirus protein (*e.g.*, selected from the group of Zaire-Mayinga, Zaire-Kikwit, Zaire-Gabon, Cote d'Ivoire Ebola virus, Sudan-Boniface, Sudan-Maleo, Sudan-Gulu, Marburg-Ravn, Marburg-Ozolin, Marburg-Ratayczak, Marburg-Musoke, Marburg-Angola) is selected from the group consisting of an envelope glycoprotein (GP), nucleoprotein (NP), virion protein 35 (VP35), virion protein 40 (VP40), virion protein 30 (VP30), virion protein 24 (VP24), and RNA-directed RNA polymerase protein (L).

In another embodiment, the antigenic determinant of the filovirus protein is an envelope glycoprotein (GP), preferably at least an envelope glycoprotein (GP) and a virion protein 40 (VP40).

In another embodiment, the antigenic determinant of the filovirus protein is an envelope glycoprotein (GP) selected from the group of ZEVOV and SEBOV, preferably at least an envelope glycoprotein (GP) and a virion protein 40 (VP40), wherein the GP and VP40 are derived from the same strain, preferably wherein the same strain is selected from the group of ZEBOV and SEBOV.

In another embodiment, the antigenic determinant of the filovirus protein is at least an envelope glycoprotein (GP) and a virion protein 40 (VP40), wherein the GP and VP40 are derived from a different isolate or the same isolate, preferably wherein the different or the same isolate is selected from the group of Zaire-Mayinga, Zaire-Kikwit, Zaire-Gabon, Cote d'Ivoire Ebola virus, Sudan-Boniface, Sudan-Maleo, Sudan-Gulu, Marburg-Ravn, Marburg-Ozolin, Marburg-Ratayczak, Marburg-Musoke and Marburg-Angola, preferably wherein the isolate is selected from the group of Zaire-Mayinga, Sudan-Gulu, Marburg-Musoke and Marburg-Angola, most preferably wherein the isolate is selected from the group of Zaire-Mayinga, Sudan-Gulu and Marburg-Musoke.

In another preferred embodiment, the recombinant MVA/FPV vector according to the invention comprises a nucleotide sequence encoding an antigenic determinant of two, three, four or more Ebola and/or Marburg subtypes.

Another preferred embodiment covers the recombinant MVA/FPV vector according to any of the embodiments of the invention which comprises an antigenic determinant of two, three, four or more filovirus proteins selected from the group consisting of envelope glycoprotein (GP), nucleoprotein (NP), virion protein 35 (VP35), virion protein 40 (VP40), virion protein 30 (VP30), virion protein 24 (VP24), and RNA-directed RNA polymerase protein (L).

In a preferred embodiment, the recombinant MVA/FPV vector according to any of the embodiments of the invention comprises an antigenic determinant of one, two, three, four or more filovirus protein selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:20, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:34 and SEQ ID NO:37.

In a preferred embodiment, the recombinant MVA/FPV vector according to any of the embodiments of the invention comprises an antigenic determinant of a filovirus protein selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:20, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:34 and SEQ ID NO:37.

In another embodiment, the recombinant MVA/FPV vector according to any of the embodiments of the invention comprises an antigenic determinant of a filovirus protein consisting of SEQ ID NO: 20.

In another embodiment, the recombinant MVA/FPV vector according to any of the embodiments of the invention comprises an antigenic determinant of a filovirus protein selected from the group consisting of SEQ ID NO: 20 and SEQ ID NO: 34.

In another embodiment, the recombinant MVA/FPV vector according to any of the embodiments of the invention comprises an antigenic determinant of a filovirus protein selected from the group consisting of SEQ ID NO:6, SEQ ID NO:20, SEQ ID NO:31 and SEQ ID NO:34.

In another embodiment, the recombinant MVA/FPV vector according to any of the embodiments of the invention comprises an antigenic determinant of a filovirus protein selected from the group consisting of SEQ ID NO:6, SEQ ID NO: 20, SEQ ID NO:29, SEQ ID NO:31 and SEQ ID NO:34.

In another preferred embodiment, the recombinant MVA/FPV vector according to any of the embodiments of the invention comprises an antigenic determinant of a filovirus protein selected from the group consisting of SEQ ID NO:6, SEQ ID NO:20, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:34 and SEQ ID NO:37.

In another preferred embodiment, the MVA vector according to any of the embodiments of the invention comprises a heterologous nucleotide sequence encoding an antigenic determinant of a filovirus protein consisting of SEQ ID NO:29 and/or SEQ ID NO:6, SEQ ID NO:20, SEQ ID NO:31.

In another preferred embodiment, the MVA vector according to any of the embodiments of the invention comprises a nucleotide sequence comprising SEQ ID NO:28 and/or SEQ ID NO:5, SEQ ID NO:19, SEQ ID NO:30.

In another preferred embodiment, the MVA vector according to any of the embodiments of the invention comprises a nucleotide sequence encoding an antigenic protein of a filovirus virion protein 40 (VP40) comprising SEQ ID NO:33 or a nucleotide sequence encoding the protein sequence comprising SEQ ID NO:34.

In another preferred embodiment, the recombinant MVA vector according to any of the embodiments of the invention comprising a nucleotide sequence selected from the group of a) SEQ ID NO:5, SEQ ID NO:19 and SEQ ID NO:30, b) SEQ ID NO:5, SEQ ID NO:19, SEQ ID NO:28 and SEQ ID NO:30 and c) SEQ ID NO:19 and SEQ ID NO:33.

In another aspect, the present invention comprises a recombinant MVA vector or FPV vector comprising a heterologous nucleotide sequence encoding an antigenic determinant of a filovirus glycoprotein, in particular a filovirus envelope glycoprotein.

The filovirus glycoprotein may encode a GP-MARV or a GP-EBOV.

For the embodiments as described herein the glycoprotein of MARV may be derived from MARV-Musoke, preferably the full-length MARV-Musoke, which, in turn, may be derived from the Lake Victoria strain or isolate of MARV-Musoke. The GP-MARV may also be derived from MARV-Ravn MARV-Ozolin, MARV-Ratayczak or from MARV-Angola. A nucleotide sequence encoding a full-length GP-MARV-Musoke is shown in SEQ ID NO:5 encoding amino acids 1 to 681 or 19 to 681 of SEQ ID NO:6. In a preferred embodiment, the GP-MARV-Musoke comprises the nucleotide sequence of SEQ ID NO:5 preferably encoding the protein of SEQ ID NO:6. In certain embodiments, the GP-MARV-Musoke is truncated wherein the truncated GP-MARV-Musoke may comprise only the extracellular domain of the envelope glycoprotein, comprising amino acids 1 to 648 or amino acids 19 to 648 of SEQ ID NO:6 (GenBank Accession No. ABA87127.1). In other embodiments as described herein the glycoprotein of MARV may be derived from MARV-Angola, preferably the full-length GP-MARV-Angola. In a preferred embodiment, the GP-MARV-Angola comprises the nucleotide sequence of SEQ ID NO:36 encoding amino acids of SEQ ID NO:37.

The glycoprotein of EBOV may be GP-SEBOV or may be derived from GP-ZEBOV, in particular from the Mayinga strain of GP-ZEBOV (GP-ZEBOV-Mayinga). The full-length GP-ZEBOV-Mayinga comprises the nucleotide sequence of SEQ ID NO:19 encoding the amino acid sequence of SEQ ID NO:20. In a preferred embodiment, the GP-ZEBOV-Mayinga comprises the nucleotide sequence of SEQ ID NO:19 preferably encoding the protein of SEQ ID NO:20. The GP-EBOV may also be GP-BEBOV, GP-EBOV-Cdl or GP-EBOV-Reston. The GP-ZEBOV may be truncated and may comprise the nucleotide sequence of SEQ ID NO:19 modified to encode amino acids 1-636 of SEQ ID NO:20 or modified to delete the mucin domain spanning amino acids 314 to 464 of SEQ ID NO:20.

The GP-SEBOV may be derived from the Gulu strain of GP-SEBOV (GP-SEBOV-Gulu). In certain embodiments, the GP-SEBOV comprises the nucleotide sequence of SEQ ID NO:30, preferably encoding the amino acid sequence of SEQ ID NO:31.

The recombinant MVA/FPV according to the present invention can also further comprise tetanus toxoid fragment C sequence. In a preferred embodiment, GP-MARV-Musoke, in particular the full-length MARV-Musoke GP, further comprises tetanus

toxoid fragment C. A tetanus toxoid fragment C may comprise the nucleotide sequence of SEQ ID NO:7 encoding the amino acid sequence of SEQ ID NO:8. In certain embodiments, the truncated GP-MARV-Musoke further comprises tetanus toxoid fragment C (TTC) which may comprise the nucleotides 2281-3642 of the nucleotide sequence of SEQ ID NO:7 encoding amino acids 760-1213 of the amino acid sequence of SEQ ID NO:8.

The recombinant MVA/FPV vector according to the present invention can additionally comprise an immunostimulatory or co-stimulatory molecule. In a preferred embodiment, the heterologous nucleotide sequence encoding an antigenic determinant of a GP-MARV-Musoke further comprises one or more immunostimulatory molecules. In certain embodiments, the one or more immunostimulatory molecules is human CD40 ligand (hCD40L) which may comprise SEQ ID NO:9 encoding the amino acid sequence of SEQ ID NO:10. In certain embodiments, the one or more immunostimulatory molecule(s) is a fusion protein comprising the sushi domain of human interleukin-15 receptor (hIL15R-Sushi) which may comprise SEQ ID NO:11 encoding the amino acid sequence of SEQ ID NO:12.

The one or more immunostimulatory molecules may also be lymphocyte function-associated antigen 3 (LFA-3, or CD58), intercellular adhesion molecule 1 (ICAM-1, or CD54) and B7.1 (CD80), collectively known as the triad of costimulatory molecules (*i.e.*, 'TRICOM'). "TRICOM" as used herein is an abbreviation for Triad of COstimlatory Molecules consisting of B7-1 (also known as CD80), intracellular adhesion molecule-1 (ICAM-1, also known as CD54) and lymphocyte function-associated antigen-3 (LFA-3, also known as CD58), included in the recombinant viral vectors (*e.g.*, poxviral vectors) expressing a specific antigen in order to increase the antigen-specific immune response. The individual components of TRICOM can be under the control of the same or different promoters, and can be provided on the same vector with the specific antigen or on a separate vector. Exemplary vectors are disclosed, for example, in Hodge et al., "A Triad of Costimulatory Molecules Synergize to Amplify T-Cell Activation," *Cancer Res.* 59:5800-5807 (1999) and U.S. Patent No. 7,211,432 B2. The LFA-3 may comprise the nucleotide sequence of SEQ ID NO:13 encoding the amino acid sequence of SEQ ID NO:14, the ICAM-1 may comprise the nucleotide sequence of SEQ ID NO:15 encoding the amino acid sequence of SEQ ID NO:16, and the B7.1 may comprise the nucleotide sequence of SEQ ID NO:17 encoding the amino acid sequence of SEQ ID NO:18.

The recombinant MVA/FPV according to the present invention may also additionally comprise a membrane anchor sequence such as the vaccinia virus gene B5m

comprising the nucleotide sequence of SEQ ID NO:21 encoding the amino acid sequence of SEQ ID NO:22. In particular, the antigenic determinant as described herein may preferably be operably linked to a membrane anchor such as the B5m. Thus, when used herein that the recombinant MVA/FPV comprises a membrane anchor sequence, it is meant that an antigenic determinant comprised by the recombinant MVA/FPV is preferably operably linked to the membrane anchor. A membrane anchor refers to any polypeptide capable of anchoring heterologous polypeptides to the outer face of the cell membrane. Preferably, the membrane anchor comprises the cytoplasmic and transmembrane domains of Vaccinia virus B5R protein, termed herein as the "B5R anchor" or "B5m" As defined, a B5R anchor refers to the 42-amino-acid C-terminal segment of the B5R protein from any type of Vaccinia virus, for example, the WR strain (Katz et al. *J Virol.* 71(4):3178-87 (1997)) or more preferably a MVA. In addition, B5R anchor variants having at least 80%, such as at least 85%, for example at least 90%, or at least 95%, such as at least 98% sequence identity with respect to the reference B5R anchor sequence are also included in the present invention. A preferred anchor sequence is shown in SEQ ID NO: 21, its translation product is also shown in SEQ ID NO: 22.

In a preferred embodiment, the full-length and/or truncated GP-ZEBOV further comprises vaccinia virus gene B5m.

In another aspect, the present invention comprises a recombinant MVA/FPV vector comprising a heterologous nucleotide sequence encoding an antigenic determinant of a filovirus glycoprotein as described above, and further comprises heterologous nucleotide sequences encoding additional filovirus proteins required to form virus-like particles (VLP). In one embodiment, the additional heterologous nucleotide sequence encoding filovirus protein required to form VLPs can be VP40. In certain embodiments, the additional filovirus proteins required to form virus-like particles or enhancing formation of VLPs are NP-EBOV and VP40-EBOV wherein these proteins may be derived from the strains as indicated above. Preferably, the filovirus nucleoprotein (*e.g.*, NP-EBOV) and a filovirus virion protein 40 (*e.g.*, VP40-EBOV) are derived from the same filovirus strain. By vaccinating non-human primates with a recombinant MVA expressing GP and VP40 (either in addition or without expressing NP) and which is capable of generating GP-containing EBOV-VLPs from infected cells the inventors could achieve protection against filovirus challenge in non-human primates. The production of virus-like particles in the animals being vaccinated creates an additional vaccine modality closely mimicking the viral particles present in a *bona fide* filoviral infection. Such recombinant MVA filo VLP vaccination stimulated both the humoral and

cellular immune response and thus protected against filovirus challenge. A further advantage of vaccination with an attenuated MVA virus providing filoviral VLPs is to circumvent the need for purification of virus-like particles for inoculation and the additional MVA mediated immune stimulation. The use of a filovirus nucleoprotein (*e.g.*, NP-EBOV) and a filovirus virion protein 40 (*e.g.*, VP40-EBOV) derived from the same strain is of advantage for enhancing the formation of VLPs, preferably for generating homogenous GP spike decorated VLPs with a homogenous diameter for closely mimicking the viral particles and improving protection against a filovirus infection.

The present invention also relates to a recombinant MVA/FPV vector comprising a heterologous nucleotide sequence encoding an antigenic determinant of a filovirus glycoprotein and a heterologous nucleotide sequence encoding an antigenic determinant of a further filovirus protein. The nucleotide sequence encoding an antigenic determinant of a further filovirus protein may encode one or more filovirus proteins selected from the group consisting of nucleoprotein (NP), virion protein 35 (VP35), virion protein 40 (VP40), virion protein 30 (VP30), virion protein 24 (VP24), and RNA-directed RNA polymerase protein (L). Said genes and proteins, respectively, can be derived from the one or more filovirus strains described above. The NP-EBOV-CdI of certain embodiments comprises the nucleotide sequence of SEQ ID NO:28 encoding the amino acid sequence of SEQ ID NO:29.

In certain embodiments, VP40 is selected from MARV or EBOV, preferably VP40 is selected from one or more EBOV subtypes selected from the group consisting of Zaire Ebola virus (ZEBOV), Sudan Ebola virus (SEBOV), Cote d'Ivoire Ebola virus (EBOV-CdI, also called Tai Forest virus or TAFV), Reston Ebola virus (EBOV-Reston) and Bundibugyo Ebola virus (BEBOV). In a preferred embodiment, VP40 is selected from one or more of ZEBOV, SEBOV and MARV. In certain embodiments, the filovirus glycoprotein and the filovirus VP40 are selected from the same filovirus strain. In a further preferred embodiment, VP40 and/or the filovirus glycoprotein are selected from one or more of Zaire-Mayinga, Zaire-Kikwit, Zaire-Gabon, Cote d'Ivoire Ebola virus, Sudan-Boniface, Sudan-Maleo, Sudan-Gulu, Marburg-Ravn, Marburg-Ozolin, Marburg-Ratayczak, Marburg-Musoke and Marburg-Angola, more preferably selected from one or more of Zaire-Mayinga (VP40-ZEBOV-Mayinga), Sudan-Gulu (VP40-SEBOV-Gulu), Marburg-Musoke (VP40-MARV-Musoke) and Marburg-Angola (VP40-MARV-Angola). In a further embodiment, the MVA vector of any of the embodiments further comprises a filovirus nucleoprotein (NP), preferably wherein the filovirus nucleoprotein and the filovirus VP40 are derived from the same filovirus strain. In a further embodiment, VP40 comprises the nucleic sequence encoding VP40-ZEBOV-Mayinga or VP40-MARV-

Musoke. In other embodiments, filovirus VP40 comprises the nucleotide sequence of SEQ ID NO:33. In a further embodiment, VP40 comprises a nucleic acid encoding the protein sequence of SEQ ID NO:34. In a further preferred embodiment, VP40 comprises the nucleotide sequence of SEQ ID NO:33 encoding the amino acid sequence of SEQ ID NO:34.

In a further preferred embodiment, the recombinant MVA/FPV vector comprises two heterologous nucleotide sequences encoding an antigenic determinant of a filovirus envelope glycoprotein and at least one heterologous nucleotide sequence encoding an antigenic determinant of a further filovirus protein. In certain embodiments, the first heterologous nucleotide sequence encoding an antigenic determinant of a filovirus envelope glycoprotein encodes a GP-MARV, and the second heterologous nucleotide sequence encoding an antigenic determinant of a filovirus envelope glycoprotein encodes a GP-EBOV. The recombinant MVA/FPV vector comprises, according to a further preferred embodiment of the present invention, three heterologous nucleotide sequences encoding an antigenic determinant of a filovirus envelope glycoprotein and at least one heterologous nucleotide sequence encoding an antigenic determinant of a further filovirus protein. Preferably, the first heterologous nucleotide sequence encoding an antigenic determinant of a filovirus envelope glycoprotein encodes a GP-MARV, the second heterologous nucleotide sequence encoding an antigenic determinant of a filovirus envelope glycoprotein encodes a GP-EBOV, and the third heterologous nucleotide sequence encoding an antigenic determinant of a filovirus envelope glycoprotein encodes a GP-EBOV derived from an EBOV strain or isolate different than the GP-EBOV encoded by the second heterologous nucleotide sequence. Accordingly, one heterologous nucleotide sequence encoding an antigenic determinant of a filovirus envelope glycoprotein may encode GP-SEBOV-Gulu and the other one GP-ZEBOV-Mayinga.

In another embodiment, the recombinant MVA/FPV vector comprises two heterologous nucleotide sequences encoding an antigenic determinant of a GP-EBOV from an EBOV strain or isolate and two heterologous nucleotide sequence encoding an antigenic determinant of a GP-MARV from an MARV strain or isolate, preferably the MARV strain is MARV-Angola and MARV-Musoke and the EBOV strain is ZEBOV and/or SEBOV, preferably ZEBOV-Mayinga and SEBOV-Gulu. Of course, the further nucleotide sequence encoding an antigenic determinant of a further filovirus protein may encode also filovirus proteins selected from the group consisting of nucleoprotein (NP), virion protein 35 (VP35), virion protein 40 (VP40), virion protein 30 (VP30), virion protein 24 (VP24), and RNA-directed RNA polymerase protein (L), as already

mentioned above which may also be derived from the different strains as already indicated above.

The recombinant MVA/FPV vector according to a further preferred embodiment of the present invention comprises two heterologous nucleotide sequences encoding an antigenic determinant of a filovirus envelope glycoprotein of GP-MARV and GP-EBOV and a third heterologous nucleotide sequence encoding an antigenic determinant of VP40. Such VP40 can be any of the VP40 as described *supra* or *infra*. Accordingly one heterologous nucleotide sequence encoding an antigenic determinant of a filovirus envelope glycoprotein may encode GP-SEBOV-Gulu, the other one GP-ZEBOV-Mayinga and the third heterologous nucleotide sequence may encode an antigenic determinant of filovirus protein VP40-ZEBOV, VP40-SEBOV or VP40-MARV, preferably VP40-ZEBOV-Mayinga or VP40-MARV-Musoke.

In a further preferred embodiment, the recombinant MVA/FPV vector comprises two heterologous nucleotide sequences encoding an antigenic determinant of a filovirus envelope glycoprotein GP-EBOV, preferably GP-ZEBOV and/or GP-SEBOV, more preferably GP-ZEBOV-Mayinga and GP-SEBOV-Gulu, one filovirus envelope glycoprotein of GP-MARV, preferably GP-MARV-Musoke or GP-MARV-Angola and at least one filovirus nucleoprotein, preferably selected from the group of NP-EBOV-Cdl, NP-ZEBOV and NP-MARV, preferably NP-MARV-Musoke or NP-MARV-Angola.

Integration sites into MVA/FPV

Heterologous nucleotide sequences encoding antigenic determinants of a filovirus glycoprotein, optionally further comprising at least one heterologous nucleotide sequence encoding a further filovirus protein may be inserted into one or more intergenic regions (IGR) of the MVA. In certain embodiments, the IGR is selected from IGR07/08, IGR 44/45, IGR 64/65, IGR 88/89, IGR 136/137, and IGR 148/149. In certain embodiments, less than 5, 4, 3, or 2 IGRs of the recombinant MVA comprise heterologous nucleotide sequences encoding antigenic determinants of a filovirus envelope glycoprotein and/or a further filovirus protein. The heterologous nucleotide sequences may, additionally or alternatively, be inserted into one or more of the naturally occurring deletion sites, in particular into the main deletion sites I, II, III, IV, V, or VI of the MVA genome. In certain embodiments, less than 5, 4, 3, or 2 of the naturally occurring deletion sites of the recombinant MVA comprise heterologous nucleotide sequences encoding antigenic determinants of a filovirus envelope glycoprotein and/or a further filovirus protein.

The number of insertion sites of MVA comprising heterologous nucleotide sequences encoding antigenic determinants of a filovirus protein can be 1, 2, 3, 4, 5, 6, 7, or more. In certain embodiments, the heterologous nucleotide sequences are inserted into 4, 3, 2, or fewer insertion sites. Preferably, two insertion sites are used. In certain embodiments, three insertion sites are used. Preferably, the recombinant MVA comprises at least 2, 3, 4, 5, 6, or 7 genes inserted into 2 or 3 insertion sites.

Heterologous nucleotide sequences encoding antigenic determinants of a filovirus glycoprotein, optionally further comprising at least one heterologous nucleotide sequence encoding a further filovirus protein may be inserted into one or more intergenic regions (IGR) of the FPV. In a preferred embodiment, the IGR is situated between ORFs 7 and 9 of the 1.3-kbp HindIII fragment of the genome (see Drillien et al, *Virology* 160:203-209 (1987) (US 5,180,675) and Spehner et al, *J. Virol.* 64:527-533 (1990)). In certain embodiments, heterologous nucleotide sequences may be inserted in fowlpox insertion sites as described in EP 0 538 496 A1 and WO 05/048957. Also preferred fowlpox insertion sites of the present invention are the LUS insertion site, the FP14 insertion site, and the 43K insertion site. These sites are also referred to sometimes as FPN006/FPN007 (LUS insertion site), FPN254/FPN255 (LUS insertion site), FPV060/FPV061 (FP14 insertion site), and FPV107/FPV108 (43K insertion site).

In one preferred embodiment, the insertion site in fowlpox is the LUS insertion site. There are two long unique sequences (LUS) at each end of the fowlpox viral genome (Genbank Accession NO: AF 198100.1), and thus two LUS insertion sites in each genome. The LUS insertion site at the left end of the genome lies 3' of FPV006 and 5' of FPV007 125L, preferably between position 7470 and 7475 in the fowlpox genomic sequence as annotated in GenBank Accession No. AF198100.1. The LUS insertion site at the right end of the genome lies 3' of FPV254 and 5' of FPV255, preferably between position 281065 and 281070 in the fowlpox genomic sequence *e.g.*, of GenBank Accession No. AF198100.1. In one embodiment, the heterologous nucleotide sequence can be inserted at any position within the nucleotide position 281065 and 281070.

In another preferred embodiment, the insertion site in fowlpox is the FP14 insertion site. This site lies 3' of FPV060 and 5' of FPV061 in the fowlpox genomic sequence, preferably between position 67080 and 67097 of the fowlpox genome *e.g.*, of GenBank Accession No. AF198100.1. In one embodiment, the nucleotides between position 67080 and 67097 of the DNA sequence are deleted in the recombinant virus and replaced with defined inserts representing a sequence of interest. In one embodiment,

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the FP14 insertion site is between the orthologue of the FPV060 gene and the orthologue of FPV061 *e.g.*, of AF198100.1. The term "FPV060, FPV061, FPV254" etc. refers to the position of the corresponding coding sequence (*i.e.*, CDS) of the respective gene numbered from 5´ to 3´ as annotated in GenBank Accession No. AF198100.1. In a preferred embodiment, the FP14 insertion site is between position 67091 and 67092 in the fowlpox genomic sequence (referred to also as IGR60/61 insertion site as annotated in GenBank Accession No. AF198100.1).

In yet another preferred embodiment, the insertion site in fowlpox is designated the 43K insertion site. This site lies 3' of FPV107 and 5' of FPV108, preferably at position 128178 of the fowlpox genomic sequence as annotated in GenBank Accession No. AF198100.1.

In a preferred embodiment, the integration site is FP14 (IGR60/61) and/or the BamHI J region. The BamH1 J region is further described in S. Jenkins et al. (1991), *Aids Research and Human Retroviruses* 7(12):991:998.

In a certain embodiment, the IGR is IGR BamHI J FPV.

The number of insertion sites of the FPV comprising heterologous nucleotide sequences encoding antigenic determinants of a filovirus protein can be one or two. Preferably, two insertion sites are used. In another preferred embodiment, the recombinant FPV comprises at least 1, 2, 3, 4 or 5 genes inserted into one or two insertion sites.

The recombinant MVA/FPV viruses provided herein can be generated by routine methods known in the art. Methods to obtain recombinant poxviruses or to insert exogenous coding sequences into a poxviral genome are well known to the person skilled in the art. For example, methods for standard molecular biology techniques such as cloning of DNA, DNA and RNA isolation, Western blot analysis, RT-PCR and PCR amplification techniques are described in Molecular Cloning, A laboratory Manual (2nd Ed.) (J. Sambrook et al., Cold Spring Harbor Laboratory Press (1989)), and techniques for the handling and manipulation of viruses are described in Virology Methods Manual (B.W.J. Mahy et al. (eds.), Academic Press (1996)). Similarly, techniques and know-how for the handling, manipulation and genetic engineering of MVA are described in Molecular Virology: A Practical Approach (A.J. Davison & R.M. Elliott (Eds.), The Practical Approach Series, IRL Press at Oxford University Press, Oxford, UK (1993) (see, *e.g.*, Chapter 9: Expression of genes by Vaccinia virus vectors)) and Current Protocols in Molecular Biology (John Wiley & Son, Inc. (1998) (see, *e.g.*, Chapter 16, Section IV: Expression of proteins in mammalian cells using vaccinia viral vector)).

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For the generation of the various recombinant MVAs/FPVs disclosed herein, different methods may be applicable. The DNA sequence to be inserted into the virus can be placed into an *E. coli* plasmid construct into which DNA homologous to a section of DNA of the MVA/FPV has been inserted. Separately, the DNA sequence to be inserted can be ligated to a promoter. The promoter-gene linkage can be positioned in the plasmid construct so that the promoter-gene linkage is flanked on both ends by DNA homologous to a DNA sequence flanking a region of MVA/FPV DNA containing a non-essential locus. The resulting plasmid construct can be amplified by propagation within *E. coli* bacteria and isolated. The isolated plasmid containing the DNA gene sequence to be inserted can be transfected into a cell culture, *e.g.*, of chicken embryo fibroblasts (CEFs), at the same time the culture is infected with MVA. Recombination between homologous MVA DNA in the plasmid and the viral genome, respectively, can generate a MVA modified by the presence of foreign DNA sequences.

According to a preferred embodiment, a cell of a suitable cell culture as, *e.g.*, CEF cells, can be infected with a poxvirus. The infected cell can be, subsequently, transfected with a first plasmid vector comprising a foreign or heterologous gene or genes, preferably under the transcriptional control of a poxvirus expression control element. As explained above, the plasmid vector also comprises sequences capable of directing the insertion of the exogenous sequence into a selected part of the poxviral genome. Optionally, the plasmid vector also contains a cassette comprising a marker and/or selection gene operably linked to a poxviral promoter. Suitable marker or selection genes are, *e.g.*, the genes encoding the green fluorescent protein, β-galactosidase, neomycin-phosphoribosyltransferase or other markers. The use of selection or marker cassettes simplifies the identification and isolation of the generated recombinant poxvirus. However, a recombinant poxvirus can also be identified by PCR technology. Subsequently, a further cell can be infected with the recombinant poxvirus obtained as described above and transfected with a second vector comprising a second foreign or heterologous gene or genes. In case, this gene shall be introduced into a different insertion site of the poxviral genome, the second vector also differs in the poxvirus-homologous sequences directing the integration of the second foreign gene or genes into the genome of the poxvirus. After homologous recombination has occurred, the recombinant virus comprising two or more foreign or heterologous genes can be isolated. For introducing additional foreign genes into the recombinant virus, the steps of infection and transfection can be repeated by using the recombinant virus isolated in previous steps for infection and by using a further vector comprising a further foreign gene or genes for transfection.

Alternatively, the steps of infection and transfection as described above are interchangeable, *i.e.*, a suitable cell can at first be transfected by the plasmid vector comprising the foreign gene and, then, infected with the poxvirus. As a further alternative, it is also possible to introduce each foreign gene into different viruses, coinfect a cell with all the obtained recombinant viruses and screen for a recombinant including all foreign genes. A third alternative is ligation of DNA genome and foreign sequences in vitro and reconstitution of the recombined vaccinia virus DNA genome using a helper virus. A fourth alternative is homologous recombination in *E.coli* or another bacterial species between a vaccinia virus genome cloned as a bacterial artificial chromosome (BAC) and a linear foreign sequence flanked with DNA sequences homologous to sequences flanking the desired site of integration in the vaccinia virus genome.

Expression of heterologous filovirus genes

A heterologous nucleotide sequence encoding an antigenic determinant of a filovirus protein can be expressed as a single transcriptional unit. For example, a heterologous nucleotide sequence encoding an antigenic determinant of a filovirus protein can be operably linked to a poxvirus *e.g.,* vaccinia virus promoter and/or linked to a poxvirus *e.g.*, vaccinia virus transcriptional terminator.

In certain embodiments, the "transcriptional unit" is inserted by itself into an insertion site in the MVA/FPV genome. In certain embodiments, the "transcriptional unit" is inserted with other transcriptional unit(s) into an insertion site in the MVA/FPV genome. The "transcriptional unit" is not naturally occurring (*i.e.*, it is heterologous, exogenous or foreign) in the MVA/FPV genome and is capable of transcription in infected cells.

Preferably, the recombinant MVA/FPV comprises 1, 2, 3, 4, 5, or more transcriptional units inserted into the MVA/FPV genome. In certain embodiments, the recombinant MVA/FPV stably expresses heterologous nucleotide sequences encoding antigenic determinants of a filovirus protein encoded by 1, 2, 3, 4, 5, or more transcriptional units. In certain embodiments, the recombinant MVA/FPV comprises 2, 3, 4, 5, or more transcriptional units inserted into the MVA/FPV genome at 1, 2, 3, or more insertion sites in the MVA/FPV genome.

In certain embodiments, expression of one, more, or all of the heterologous nucleotide sequences encoding antigenic determinants of a filovirus protein is under the control of one or more poxvirus promoters. In certain embodiments, the poxvirus promoter is a Pr7.5 promoter, a hybrid early/late promoter, a PrS promoter, a PrS5E promoter, a synthetic or natural early or late promoter, or a cowpox virus ATI promoter. Suitable

promoters are further described in WO 2010/060632, WO 2010/102822, WO 2013/189611 and WO 2014/063832. In certain embodiments, the poxvirus promoter is selected from the group consisting of the PrS promoter (SEQ ID NO:23), the PrS5E promoter (SEQ ID NO:24), the Pr7.5 (SEQ ID NO:25), the PrLE1 promoter (SEQ ID NO:27), the Pr13.5 long promoter (SEQ ID NO:35) and the FPV-40K promoter (SEQ ID NO:26), more preferably selected from the group consisting of the PrS promoter (SEQ ID NO:23), the PrS5E promoter (SEQ ID NO:24), the Pr7.5 (SEQ ID NO:25) and the PrLE1 promoter (SEQ ID NO:27).

In certain embodiments, the nucleotide sequence encoding the antigenic determinant of the filovirus protein preferably the ZEBOV, SEBOV, EBOV-Cdl, MARV and NP-ZEBOV protein, more preferably the GP-ZEBOV-Mayinga, GP-SEBOV-Gulu, GP-MARV and NP-ZEBOV, most preferably the GP-MARV-Musoke or GP-MARV-Angola are under the control of the promoter selected from the group consisting of PrS, PrLE1 and Pr7.5. In a preferred embodiment, the nucleotide sequence encoding the antigenic determinant of the filovirus protein GP-SEBOV and GP-MARV-Musoke are expressed under the control of the PrS promoter (*e.g.*, SEQ ID NO:23), NP-EBOV-Cdl is expressed under the control of the PrLE1 or modified PrLE1 promoter (*e.g.,* SEQ ID NO:27 and SEQ ID NO:32), and GP-ZEBOV-Mayinga is expressed under the control of the Pr7.5 promoter (*e.g.*, SEQ ID NO:25).

In another preferred embodiment, the nucleotide sequence encoding the antigenic determinant of the filovirus protein of the FPV of any of the embodiments is under the control of the promoter, preferably including or having SEQ ID NO:26.

Filovirus Vaccines and Pharmaceutical Compositions

Since the recombinant MVA viruses described herein are highly replication restricted and, thus, highly attenuated, they are ideal candidates for the treatment of a wide range of mammals including humans and even immune-compromised humans. Hence, provided herein are pharmaceutical compositions and vaccines for inducing an immune response in a living animal body, including a human. Additionally provided is a recombinant MVA vector comprising a nucleotide sequence encoding an antigenic determinant of a filovirus glycoprotein for use in the treatment and/or prevention of a filovirus-caused disease.

The vaccine preferably comprises any of the recombinant MVA viruses described herein formulated in solution in a concentration range of 10^4 to 10^9 $TCID_{50}$/ml, 10^5 to $5x10^8$ $TCID_{50}$/ml, 10^6 to 10^8 $TCID_{50}$/ml, or 10^7 to 10^8 $TCID_{50}$/ml. A preferred vaccination

dose for humans comprises between 10^6 to 10^9 $TCID_{50}$, including a dose of 10^6 $TCID_{50}$, 10^7 $TCID_{50}$, or 10^8 $TCID_{50}$.

The pharmaceutical compositions provided herein may generally include one or more pharmaceutically acceptable and/or approved carriers, additives, antibiotics, preservatives, adjuvants, diluents and/or stabilizers. Such auxiliary substances can be water, saline, glycerol, ethanol, wetting or emulsifying agents, pH buffering substances, or the like. Suitable carriers are typically large, slowly metabolized molecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates, or the like.

For the preparation of vaccines, the recombinant MVA viruses provided herein can be converted into a physiologically acceptable form. This can be done based on experience in the preparation of poxvirus vaccines used for vaccination against smallpox as described by H. Stickl et al., *Dtsch. med. Wschr.* 99:2386-2392 (1974).

For example, purified viruses can be stored at -80ºC with a titer of $5x10^8$ $TCID_{50}$/ml formulated in about 10 mM Tris, 140 mM NaCl pH 7.4. For the preparation of vaccine shots, *e.g.*, 10^2-10^8 or 10^2-10^9 particles of the virus can be lyophilized in 100 ml of phosphate-buffered saline (PBS) in the presence of 2% peptone and 1% human albumin in an ampoule, preferably a glass ampoule. Alternatively, the vaccine shots can be produced by stepwise freeze-drying of the virus in a formulation. This formulation can contain additional additives such as mannitol, dextran, sugar, glycine, lactose or polyvinylpyrrolidone or other aids such as antioxidants or inert gas, stabilizers or recombinant proteins (*e.g.*, human serum albumin) suitable for in vivo administration. The glass ampoule is then sealed and can be stored between 4ºC and room temperature for several months. However, as long as no need exists, the ampoule is stored preferably at temperatures below -20ºC.

For vaccination or therapy, the lyophilisate can be dissolved in an aqueous solution, preferably physiological saline or Tris buffer, and administered either systemically or locally, *i.e.*, parenteral, subcutaneous, intravenous, intramuscular, intranasal, or any other path of administration known to the skilled practitioner. The mode of administration, the dose and the number of administrations can be optimized by those skilled in the art in a known manner. However, most commonly a patient is vaccinated with a second shot about one month to six weeks after the first vaccination shot.

Combination Vaccines Using Homologous/Heterologous Prime-Boost Regimens

The Combination Vaccines and methods described herein may also be used as part of a homologous prime-boost regimen. In the homologous prime-boost, a first priming

vaccination is given followed by one or more subsequent boosting vaccinations. The boosting vaccinations are configured to boost the immune response generated in the first vaccination by administration of the same recombinant poxvirus that was used in the first vaccination.

In one exemplary embodiment a homologous prime-boost regimen may be employed wherein a MVA viral vector as defined herein is administered in a first dosage. One or more subsequent administrations of a MVA viral vector as defined herein can be given to boost the immune response provided in the first administration. Preferably, the one or more antigenic determinants are the same or similar to those of the first administration.

The MVA and FPV recombinant viral vectors according to the present invention may also be used in heterologous prime-boost regimens in which one or more of the initial prime vaccinations are done with either the MVA or the FPV vector as defined herein and one or more subsequent boosting vaccinations are done with the poxviral vector not used in the prime vaccination, *e.g.*, if a MVA vector defined herein is given in a prime boost, then subsequent boosting vaccinations would be FPV vectors and vice versa.

In a preferred embodiment the prime vaccination is done with the MVA vector and the boosting vaccination with the FPV. Accordingly, one aspect of the invention relates to a combination vaccine comprising:

a) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding an antigenic protein of at least one filovirus subtype, together with a pharmaceutically acceptable carrier; and

b) a second composition comprising an immunologically effective amount of a fowlpox vector comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein the first compositions is a priming composition and the second composition is a boosting composition, preferably wherein the boosting composition comprises two or more doses of the vector of the boosting composition.

Vaccines and Kits Comprising Recombinant MVA and FPV Viruses

Also provided herein are vaccines and kits comprising any one or more of the recombinant FPVs and/or MVAs described herein. The kit can comprise one or multiple containers or vials of the recombinant MVA or FPV, together with instructions for the administration of the recombinant MVA and FPV to a subject at risk of filovirus

infection. In certain embodiments, the subject is a human. In certain embodiments, the instructions indicate that the recombinant MVA is administered to the subject in a single dose, or in multiple (*i.e.*, 2, 3, 4, etc.) doses. In certain embodiments, the instructions indicate that the recombinant MVA or FPV virus is administered in a first (priming) and second (boosting) administration to naïve or non-naïve subjects. Preferably, a kit comprises at least two vials for prime/boost immunization comprising the recombinant MVAs as described herein for a first inoculation ("priming inoculation") in a first vial/container and for an at least second and/or third and/or further inoculation ("boosting inoculation") in a second and/or further vial/container.

In a preferred embodiment the vaccines and kits provided herein comprise a first composition which comprises a MVA vector comprising a nucleic acid encoding an antigenic protein of a second filovirus subtype, of a third filovirus subtype or at least four filovirus subtypes.

In a preferred embodiment, the vaccines and kits provided herein comprise a MVA vector in the first composition, which comprises a nucleic acid encoding an antigenic protein selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 20, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:34 and SEQ ID NO:37.

In a further embodiment the vaccines and kits provided herein comprise a MVA vector in the first composition comprising a nucleic acid encoding an antigenic protein selected from the group having SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:20, SEQ ID NO:29 and SEQ ID NO:31, preferably comprising a nucleic acid encoding an antigenic protein selected from the group having: SEQ ID NO:6, SEQ ID NO:20, SEQ ID NO:29 and SEQ ID NO:31.

In a further embodiment the vaccines and kits provided herein comprise a first composition which comprises a MVA vector comprising a nucleic acid encoding an antigenic protein of at least four filovirus subtypes, preferably wherein the four different filovirus subtypes are selected from the group having SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 20, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:34 and SEQ ID NO:37.

In a further preferred embodiment the vaccines and kits provided herein are for use in generating a protective immune response against at least one filovirus subtype, wherein the first composition is used for priming said immune response and the second composition is used for boosting said immune response or for use in generating a protective immune response against at least one filovirus subtype, wherein the second

composition is used for priming said immune response and the first composition is used for boosting said immune response. In any of the vaccines and kits provided herein the boosting composition can comprise two or more doses of the vector of the boosting composition.

As discussed previously above, the present invention also relates to heterologous vaccination regimes using two different non-replicating viral vectors.

For heterologous vaccine programs, the present invention provides a combination vaccine and/or vaccination kit which comprises:

(a) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding antigenic proteins of at least two filovirus subtypes, together with a pharmaceutically acceptable carrier; and
(b) a second composition comprising an immunologically effective amount of a fowlpox vector comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein one of the compositions is a priming composition and the other composition is a boosting composition.

The present invention also provides a combination vaccine and/or vaccination kit which comprises:

(a) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding antigenic proteins of at least two filovirus subtypes, together with a pharmaceutically acceptable carrier; and
(b) a second composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein one of the compositions is a priming composition and the other composition is a boosting composition.

In this embodiment, the combination vaccines and/or kit comprises at least two vials for prime/boost immunization comprising the recombinant MVAs/FPVs as described herein for a first inoculation ("priming inoculation") in a first vial/container and for an at least second and/or third and/or further inoculation ("boosting inoculation") in a second and/or further vial/container.

The combination vaccine and/or kit can comprise multiple containers or vials of the recombinant MVA/FPV, together with instructions for the administration of the recombinant MVA/FPV to a subject at risk of filovirus infection. In certain embodiments,

the subject is a human. In certain embodiments, the instructions indicate that the recombinant MVA/FPV is administered to the subject in a single dose, or in multiple (*i.e.*, 2, 3, 4, etc.) doses. In certain embodiments, the instructions indicate that the recombinant MVA/FPV virus is administered in a first (priming) and second (boosting) administration to naïve or non-naïve subjects.

The first and/or second composition or MVA and/or FPV of any combination vaccine, vaccination kit and/or any heterologous vaccine program of the invention can comprise any of the MVA and/or FPV vector described herein or as further defined under "Recombinant MVA and FPV" and any combination thereof.

In a preferred embodiment, the combination vaccines as provided herein comprise a first composition comprising a MVA vector comprising a nucleic acid encoding an antigenic protein of a second filovirus subtype, an antigenic determinant of a third filovirus subtype, an antigenic determinant of four filovirus subtypes or an antigenic determinant of at least four filovirus subtypes.

In another embodiment, the combination vaccines as provided herein comprise a filovirus subtype selected from an Ebola virus (EBOV) or a Marburg virus (MARV).

In another embodiment, the combination vaccines as provided herein comprises an antigenic determinant from one or more EBOV subtypes selected from the group consisting of Zaire Ebola virus (ZEBOV), Sudan Ebola virus (SEBOV), Cote d'Ivoire Ebola virus (EBOV-CdI), Reston Ebola virus (EBOV-Reston) and Bundibugyo Ebola virus (BEBOV).

In another embodiment, the combination vaccines as provided herein comprise an antigenic determinant of the filovirus protein is selected from the group consisting of an envelope glycoprotein (GP), nucleoprotein (NP), virion protein 35 (VP35), virion protein 40 (VP40), virion protein 30 (VP30), virion protein 24 (VP24), and RNA-directed RNA polymerase protein (L).

In a further preferred embodiment, the combination vaccines as provided herein comprise a MVA vector in the first composition comprising a nucleic acid encoding an antigenic protein selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 20, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:34 and SEQ ID NO:37.

In a further embodiment, the combination vaccines provided herein comprise a first composition which comprises a MVA vector comprising a nucleic acid encoding an antigenic protein of at least four filovirus subtypes, preferably wherein the four different filovirus subtypes are selected from the group having SEQ ID NO:2, SEQ ID NO:4,

SEQ ID NO:6, SEQ ID NO: 20, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:34 and SEQ ID NO:37.

In a further preferred embodiment, the combination vaccines provided herein comprise a first composition which comprises a MVA vector comprising a nucleic acid encoding antigenic proteins from four different filovirus subtypes selected from the group having: SEQ ID NO:6, SEQ ID NO:20, SEQ ID NO:29 and SEQ ID NO:31.

In another embodiment, the combination vaccines provided herein are for use in generating a protective immune response against at least one filovirus subtype, preferably at least two, more preferably at least four filovirus subtype.

In another embodiment of the present invention, the present invention relates to a combination vaccine or the recombinant MVA of any of the embodiments for use as a medicament or vaccine for generating a protective immune response or for inducing an enhanced immune response against at least one filovirus subtype, at least two filovirus subtypes, at least three or at least four filovirus subtypes, wherein the MVA is capable of producing filovirus-like particles in the subject to be treated, preferably, wherein the MVA is producing filovirus-like particles in the subject to be treated.

Methods and Uses of Recombinant MVA/FPV Viruses

Also provided herein are methods and/or any of the recombinant MVAs/FPVs as described herein for use in a method of immunizing a subject animal or for affecting an immune response in a subject. Also covered are uses of the recombinant MVAs/FPVs described herein for the preparation of a medicament or pharmaceutical for the immunization of a subject animal, in particular for the preparation of a medicament or vaccine for treating and/or preventing a filovirus-caused disease in a subject. Provided are also recombinant MVA/FPV according to any embodiment herein for use in priming or boosting an immune response against a filovirus, preferably wherein the recombinant MVA and/or recombinant FPV is administered once, twice, three times or four times.

Further covered herein are vaccine combinations or recombinant MVA of any of the embodiments for use as a medicament or vaccine for inducing an enhanced immune response against a filovirus infection wherein the MVA is capable of producing filovirus-like particles in the subject to be treated, preferably, wherein the MVA is producing filovirus-like particles in the subject to be treated. Also covered are vaccine combinations or recombinant MVA of any of the embodiments for use as a medicament or vaccine for treating and/or preventing a filovirus disease, wherein the MVA is capable of producing filovirus-like particles in the subject to be treated, preferably,

wherein the MVA is producing filovirus-like particles in the subject to be treated.

Accordingly, in one embodiment, the present invention provides a method of inducing an immune response against filovirus in a subject, the method comprising administering to the subject:

(a) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding antigenic proteins of at least two filovirus subtypes, together with a pharmaceutically acceptable carrier; and

(b) a second composition comprising an immunologically effective amount of a fowlpox vector comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein one of the compositions is a priming composition and the other composition is a boosting composition.

In another embodiment, the invention provides a method of inducing an immune response against a filovirus in a subject, the method comprising administering to the subject:

(a) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding antigenic proteins of at least two filovirus subtypes, together with a pharmaceutically acceptable carrier; and

(b) a second composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein one of the compositions is a priming composition and the other composition is a boosting composition.

In another embodiment, the method of inducing an immune response against a filovirus, the uses of the recombinant MVAs/FPVs described herein for the preparation of a medicament for immunization of a subject animal, in particular for the preparation of a medicament or vaccine for treating and/or preventing a filovirus-caused disease in a subject or the combination vaccine of any of the embodiments for use of providing a protective immune response against a filovirus infection as provided herein comprises a first composition which comprises a MVA vector comprising a nucleic acid encoding an antigenic protein of a second filovirus subtype, of a third filovirus subtype or of at least four filovirus subtypes.

In another embodiment, the method of inducing an immune response against a filovirus, the uses of the recombinant MVAs/FPVs described herein for the preparation

of a medicament for immunization of a subject animal, in particular for the preparation of a medicament or vaccine for treating and/or preventing a filovirus-caused disease in a subject or the combination vaccine of any of the embodiments for use of providing a protective immune response against a filovirus infection as provided herein comprise a MVA vector in the first composition which comprises a nucleic acid encoding an antigenic protein selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 20, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:34 and SEQ ID NO:37.

In a further embodiment, the method of inducing an immune response against a filovirus, the uses of the recombinant MVAs/FPVs described herein for the preparation of a medicament for immunization of a subject animal, in particular for the preparation of a medicament or vaccine for treating and/or preventing a filovirus-caused disease in a subject or the combination vaccine of any of the embodiments for use of providing a protective immune response against a filovirus infection as provided herein comprises a first composition which comprises a MVA vector comprising a nucleic acid encoding an antigenic protein of at least four filovirus subtypes, preferably wherein the four different filovirus subtypes are selected from the group having SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 20, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:34 and SEQ ID NO:37.

In another embodiment, the present invention provides a method of providing protective immunity and/or a protective immune response against a filovirus infection in a subject. In another embodiment, the invention provides a method of providing protective immunity and/or a protective immune response against a filovirus infection in a subject:

(a) a first composition comprising an immunologically effective amount of a MVA vector comprising a nucleic acid encoding antigenic proteins of at least two filovirus subtypes, preferably at least three or at least four different filovirus subtypes, together with a pharmaceutically acceptable carrier; and

(b) a second composition comprising an immunologically effective amount of a FPV vector comprising a nucleic acid encoding an antigenic protein of a first filovirus subtype, together with a pharmaceutically acceptable carrier;

wherein one of the compositions is a priming composition and the other composition is a boosting composition, preferably wherein the second composition is a boosting composition, preferably to be administered once, twice, three times or four times.

In another embodiment, the method of providing protective immunity and/or a protective immune response against a filovirus infection of any of the embodiments comprises a MVA vector in the first composition comprising a nucleic acid encoding an antigenic protein selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 20, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:34 and SEQ ID NO:37.

In another embodiment, the method of providing protective immunity and/or a protective immune response against a filovirus infection of any of the embodiments comprises a MVA vector in the first composition comprising a nucleic acid encoding antigenic proteins from four different filovirus subtypes having SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 20, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:34 and SEQ ID NO:37.

In another embodiment, the present invention provides a method for production of filovirus-like particles or a method of inducing an enhanced immune response against a filovirus in a subject, the method comprising production of filovirus-like particles in the subject of any of the embodiments, wherein the filovirus VP40 is selected from the group consisting of Zaire Ebola virus (ZEBOV), Sudan Ebola virus (SEBOV), Cote d'Ivoire Ebola virus (EBOV-CdI), Reston Ebola virus (EBOV-Reston) and Bundibugyo Ebola virus (BEBOV), preferably wherein the filovirus VP40 is selected from one or more ZEBOV, SEBOV and MARV.

In another embodiment, the present invention provides a method for production of filovirus-like particles or a method of inducing an enhanced immune response against a filovirus in a subject, the method comprising production of filovirus-like particles in the subject of any of the embodiments, wherein the filovirus glycoprotein and the filovirus VP40 are selected from the same filovirus strain.

In another embodiment, the present invention provides a method for production of filovirus-like particles or a method of inducing an enhanced immune response against a filovirus in a subject, the method comprising production of filovirus-like particles in the subject of any of the embodiments, wherein the MVA vector further comprises a nucleic acid encoding a filovirus nucleoprotein (NP), preferably wherein the filovirus nucleoprotein and the filovirus VP40 are derived from the same filovirus strain.

In another embodiment, the filovirus strain of any of the above methods is selected from the group of Zaire-Mayinga, Zaire-Kikwit, Zaire-Gabon, Cote d'Ivoire Ebola virus, Sudan-Boniface, Sudan-Maleo, Sudan-Gulu, Marburg-Ravn, Marburg-Ozolin, Marburg-Ratayczak, Marburg-Musoke, Marburg-Angola, preferably Zaire-Mayinga or Cote

d'Ivoire Ebola virus, preferably wherein the filovirus VP40 is selected from the group of Zaire-Mayinga or Marburg-Musoke, more preferably wherein the filovirus VP40 comprises a nucleic acid encoding the protein sequence of SEQ ID NO:34 or wherein the nucleic acid encoding the antigenic protein of the filovirus VP40 comprises SEQ ID NO:33.

As used herein, the term "protective immunity" or "protective immune response" means that the vaccinated subject is able to control an infection with the pathogenic agent against which the vaccination was done. Usually, the subject having developed a "protective immune response" develops only mild to moderate clinical symptoms or no symptoms at all. Usually, a subject having a "protective immune response" or "protective immunity" against a certain agent will not die as a result of the infection with said agent. In certain embodiments, the subject animal is a mammal. The mammal may be an adult cow, a calf, in particular a juvenile calf, a rat, rabbit, pig, mouse, but preferably a human, and the method comprises administering a dose of any one or more of the recombinant MVAs/FPVs provided herein to the subject.

In certain embodiments, the subject is a human. In certain embodiments, the subject is an adult. In certain embodiments, the adult is immune-compromised. In certain embodiments, the adult is over the age of 10, 15, 20, 25, 30, 25, 40, 45, 50, 55, 60, 65, 70, 75, 80, or 85 years. In certain embodiments, the subject's age is less than 5 years, less than 3 years, less than 2 years, less than 15 months, less than 12 months, less than 9 months, less than 6, or less than 3 months. In certain embodiments, the subject's age is from 0-3 months, 3-6 months, 6-9 months, 9-12 months, 1-2 years, or 2-5 years.

Any of the recombinant MVAs/FPVs provided herein may be administered to the subject at a dose of 10^6 to 10^{10} $TCID_{50}$, preferably 10^6 to 10^9 $TCID_{50}$ as, *e.g.*, at a dose of 10^6 to 10^9 $TCID_{50}$, 10^6 to 5×10^8 $TCID_{50}$, 10^7 to 10^8 $TCID_{50}$, 5×10^7 $TCID_{50}$ to 5×10^8 $TCID_{50}$, 10^7 $TCID_{50}$ or 10^8 $TCID_{50}$. In a certain embodiment, the recombinant MVA/FPV vector is administered in an amount of 1×10^8 $TCID_{50}$ to 1×10^{10} $TCID_{50}$. In another embodiment, the recombinant MVA/FPV is administered in an amount of 1×10^8 $TCID_{50}$ to 5×10^9, preferably in an amount of 5×10^8 $TCID_{50}$ to 6×10^9. In certain embodiments, any of the recombinant MVAs provided herein are administered to a human subject at a dose of 10^7 $TCID_{50}$ or 10^8 $TCID_{50}$ or 5×10^8 $TCID_{50}$. In certain embodiments, any of the recombinant FPVs provided herein is administered to a human subject at a dose of 5×10^8, 6.3×10^8 or 1×10^9 $TCID_{50}$.

In another embodiment, the recombinant MVAs provided herein are administered to a human subject at a dose lower than the recombinant FPVs. In certain embodiments,

any of the recombinant MVAs/FPVs provided herein are administered to the subject at any of the doses provided herein prior to filovirus exposure as, *e.g.*, 1, 2, 3, or 4 weeks or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months before filovirus exposure. In certain embodiments, any of the recombinant MVAs/FPVs provided herein is administered to the subject at any of the doses provided herein after filovirus exposure as, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9. 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 hours or 1, 2, 3, 4, 5, 6, or 7 days after filovirus exposure.

In certain embodiments, the recombinant MVAs/FPVs provided herein are administered to the subject in a single dose, or in multiple (*i.e.*, 2, 3, 4, etc.) doses. In certain embodiments, the recombinant MVAs/FPVs provided herein are administered in a first (priming) and second (boosting) administration. The first dose may comprise 10^7 to 10^8 $TCID_{50}$ of recombinant MVA/FPV virus and the second dose may comprise 10^7 to 10^8 $TCID_{50}$ of recombinant MVA/FPV virus.

Boosting compositions are generally administered once or multiple times weeks or months after administration of the priming composition, for example, about 1 or 2 weeks or 3 weeks, or 4 weeks, or 6 weeks, or 8 weeks, or 16 weeks, or 20 weeks, or 24 weeks, or 28 weeks, or 32 weeks or one to two years.

Preferably, the initial boosting inoculation is administered 1-12 weeks or 2-12 weeks after priming, more preferably 1, 2, 4 or 8 weeks after priming. In a preferred embodiment, the initial boosting inoculation is administered 4 or 8 weeks after priming. In additional preferred embodiments, the initial boosting is conducted at least 2 weeks or at least 4 weeks after priming. In still another preferred embodiment, the initial boosting is conducted 4-12 weeks or 4-8 weeks after priming.

The recombinant MVAs/FPVs provided herein can be administered systemically or locally. In certain embodiments, the recombinant MVAs/FPVs are administered parenterally, subcutaneously, intravenously, intramuscularly, or intranasally, in particular subcutaneously. Preferably, the recombinant MVAs/FPVs are administered intranasally. In other embodiments, the recombinant MVAs/FPVs are administered by any other path of administration known to the skilled practitioner. In a further preferred embodiment, the recombinant MVA/FPV is administered intramuscularly, preferably the recombinant MVA/FPV is administered intramuscularly in a volume ranging between about 100 µl to about 10 ml preferably containing concentrations of *e.g.,* about 10^4 to 10^{10} virus particles/ml. Preferably, the recombinant MVA/FPV vector is administered in a volume ranging between 0.25 and 1.0 ml. More preferably, the recombinant MVA/FPV vector is administered in a volume of about 0.5 ml.

Method for producing a recombinant MVA/FPV vector

Further embodiments comprise a method for producing a recombinant MVA vector of any of the embodiments of the invention or the antigenic determinant expressed from the genome of said recombinant MVA vector, comprising the steps of

(a) infecting a host cell with the recombinant MVA virus of any of the embodiments or transfecting the cell with the recombinant DNA of the recombinant MVA virus of any of the embodiments preferably with the addition of a helper virus for production of MVA virus particles,

(b) cultivating the infected or transfected cell, and

(c) isolating the MVA virus and/or the antigenic determinant from said cell.

In another embodiment, the invention relates to a recombinant MVA virus and/or an antigenic determinant obtained from the method for producing a recombinant vector.

Further embodiments comprise a method for producing a recombinant FPV vector of any of the embodiments of the invention or the antigenic determinant expressed from the genome of said recombinant FPV vector, comprising the steps of

(a) infecting a host cell with the recombinant FPV virus of any of the embodiments or transfecting the cell with the recombinant DNA of the recombinant FPV virus of any of the embodiments preferably with the addition of a helper virus for production of FPV virus particles,

(b) cultivating the infected or transfected cell, and

(c) isolating the FPV virus and/or the antigenic determinant from said cell.

In another embodiment, the invention relates to a recombinant FPV virus and/or an antigenic determinant obtained from the method for producing a recombinant vector.

In another embodiment, the invention relates to a method of generating a recombinant MVA vector comprising the steps of:

(a) infecting a host cell with a MVA virus,

(b) transfecting the infected cell with a recombinant vector comprising at least one nucleotide sequence encoding an antigenic determinant of any of the proteins, said nucleic acid sequence further comprising a genomic MVA virus sequence capable of directing the integration of the at least one nucleotide sequence into the MVA virus genome, and

(c) identifying, isolating and optionally purifying the generated recombinant MVA virus.

In another embodiment, the invention relates to a method of generating a recombinant FPV vector comprising the steps of:

(a) infecting a host cell with an FPV virus,

(b) transfecting the infected cell with a recombinant vector comprising at least one nucleotide sequence encoding an antigenic determinant of any of the proteins, said nucleic acid sequence further comprising a genomic FPV virus sequence capable of directing the integration of the at least one nucleotide sequence into the FPV virus genome, and

(c) identifying, isolating and optionally purifying the generated recombinant FPV virus.

Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the appended claims.

EXAMPLES

The detailed examples which follow are intended to contribute to a better understanding of the present invention. However, the invention is not limited by the examples. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein.

Example 1: Construction of Recombinant MVA

The following sections describe construction of recombinant MVAs comprising one or more heterologous nucleic acids expressing an antigenic determinant of a filovirus envelop glycoprotein and/or a further filovirus protein. All other constructs described herein are made using similar methods.

Construction of MVA-mBN252B (PrS-GP-MARV-Musoke)

The full-length DNA sequence of the naturally-occurring GP-MARV-Musoke gene (Lake Victoria isolate) served as reference sequence for construction of MARV vaccine candidate MVA-mBN252B. A nucleotide sequence encoding full-length GP-MARV-Musoke was synthesized by Geneart AG (Regensburg, Germany) with codon usage optimized for expression in humans and to minimize or prevent internal homologous recombination events. Although the codon optimization changed the wild-type DNA sequence, the codon-optimized sequence encodes an amino acid sequence identical to the wild-type GP-MARV-Musoke (SEQ ID NO:6; NCBI accession number ABA87127.1). Expression of GP-MARV-Musoke is driven by the promoter PrS, a synthetic promoter designed from early and late elements of vaccinia virus promoters (SEQ ID NO:23; *see also* S. Chakrabarti *et al.*, "Compact, Synthetic Vaccinia Virus Early/Late Promoter for Protein Expression", *BioTechniques* 23(6):1094-1097 (1997)). The codon-optimized GP-MARV-Musoke gene was inserted into the MVA-BN genome by standard methods (see below) using one of several customized recombination plasmids targeting different specific regions of the MVA-BN genome, including the deletion sites or the intergenic (non-coding) regions (IGR).

To insert the codon-optimized GP-MARV-Musoke gene into the MVA-BN genome, chicken embryonic fibroblast cells (CEF cells) were infected with MVA-BN and subsequently transfected with the recombination plasmid pBN433 (Figure 5A). pBN433 contained the codon-optimized GP-MARV-Musoke gene (SEQ ID NO:5 (DNA) encoding SEQ ID NO:6 (amino acid)) under control of the synthetic PrS promoter inserted via BspEI/NheI restriction into plasmid pBNX197 (Figure 4B). Plasmid pBN433 also contains MVA-BN DNA sequences flanking IGR 148/149 in the MVA-BN genome and a selection cassette flanked by loxP sites, which allows later elimination of the selection cassette by Cre recombinase-mediated recombination. Following homologous recombination between flanking sequences in the plasmid and homologous sequences at the desired insertion site in the MVA-BN genome (*i.e.*, IGR 148/149), the coding portion of the plasmid was inserted into the desired site in the MVA-BN genome.

After amplification and plaque purification (nine passages; three of them including plaque purification) under selective conditions (mycophenolic acid/xanthine and hypoxanthine), the recombinant MVA-BN product designated MVA-mBN252A (PreMaster A), containing the gene for GP-MARV-Musoke was obtained. Recombinant MVA-mBN252A PreMaster virus stock was examined for elimination of MVA-BN (parental virus; data not shown), for correct sequence of the inserted gene together

with the insertion flanking regions (by gene-specific PCR using primers specific for the MVA-BN genomic sequence into which the foreign gene was inserted; data not shown), for absence of microbes (sterility test; data not shown), and for the presence and correct size of the insert (by sequencing; data not shown). The titer of the MVA-mBN252A PreMaster virus stock was also determined.

The presence of a selection cassette in the inserted sequence permits positive selection for recombinant MVA-BN viruses in culture. To generate the final recombinant MVA-mBN252B, the selection cassette was removed from MVA-mBN252A PreMaster virus stock using the Cre/loxP system. To remove the selection cassette, CEF cells infected with recombinant MVA-BN containing the insert of plasmid pBN433 (*i.e.*, GP-MARV-Musoke under the control of the PrS promoter, plus a selection cassette flanked by loxP sites) were further transfected with pBN274, an expression plasmid encoding the CRE recombinase (Figure 4C). The site-specific Cre-recombinase catalyzed the precise excision of the selection cassette DNA sequences flanked by the target loxP sequence, completely removing the selection cassette. The resulting virus was plaque purified under non-selective conditions (twenty seven passages; nine of them including plaque purification), and the recombinant virus MVA-mBN252B devoid of selection cassette was isolated. Complete elimination of the selection cassette was confirmed by nested PCR (data not shown). Finally, expression of GP-MARV-Musoke by recombinant MVA-mBN252B was confirmed by reverse-transcriptase PCR (RT-PCR; data not shown).

Construction of MVA-mBN226B (Multi-antigen MVA-Filo)

For all transgenes expressed from MVA-mBN226B, the full-length DNA sequences of the naturally-occurring genes served as reference sequences. Those were synthesized by Geneart AG (Regensburg, Germany) with codon usage optimized for expression in humans and to minimize or prevent internal homologous recombination events. The codon optimization changed the wild-type DNA sequence without altering the amino acid sequence. MVA-mBN226B contains the following filoviral genes: GP-SEBOV (SEQ ID NO:30); NP-EBOV-CdI (SEQ ID NO:28); GP-ZEBOV, Mayinga strain (GP-ZEBOV-Mayinga, SEQ ID NO:19) and GP-MARV-Musoke (SEQ ID NO:5). GP-SEBOV and GP-MARV-Musoke are expressed under the control of the PrS promoter (SEQ ID NO:23), NP-EBOV-CdI is expressed under the control of the PrLE1 or modified PrLE1 promoter (SEQ ID NO:27 and SEQ ID NO:32), and GP-ZEBOV-Mayinga is expressed under the control of the Pr7.5 promoter (SEQ ID NO:25).

The PrS promoter is a synthetic promoter designed from early and late elements of Vaccinia virus promoters, which ensures transgene expression during both the early and late phases of gene expression. Similarly, the Pr7.5 promoter from the vaccinia virus 7.5 kDa gene is a strong early and late promoter, meaning transgenes under its control will also be expressed during both the early and late phases of gene expression (SEQ ID NO:25; *see also* M.A. Cochran *et al.*, "*In vitro* mutagenesis of the promoter region for a vaccinia virus gene: evidence for tandem early and late regulatory signals", *J. Virol.* 54(1):30-37 (1985)). The promoter PrLE1 is a synthetic promoter consisting of the A-type inclusion body promoter of cowpox virus (ATI) fused to five optimized early elements derived from Pr7.5 (SEQ ID NO:27; *see also* K. Baur *et al.*, " Immediate-Early Expression of a Recombinant Antigen by Modified Vaccinia Virus Ankara Breaks the Immunodominance of Strong Vector-Specific B8R Antigen in Acute and Memory CD8 T-Cell Responses", *J. Virol.* 84(17):8743-8752 (2010)). Consequently, NP- EBOV-CdI will be expressed during both the early and late phases of expression. Moreover, PrLE1 was shown to induce especially strong cell-mediated immune responses. During passaging of MVA-mBN226B, one of the five early elements derived from Pr7.5 was lost, likely by homologous recombination; analysis showed sufficient expression levels of NP-EBOV-CdI, however (data not shown), so the modified construct was used without replacing the modified PrLE1 promoter.

For the insertion of foreign genes into the MVA-BN genome several recombination plasmids that target the different deletions and intergenic regions (IGR) of the MVA-BN genome were generated. To generate recombinant MVA-BN products, foreign sequences of interest can be inserted into any of these basic vectors, *e.g.,* pBNX186 targeting IGR 88/89 (*see* Figure 4A) or pBNX197 targeting IGR 148/149 (*see* Figure 4B), using commonly available restriction enzymes and conventional molecular biology techniques. To produce recombinant MVA-BN isolates expressing the desired transgenes, CEF cells are then infected with MVA-BN and subsequently transfected with one or more recombination plasmids expressing the desired transgene or transgenes and including a selection cassette enabling positive selection for recombinant viruses. During homologous recombination, the plasmid flanking sequences recombine with the homologous sequences of the insertion site in the MVA-BN virus genome. This inserts the plasmid sequences into the site targeted by the basic vector used as starting material (*e.g.*, IGR 148/149, IGR 88/89, etc.) in the MVA-BN genome. pBNX197 targets IGR 148/149 (Figure 4B) and was used as starting plasmid for construction of the final recombination plasmid pBN384 (Figure 5B). Plasmid pBN384 expresses GP-ZEBOV-Mayinga and GP-MARV-Musoke. pBNX 186

targets IGR 88/89 (Figure 4A) and was used as starting plasmid for construction of the final recombination plasmid pBN385 (Figure 5C). Plasmid pBN385 expresses GP-SEBOV and NP-EBOV-CdI.

To insert the GP-SEBOV, NP-EBOV-CdI, GP-ZEBOV-Mayinga, and GP-MARV-Musoke transgenes into MVA-BN, CEF cells were infected with MVA-BN and subsequently transfected with the recombination plasmids pBN384 and pBN385. After amplification and plaque purification (ten passages; three including plaque purification) under double selective conditions (mycophenolic acid/xanthine and hypoxanthine as well as Geneticin) the recombinant MVA-BN product designated MVA-mBN226A (Interim Premaster), containing the genes for three glycoproteins, one nucleoprotein and two selection cassettes, was obtained. Recombinant MVA-mBN226A PreMaster virus stock was examined for elimination of MVA-BN (parental virus; data not shown), for correct sequence of the inserted genes together with the insertion flanking regions (by gene-specific PCR using primers specific for the MVA-BN genomic sequences into which the foreign gene was inserted; data not shown), for absence of microbes (sterility test; data not shown), and for the presence and correct size of the inserts (by sequencing; data not shown). The titer of the MVA-mBN252A PreMaster virus stock was also determined.

After further amplification, removal of the selection cassettes and plaque purification under non-selective conditions (twenty passages; six including plaque purification) recombinant virus MVA-mBN226B devoid of selection cassettes was isolated. Complete elimination of the selection cassettes was confirmed by nested PCR (data not shown). Finally, transgene expression by recombinant MVA-mBN226B was confirmed by reverse-transcriptase PCR (RT-PCR; data not shown).

Construction of MVA-mBN254A (MVA-GP-ZEBOV)

For the GP-ZEBOV transgene expressed from MVA-mBN254A, the full-length DNA sequence of the naturally-occurring gene served as reference sequences. The GP-ZEBOV gene was synthesized by Geneart AG (Regensburg, Germany) with codon usage optimized for expression in humans and to minimize or prevent internal homologous recombination events as described above in “Construction of MVA-mBN226B”. The codon optimization changed the wild-type DNA sequence without altering the amino acid sequence. GP-ZEBOV-Mayinga is expressed under the control of the PrS5E promoter (SEQ ID NO:24).

The PrS5E (SEQ ID NO:24) is a synthetic strong early and late promoter designed from the synthetic early and late promoter (Chakrabarti *et al.*, 1997) followed by 5 early elements of the Pr7.5 promoter from the vaccinia virus 7.5 kDa gene (SEQ ID NO:25; *see also* M.A. Cochran *et al.*, "*In vitro* mutagenesis of the promoter region for a vaccinia virus gene: evidence for tandem early and late regulatory signals", *J. Virol.* 54(1):30-37 (1985)). The PrS5E promoter is described in more detail in the patent application WO 2013/189611A1.

For the insertion of foreign genes into the MVA-BN genome several recombination plasmids that target the different deletions and intergenic regions (IGR) of the MVA-BN genome were constructed. To generate recombinant MVA-BN products, foreign sequences of interest can be inserted into any of these basic vectors, *e.g.,* pBNX197 targeting IGR 148/149 (*see* Figure 4B), using commonly available restriction enzymes and conventional molecular biology techniques. To produce recombinant MVA-BN isolates expressing the desired transgenes, CEF cells are then infected with MVA-BN and subsequently transfected with one or more recombination plasmids expressing the desired transgene or transgenes and including a selection cassette enabling positive selection for recombinant viruses. During homologous recombination, the plasmid flanking sequences recombine with the homologous sequences of the insertion site in the MVA-BN virus genome. This inserts the target sequences into the site targeted by the basic vector used as starting material (*e.g.*, IGR 148/149) in the MVA-BN genome. pBNX197 targets IGR 148/149 (Figure 4B) and was used as starting plasmid for construction of the final recombination plasmid pBN436 (Figure 5D). Plasmid pBN436 contains GP-ZEBOV-Mayinga.

To insert the GP-ZEBOV-Mayinga transgene into MVA-BN, CEF cells were infected with MVA-BN and subsequently transfected with the recombination plasmid pBN436 (Figure 5D). After amplification and plaque purification (nine passages; including three plaque purifications) under selective conditions (mycophenolic acid/xanthine and hypoxanthine) the recombinant MVA-BN product designated MVA-mBN254A (Premaster), containing the gene for GP-ZEBOV-Mayinga and the selection marker GPT-RFP fusion gene (Figure 3C). Recombinant MVA-mBN254A PreMaster virus stock was examined for elimination of MVA-BN (parental virus; data not shown), for correct sequence of the inserted genes together with the insertion flanking regions (by gene-specific PCR using primers specific for the MVA-BN genomic sequences into which the foreign gene was inserted; data not shown), for absence of microbes (sterility test; data not shown), and for the presence and correct size of the inserts (by sequencing; data not shown). The titer of the MVA-mBN254A PreMaster virus stock

was also determined. Finally, transgene expression by recombinant MVA-mBN254A was confirmed by reverse-transcriptase PCR (RT-PCR; data not shown).

Other constructs were generated accordingly. In particular, MVA-mBN255 expressed NP- EBOV-Cdl (SEQ ID NO:28) under the control of the PrS promoter integrated into the IGR 88/99, VP40 ZEBOV (SEQ ID NO:33) under control of the PrS promoter into the IGR 136/137 and GP-ZEBOV (SEQ ID NO:19) under control of the PrS5E promoter into the IGR 148/149 (Figure 13).

Construction of FPV-mBN368A (FPV-GP-ZEBOV) and FPV-mBN391 (FPV-multi-filo)

For the GP-ZEBOV transgene expressed from FPV-mBN368A, the full-length DNA sequence of the naturally-occurring gene served as reference sequences. The GP-ZEBOV gene was synthesized by Geneart AG (Regensburg, Germany) with codon usage optimized for expression in humans as described in "**Construction of MVA-mBN226B**". The codon optimization changed the wild-type DNA sequence without altering the amino acid sequence. GP-ZEBOV-Mayinga is expressed under the control of the FPV-40K promoter (SEQ ID NO:26). The FPV-40K promoter is the FPV promoter sequence of the 40K protein coding sequence in FPV.

For the insertion of foreign genes into the FPV genome, several recombination plasmids that target the different integration sites into the FPV genome were constructed. To generate recombinant FPV products, foreign sequences of interest can be inserted into any of these basic vectors, *e.g.,* pBNX221 targeting insertion site BamHI J (*see* Figure 4D), using commonly available restriction enzymes and conventional molecular biology techniques. To produce recombinant FPV isolates expressing the desired transgenes, CEF cells are then infected with FPV and subsequently transfected with one or more recombination plasmids expressing the desired transgene or transgenes and including a selection cassette enabling positive selection for recombinant viruses. During homologous recombination, the plasmid flanking sequences recombine with the homologous sequences of the insertion site in the FPV virus genome. This inserts the target sequences into the site targeted by the basic vector used as starting material (*e.g.*, insertion site BamHI J) in the FPV genome. pBNX221 targets insertion site BamHI J (Figure 4D) and was used as starting plasmid for construction of the final recombination plasmid pBN555 (Figure 5E). Plasmid pBN555 contains GP-ZEBOV-Mayinga under control of the FPV-40K promoter.

To insert the GP-ZEBOV-Mayinga transgene into FPV, CEF cells were infected with FPV and subsequently transfected with the recombination plasmid pBN555 (Figure 5E). After amplification and plaque purification (13 passages; including four plaque purifications) under selective conditions (mycophenolic acid/xanthine and hypoxanthine) the recombinant MVA-BN product designated FPV-mBN368A (Premaster), containing the gene for GP-ZEBOV-Mayinga and the selection marker GPT-RFP fusion gene (Figure 3D), was obtained. Recombinant FPV-mBN368A PreMaster virus stock was examined for elimination of FPV (parental virus; data not shown), for correct sequence of the inserted genes together with the insertion flanking regions (by gene-specific PCR using primers specific for the FPV genomic flanking sequences into which the foreign gene was inserted; data not shown), for absence of microbes (sterility test; data not shown), and for the presence and correct size of the inserts (by sequencing; data not shown). The titer of the FPV-mBN368A PreMaster virus stock was also determined. Finally, transgene expression by recombinant FPV-mBN368A was confirmed by reverse-transcriptase PCR (RT-PCR; data not shown).

Further fowlpox constructs were generated using FP14 (IGR 60/61) and the BamHI J region for integration according to the method as described above. FPV-mBN391 expressed GP-ZEBOV (SEQ ID NO:19 and 20) under the control of the FPV-40K promoter (SEQ ID NO:26), GP-MARV-Musoke (SEQ ID NO:5 and 6) under the PrS promoter (SEQ ID NO:23) both at the FP14 site and GP-MARV-Angola (SEQ ID NO:36 and 37) under the Pr13.5 long promoter (SEQ ID NO:35), GP-SEBOV (SEQ ID NO:30 and 31) under the FPV-40K promoter and NP-EBOV-Cdl (SEQ ID NO:28 and 29) under the control of the PrLE1 promoter (SEQ ID NO:27), all three inserted at the BamHI J region in the order mentioned.

Example 2: MVA-BN-Filo (MVA-mBN226B) in Non-Human Primates

Immunogenicity and protective efficacy of MVA-BN-Filo (MVA-mBN226B) was analyzed in an Ebola and Marburg challenge model in cynomolgus macaques. Monkeys were housed and fed in accord with the appropriate institutional guidelines for care and feeding of research animals.

The experimental design is set forth in Table 1 below.

Table 1: Vaccination protocol for MVA-BN-Filo in cynomolgus macaques.

		Test/Reference Item Administration				Challenge Virus Administration	
Group	Group Size	Vaccination	Dose per Admin.	Route	Schedule (Days)	Virus	Schedule (Day)
1	1	Vehicle Control (TBS)	-	s.c.	0 and 28	EBOV	42
2	1	Vehicle Control (TBS)	-	s.c.	0 and 28	MARV	42
3	3	MVA-BN®-Filo	$5x10^8$ $TCID_{50}$	s.c.	0 and 28	EBOV	42
4	3	MVA-BN®-Filo	$5x10^8$ $TCID_{50}$	s.c.	0 and 28	MARV	42

Intramuscular challenge (1,000 pfu) with either EBOV Zaire strain or MARV Musoke strain; surviving animals were euthanized on Day 63

Dose volume was 0.5 mL for both vehicle control and vaccination groups; all vaccinations were delivered by subcutaneous injection. First vaccination day is designated Day 0. All animals received a challenge dose of 1,000 pfu of ZEBOV (Groups 1 and 3) or MARV-Musoke (Groups 2 and 4) via intramuscular injection on Day 42. All surviving animals were euthanized on Day 63.

GP-specific antibodies were measured by ELISA. As expected, the non-vaccinated control animal challenged with MARV-Musoke had no detectable GP-MARV-specific antibodies at any time prior to challenge (*i.e.*, on Day 0, Day 28, and Day 36 (data not shown) and succumbed to disease. In contrast, two of the three animals vaccinated with MVA-BN-Filo (animal numbers 30766 and 30768) had low GP-MARV antibody titers 28 days after the first vaccination (post first vaccination; *see* also Figure 6) and all three vaccinated animals showed a clear boost response eight days after the second vaccination (post booster vaccination; *see* Figure 6). All three animals survived the otherwise lethal intramuscular challenge with MARV-Musoke.

Similarly, the non-vaccinated control animal challenged with ZEBOV was negative for GP-ZEBOV-specific antibodies at all time-points tested (data not shown). The control animal, as well as all vaccinated animals succumbed to infection following challenge with ZEBOV by intramuscular injection. Surprisingly, all three vaccinated animals generated GP-ZEBOV-specific antibodies prior to challenge, at levels greater in

magnitude than those measured in hyperimmune serum generated in non-human primates by vaccination with ZEBOV-GP. Complete necropsies were performed on tissue collected in 10% neutral buffered formalin at the time of death. Tissue sections were processed by routine methods, sectioned at 5 µm, and stained with hematoxylin and eosin for histological evaluation. Findings are summarized in Table 2 below.

Table 2: Histological evaluation of vaccinated and control animals.

Animal #	**30763**	**30766**	**30765**	**30768**	**30764**	**30770**	**30769**	**30767**
Necropsy # Challenge Experimental group Survival	N11-05 Marburg control 9d €	N11-04 Marburg vaccine 21d	N11-06 Marburg vaccine 21d	N11-10 Marburg vaccine 21d	N11-07 Ebola control 5d (E)	N11-08 Ebola control 7d (SD)	N11-09 Ebola control 6d (E)	N11-03 Ebola control 6d (E)
Liver:								
multifocal hepatic necrosis	++	-	-	-	+	++	++	-
vasculitis	+	++*	-	++*	-	-	-	-
Lung:								
intra alveolar edema	+	-	-	-	+	+	+	-
septal edema	+	-	-	-	+	+	+	-
hemorrhage	+	-	-	-	+	+	-	-
interstitial pneumonitis	-	-	-	-	-	+++	++	-
Spleen:								
hyperplasia	-	++	+++	+++	-	-	-	-
lymphoid depletion	++	-	-	-	++	+++	+++	++
fibrin deposition red pulp	+++	-	-	-	++	+++	+	++
Splenic vasculitis	+++	-	-	-	++	++	+	++
Inguinal lymph node:								
macrophage infiltration	++	-	++	-	+	+	+	+++
lymphoid depletion	+++	-	-	-	+++	+++	++	-
Axillary lymph node:								
Macrophage infiltration	-	-	-	-	-	-	-	-
lymphoid depletion	+	-	-	-	++	++	++	+
Mesenteric lymph node:								
Macrophage infiltration	-	-	-	-	-	-	-	-
lymphoid depletion	++	-	-	-	++	++	++	+
Adrenal gland:								
necrosis	-	-	-	-	+++	++	-	-

* vasculitis in animals 30766 and 30768 appears to be a pre-existing condition.

E- euthanized; SD- spontaneous death

Analysis confirmed typical symptoms of hemorrhagic fever in the MARV-Musoke-challenged, non-vaccinated control animals, as well as in all ZEBOV-challenged animals, while vaccinated animals challenged with MARV-Musoke showed few

histological changes, except for splenic hyperplasia consistent with a post-challenge immune response and B-cell hyperplasia.

Results of the experiment are summarized in Figure 7. Figure 7A shows that vaccination with MVA-BN-Filo protected 100% of animals from challenge with MARV-Musoke. Figure 7B shows clinical scores post-challenge; vaccinated animals challenged with MARV-Musoke showed no symptoms or histological changes associated with hemorrhagic fever and harbored no virus in liver, spleen, adrenal glands, lymph nodes, or lungs.

Example 3: MVA-BN-Filo in Non-Human Primates

This experiment tested MVA-BN-Filo in non-human primates under a similar study protocol as described in Example 2 but challenged with another Marburg virus strain, *i.e.,* Marburg Angola instead of Marburg Musoke in Example 2.

Table 3: Study design and outcome for MVA-BN-Filo in non-human primates.

Group	N	Test/Control Article Administration			Challenge	Survival
		Vaccination	Dose	Schedule		
1	2	MVA-BN® Filo	$5x10^8$ $TCID_{50}$	0, 28	Marburg Angola Day 56	2/2
2	3	MVA-BN® MARV-IL15sushi	$1x10^8$ $TCID_{50}$			2/3
3	3	MVA-BN®MARV-CD40L	$1x10^8$ $TCID_{50}$			3/3
4	3	MVA-BN® MARV-TRICOM	$1x10^8$ $TCID_{50}$			2/3
5	1	TBS	n/a			0/1

Intramuscular challenge (1,000 pfu) with MARV Angola strain; surviving animals were euthanized on Day 70.

Table 3 shows that MVA-BN-Filo completely protects non-human primates against the Angola strain of Marburg virus. In contrast, the non-vaccinated animal in group 5 succumbed to infection. It also shows that protective efficacy is dose dependent, since a 5-fold lower dose using MVA-BN-MARV, encoding also GP of Marburg virus, is only partially protective, unless it co-expresses CD40L as co-stimulatory molecule. All vaccine candidates produce antibodies specific for the MVA vector (Vaccinia specific

antibodies), as well as antibodies specific for the MARV GP insert, as outlined in Table 4.

Table 4: Antibodies induced by MVA-BN-Filo in non-human primates.

Day 35	MVA-Multivalent	MVA-MARV IL-15	MVA-MARV CD40L	MVA-MARV TRICOM
Vaccinia-Elisa	21596	17838	6560	3246
Vaccinia-PRNT	1735	507	94	85
MARV/GP-ELISA	298959	285187	199132	409941

Example 4: Heterologous prime/boost

This experiment tested the combination of recombinant MVA and recombinant fowlpox FPV in prime/boost immunizations.

H-2K^{k+} B6CBA F1 mice (Janvier Labs, France) were immunized subcutaneously (s.c.) with 5 × 10^7 $TCID_{50}$ MVA-ZEBOV-GP (MVA; MVA-mBN254A, Figure 3C) or FPV-ZEBOV-GP (FPV; FPVmBN368A, Figure 3D). The virus dose was injected at both flanks in a total volume of 100µl/flank.

For the detection of ZEBOV-GP-specific IgG, 96-well plates (Corning, MA, USA) were coated with ZEBOV GP antigen (IBT Bioservices, MD, USA) at 4°C over night. Duplicates of two-fold serum dilutions were added onto washed and blocked plates and a sheep anti-mouse IgG-HRP (AbD Serotec, UK) was used as detection antibody. TMB substrate was added for 30 minutes at RT and the reaction was stopped by the addition of 1M H_2SO_4. The absorbance was measured at 450nm. The murine monoclonal antibody 13F6 was used as a standard in order to calculate the serum concentration of ZEBOV-GP IgG.

Mouse lymphocytes were freshly isolated from spleens by gently grinding and forcing the tissue through a 70µm cell strainer (BD Bioscience, CA, USA). After erylysis, cells were incubated with 5µg/ml ZEBOV-$GP_{577-584}$ peptide (TELRTFSI) (GenScript, NJ, USA) for 6 hours at 37°C in complete RPMI in the presence of 10µg/ml brefeldin A and CD107a-FITC. For live/dead discrimination, cells were stained using the Zombie Aqua™ Fixable Viability kit (BioLegend, CA, USA). Intracellular staining of IFN-γ and TNF-α was performed after surface staining with CD4-BV605, CD8α-BV421 (BioLegend, CA, USA) and CD44-APC-eFluor780 (eBisocience, CA, USA) and fixation/permeabilization according to the manufacturers' instructions (BD Cytofix/Cytoperm, BD Biosciences). All cells were acquired using a digital flow

cytometer (LSR II, BD Biosciences, CA, USA) and data were analyzed with FlowJo software (FlowJo, OR, USA).

The four possible combinations of recombinant MVA and FPV prime/boost immunizations were tested in H-2K^{k+} CBAB6 F1 mice, because a strong CD8 T cell epitope from Zaire Ebola virus (ZEBOV) glycoprotein (GP) was described for this MHC class I haplotype, namely $GP_{577-584}$ (TELRTFSI) (Rao et al., *Vaccine* 17(23-24):2991-8 (1999)). The serum concentration of ZEBOV-GP-specific IgG was analyzed on day 21 and 41 after s.c. immunization on day 0 and 21. While on day 21 all MVA-immunized mice had robust IgG titers, only 20% of FPV-immunized mice had seroconverted. After the second immunization, all animals were seropositive for ZEBOV-GP-specific IgG. The lowest titers were observed after homologous immunization with FPV. Between the animals immunized twice with MVA and those primed with FPV and boosted with MVA no difference in the concentration of GP-specific IgG could be detected on day 41. The mice primed with MVA and boosted with FPV, however, had slightly higher titers than all other groups on day 41 (Figure 8A).

Interestingly, the same combination that resulted in the strongest antibody response also induced the strongest CD8 T cell response. Again, homologous immunization with FPV resulted in the weakest CTL response, followed by MVA-MVA and FPV-MVA immunizations. Priming with MVA followed by a boost with FPV induced ~5-fold more cytotoxic CD8 T cells than the homologous combination of 2× MVA (Figure 8B).

Taken together, these data imply that heterologous immunization with MVA first and FPV second induces the strongest ZEBOV-GP-specific antibody response and also the strongest CTL response, as shown by the presence of highly functional CD8 T cells.

Example 5: Enhanced ZEBOV-GP specific CD8 T cell response

H-2Kk+ CBA mice were immunized s.c. with 5×10^7 $TCID_{50}$ MVA or FPV on day 0 and 21. Mice (5 each per group) were sacrificed on day 42 for T cell analysis by intracellular cytokine staining of splenocytes. The following prime/boost regimens were used: 1: MVA-ZEBOV-GP (mBN254)/FPV-ZEBOV-GP (mBN368), 2: MVA-multi-filo (MVA-mBN226)/FPV-ZEBOV-GP (mBN368), 3: MVA- ZEBOV-GP-VP40 (mBN255)/FPV-ZEBOV-GP (mBN368). Data are summarized in Figure 9.

Splenic CD8 T cell responses were analysed on day 42 after standard 6 hour in vitro re-stimulation with 5 µg/ml ZEBOV-$GP_{577-584}$ peptide (TELRTFSI) in the presence of 10 µg/ml brefeldin A and anti-CD107a-FITC. Cells were surface stained with anti-CD4-BV605, anti-CD8-BV421, CD44-APC-eFluor780 and intracellularly with anti-IFN-γ-

PECy7 and anti-TNF-α-PerCP-eFluor710. Live/dead discrimination was performed by LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit according to the manufacturer's instruction (Life Technologies). Bar graphs show the total number of CD107a+, IFN-γ+ and TNF-α+ CD8 T cells. Shown is the mean ± SEM form 5 mice/group. The CD8 T cell response against the ZEBOV-GP-derived peptide TELFRTSI was enhanced approximately 2-fold when MVA-BN-ZEBOV/GP-VP40 was used as the priming construct in a MVA-FPV heterologous prime-boost regimen compared to MVA-ZEBOV-GP (mBN254) or MVA-multi-filo (MVA-mBN226) as priming constructs (Figure 9).

Example 6: Enhanced Protection of NHPs against ZEBOV after Vaccination with MVA-GP-VP40

Cynomolgus macaques (Macaca fascicularis) were vaccinated twice (on Day 0 and 28) subcutaneously with a dose of $5x10^8$ $TCID_{50}$ with either MVA-BN-ZEBOV/GP (n=3), MVA-BN-ZEBOV/GP-VP40 (n=3), or received Tris-buffered saline (TBS) as negative control (placebo group, n=2). Prior to immunization and weekly before challenge (Days 7, 14, 21, 28, 35 and 40) serum was collected for analysis by Ebola virus Zaire glycoprotein (GP)-specific and MVA-backbone-specific ELISA. Four weeks after the booster vaccination, animals were challenged with Ebola virus Zaire (Kikwit strain) by intramuscular administration of approximately 1000 pfu.

Table 5: Study design:

Group	Vaccination			Challenge		Survival
	Vaccine	Dose	Schedule (Route)	Virus	Schedule	
1	MVA-BN-ZEBOV/GP	$5x10^8$ $TCID_{50}$	Day 0 + 28 (s.c.)	ZEBOV Kikwit Approx. 1000 pfu i.m.	4 weeks post last vaccination	0/3
2	MVA-BN-ZEBOV/GP-VP40	$5x10^8$ $TCID_{50}$	Day 0 + 28 (s.c.)			2/3
3	TBS control	n/a	Day 0 + 28 (s.c.)			0/2

Zaire Ebola virus (ZEBOV)-specific ELISA

An ELISA was performed determining ZEBOV/GP-specific antibodies immobilized by recombinant ZEBOV/GP and detected by a horse radish peroxidase (HRP)-conjugated antibody against NHP IgG. The amount of bound HRP-labeled antibody was read out after a substrate reaction as optical density (OD) value at 450 nm. The antibody

concentration was calculated according to the Four Parameter regression analysis and based on a standard curve using monoclonal mouse antibody.

ELISA results are depicted in Figure 10. All animals vaccinated with the MVA-BN-ZEBOV/GP or MVA-BN-ZEBOV/GP-VP40 construct had detectable backbone- and ZEBOV-specific antibodies already after the prime vaccination and antibody responses were boosted by a second vaccination.

In a second study cynomolgus macaques (Macaca fascicularis) were vaccinated three times (on Day 0, 28 and 56) subcutaneously with a dose of $5x10^8$ $TCID_{50}$ of either MVA-BN-multi-filo (MVA-mBN226, n=2), with MVA-BN-ZEBOV/GP-VP40 (MVA-mBN255, n=2), or received Tris-buffered saline (TBS) as negative control (placebo group, n=2). Prior to immunization and weekly before challenge (Days 0, 27, 41 55, 35 and 67) serum was collected for analysis by Ebola virus Zaire glycoprotein (GP)-specific neutralizing assay (Figure 11). Four weeks after the last vaccination, animals were challenged with Ebola virus Zaire (Kikwit strain) by intramuscular administration of approximately 100 pfu.

Table 6: Study design:

Group	Vaccination				Challenge Schedule	Survival
	Vaccine	Dose	Schedule	Virus		
1	Negative control	n/a	Day 0 + 56	ZEBOV Kikwit approx. 100 pfu IM	4 weeks post last vaccination	0/2
2	MVA-BN-multi-filo	$5x10^8$ $TCID_{50}$	Day 0 + 28 + 56			0/2
3	MVA-BN-ZEBOV/GP-VP40	$5x10^8$ $TCID_{50}$	Day 0 + 28 + 56			2/2

Vaccination with MVA-BN-ZEBOV/GP-VP40 resulted in neutralizing antibodies detectable already after the prime vaccination, while MVA-BN-multi-filo did not induce detectable levels of neutralizing antibodies after prime at day 27 (Figure 11). Animals vaccinated with MVA-BN-ZEBOV/GP-VP40 had higher neutralizing antibody titers than MVA-BN-multi-filo vaccinated animals throughout all time points of analysis. After ZEBOV challenge MVA-BN-multi-filo succumbed by day 7 post challenge whereas MVA-BN-ZEBOV/GP-VP40 vaccinated animals survived with no symptoms or a transient fever episode.

Example 7: VLP formation and protein expression of GP and VP40

HeLa cells were infected with the indicated viruses at a MOI of 10. After 2 days of infection, supernatants were harvested and VLPs in the cleared supernatants (SNs) were then pelleted through a 20% sucrose cushion by ultracentrifugation (UC-SN). Cellular lysates were prepared by direct lysis of cells in 1x Laemmli buffer. Cell lysates were diluted 1:5 prior to separation by SDS-PAGE for immunoblot analysis. UC-SN was not diluted prior to SDS-PAGE. ZEBOV-GP was detected using a monoclonal mouse antibody (clone 6D8) from USAMRIID, and ZEBOV-VP40 was detected using a purified rabbit polyclonal antibody from IBT Bioservices.

Expression of GP and VP40 was confirmed in the fresh preparations by immunoblot. Both proteins were present in cellular lysates and were also enriched in the UC-SN (Figure 12C). Expression of the matrix protein VP40 is known to be sufficient for the formation of VLPs and no direct interaction of VP40 and GP protein has been reported. To show that GP is indeed incorporated into VLPs together with VP40 GP was immunoprecipitated from the SN of infected cells. For this purpose, HeLa cells were infected with MVA-ZEBOV/GP-VP40 and control cells with MVA-ZEBOV/GP and BAC-derived MVA wt. BAC-derived MVA wt has been described previously in Meisinger-Henschel et al (Meisinger-Henschel et al. (2010), *J Virol.* 84(19):9907-9919). The SNs from infected cells were subjected to immunoprecipitation (IP) using an anti-GP-specific antibody. Aliquots of SNs were treated with 1% Triton™ X-100 (TX-100) for 30 minutes prior to immunoprecipitation, which was previously shown to disrupt the majority of mature enveloped VLPs of the murine leukemia virus (Davidoff et al. (2012), *Virology* 433(2):401-409).

The IP-complexes were then analyzed by immunoblot for the presence of GP and of co-precipitated VP40. For immunoprecipitation (IP), cleared SNs were incubated with anti-ZEBOV-GP (clone 6D8, USAMRIID) antibody together with Protein G-Agarose (10 µl) at 4°C overnight. Immunoblots of the immunoprecipitates were then incubated with antibodies against ZEBOV-GP (monoclonal antibody 6D8) and ZEBOV-VP40 (from IBT). GP protein was immunoprecipitated efficiently from the SN of cells expressing only GP (Figure 12D, top panel) which was independent of the presence of VP40. Importantly, VP40 co-immunoprecipitated with GP from supernatants of MVA-ZEBOV/GP-VP40 infected cells only in the absence of TX-100 (Figure 12D, bottom panel, lane 2), indicating that GP had indeed been incorporated into VLPs. Since TX-100 is supposed to disrupt VLPs and since no direct GP-VP40 interaction exists, no VP40 could be co-precipitated in the TX-100 treated samples (Figure 12D, bottom

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panel, lane 4). Thus, it was shown that indeed GP presenting VLP were produced upon infection with recombinant MVA.

Example 8: VLP formation in 293T/17

To possibly get higher VLP concentrations after infection of cells, 293T/17 cells for the preparation of fresh VLPs. Protein contents of preparations were analyzed for GP and VP40 by Western Blot.

293T/17 cells in T175 culture dishes were infected with the indicated viruses at a MOI of 10. Supernatants were collected 24 h post infection and either directly mixed with 3x loading buffer (crude SN) or concentrated over a 20% sucrose cushion (UZ-prep). Cellular lysates (CL) were prepared in 1x loading buffer. Proteins were separated according to size by denaturing SDS-PAGE. Immunoblots were incubated with anti-GP antibody (clone 6D8, 1:2500, USAMRIID) or anti-VP40 antibody (polyclonal, 1:1000, IBT) and were developed using a chemiluminescence substrate.

Expression of EBOV glycoprotein (GP) was readily detectable after infection of cells with MVA-ZEBOV/GP and MVA-ZEBOV/GP-VP40 (MVA-filo-VLP), VP40 after infection of MVA-filo-VLP, both in cellular lysate (CL) and supernatant (SN) from infected cells. Surprisingly, MVA-ZEBOV/GP-infected cells seem to express more GP when compared to cells infected with MVA-filo-VLP, whereas with MVA-filo-VLP more GP was found in the SN. This possibly reflects the fact that with MVA-filo-VLP, the co-expression of GP and VP40 enhances GP release (in form of VLP) from infected cells. Both, GP and VP40 proteins were present in the UZ-preps, indicating that GP and VP40 were collected by UZ. Some GP and also VP40 was still present in SN after UZ, although less when compared to crude SN, especially true for VP40. Thus, VP40 - mainly present in form of VLPs, together with GP - is largely depleted from SN, whereas parts of the GP pool (possibly in form of pleomorphic particles) remain in the SN after UZ.

Transmission electron microscopy (TEM) and immuno-electron microscopy analysis of VLPs from 293T/17cells showed that MVA-filo-VLPs produced by the respective MVA recombinant were densely decorated with GP, GP spikes lining the entire surface of a filo-VLP. Additionally, preparations from cells infected with MVA-wt or MVA-ZEBOV/GP were analyzed by immuno-EM; no VLPs were detected in these samples.

Example 9: Immunogenicity of heterologous prime-boost immunization in NHP

Four cynomolgus macaques were vaccinated (s.c.) on Days 0 and 28. Two animals received as prime $5x10^8$ $TCID_{50}$ MVA-mBN226 Day 0 and as boost $1x10^9$ $TCID_{50}$ FPV-mBN368 Day 28. One animal received as prime $1x10^9$ $TCID_{50}$ FPV-mBN368 Day 0 and as boost $5x10^8$ $TCID_{50}$ MVA-mBN226. One control animal only received TBS. On Days 0, 7, 28, 35 and 49 PBMC were isolated. Blood was collected at Day 0, 28 and 49 for hematology, clinical chemistry, coagulation parameters, isolation of PBMCs or serum for analysis of T cell and antibody responses, respectively, and for viral load analysis. Serum samples were analyzed for Ebola specific humoral responses by ELISA and FRNT. GP- and Vaccinia- specific T cells were analyzed by in vitro re-stimulation of PBMC with a ZEBOV/GP peptide library with Vaccinia Wyeth, followed by detection of IFN-γ secreting cells by ELISPOT. All animals received an intramuscular (i.m.) challenge (100 pfu) of EBOV Kikwit-9510621 on Day 56. All three animals who received heterologous prime boost with MVA and FPV survived. Full seroconversion was already obtained after prime which was further improved by the boost.

Description of the SEQUENCE LISTING

SEQ ID NO:1 [DNA sequence encoding GP-SEBOV-Maleo (GenBank Accession No. U23069.1)]

SEQ ID NO:2 [amino acid sequence of GP-SEBOV-Maleo (GenBank Accession No. U23069.1)]

SEQ ID NO:3 [DNA sequence encoding NP-SEBOV-Boniface (GenBank Accession No. AF173836 .1)]

SEQ ID NO:4 [amino acid sequence of NP-SEBOV-Boniface (GenBank Accession No. AF173836)]

SEQ ID NO:5 [codon-optimized DNA sequence encoding GP-MARV-Musoke (GenBank Accession No. ABA87127.1 for protein sequence)]

SEQ ID NO:6 [amino acid sequence of GP-MARV-Musoke (GenBank Accession No. ABA87127.1)]

SEQ ID NO:7 [DNA sequence encoding TTC]

SEQ ID NO:8 [amino acid sequence of TTC]

SEQ ID NO:9 [DNA sequence encoding hCD40L]

SEQ ID NO:10 [amino acid sequence of hCD40L]

SEQ ID NO:11 [DNA sequence encoding hIL15R-Sushi]

SEQ ID NO:12 [amino acid sequence of hIL15R-Sushi]

SEQ ID NO:13 [DNA sequence encoding human LFA-3/CD58 (EMBL-CDS Accession No. CAA75083.1)]

SEQ ID NO:14 [amino acid sequence of human LFA-3/CD58 (UniProtKB/SwissProt Accession No. P19256)]

SEQ ID NO:15 [DNA sequence encoding human ICAM-1/CD54 (GenBank Accession No. BT006854)]

SEQ ID NO:16 [amino acid sequence of human ICAM-1/CD54 (UniProtKB/SwissProt Accession No. P05362)]

SEQ ID NO:17 [DNA sequence encoding human B7.1/CD80 (EMBL-CDS Accession No. AAA58390.1)]

SEQ ID NO:18 [amino acid sequence of human B7.1/CD80 (UniProtKB/SwissProt Accession No. P33681)]

SEQ ID NO:19 [codon-optimized DNA encoding GP-ZEBOV-Mayinga (GenBank Accession No. ABX75367.1)]

SEQ ID NO:20 [amino acid sequence of GP-ZEBOV-Mayinga (GenBank Accession No. ABX75367.1)]

SEQ ID NO:21 [DNA sequence encoding VV B5R anchor]

SEQ ID NO:22 [amino acid sequence of VV B5R anchor]

SEQ ID NO:23 [DNA sequence of PrS promoter]

SEQ ID NO:24 [DNA sequence of PrS5E promoter: 1x (PrS) + 5x (Pr7.5e)]

SEQ ID NO:25 [DNA sequence of Pr7.5 promoter]

SEQ ID NO:26 [DNA sequence of the FPV-40K promoter of FPV

SEQ ID NO:27 [DNA sequence of PrLE1 promoter – 1x (ATI) + 5x (Pr7.5e)]

SEQ ID NO:28 [codon-optimized DNA sequence encoding NP-EBOV-Cdl (GenBank Accession No. ACI28629.1)]

SEQ ID NO:29 [amino acid sequence of NP-EBOV-Cdl (GenBank Accession No. ACI28629.1)]

SEQ ID NO:30 [codon optimized DNA sequence encoding GP-SEBOV-Gulu (GenBank Accession No. AAU43887.1)]

SEQ ID NO:31 [amino acid sequence of GP-SEBOV-Gulu (GenBank Accession No. AAU43887.1)]

SEQ ID NO:32 [DNA sequence of PrLE1 promoter – 1x (ATI) + 4x (Pr7.5e)]

SEQ ID NO:33 [codon optimized DNA sequence DNA sequence encoding VP40-ZEBOV-Mayinga sequence]

SEQ ID NO:34 [amino acid sequence of VP40-ZEBOV-Mayinga sequence]

SEQ ID NO:35 [Pr13.5 promoter sequence]

SEQ ID NO:36 [codon optimized DNA sequence DNA sequence encoding GP-MARV-Angola]

SEQ ID NO:37 [amino acid sequence of GP-MARV-Angola]

CLAIMS

1. A recombinant Modified Vaccinia Virus (MVA) vector comprising a first nucleic acid sequence encoding at least one immunogenic protein of a Marburg virus (MARV) envelope glycoprotein (GP); a second nucleic acid encoding an immunogenic protein of Zaire Ebola virus (ZEBOV) envelope GP; a third nucleic acid encoding an immunogenic protein of Sudan Ebola virus (SEBOV) envelope GP; and a fourth nucleic acid encoding an immunogenic protein of Ebola virus Ivory Coast nucleoprotein, for use in inducing an immune response against the MARV in a subject.
2. The recombinant MVA vector for use according to claim 1, wherein the MARV envelope glycoprotein is full-length MARV-Musoke envelope glycoprotein.
3. The recombinant MVA vector for use according to claim 2, wherein the first nucleic acid encodes an immunogenic protein comprising the sequence set forth in SEQ ID NO:6.
4. The recombinant MVA vector for use according to claim 3, wherein the first nucleic acid comprises the sequence set forth in SEQ ID NO:5.
5. The recombinant MVA vector for use according to claim 1 or 2, wherein the recombinant MVA vector comprises a nucleic acid encoding an immunogenic protein comprising a sequence set forth in SEQ ID NO:2, SEQ ID NO:6, SEQ ID NO: 20, SEQ ID NO:29, SEQ ID NO:31, or SEQ ID NO:37.
6. The recombinant MVA vector for use according to claim 1 or 2, wherein the recombinant MVA vector comprises a nucleic acid encoding an immunogenic protein comprising a sequence set forth in SEQ ID NO:29, SEQ ID NO:6, SEQ ID NO:20 or SEQ ID NO:31.
7. The recombinant MVA vector for use according to claim 6, wherein the nucleic acid comprises the sequence set forth in SEQ ID NO: 28, SEQ ID NO:5, SEQ ID NO:19 or SEQ ID NO:30.
8. The recombinant MVA vector for use according to claims 1 or 2, wherein the recombinant MVA vector comprises at least one nucleic acid encoding the sequences set forth in SEQ ID NO:6, SEQ ID NO:20, SEQ ID NO:29, and SEQ ID NO:31.
9. The recombinant MVA vector for use according to any one of claims 1-8, wherein the recombinant MVA further comprises a nucleic acid encoding CD40L.
10. The recombinant MVA vector for use according to claim 9, wherein the CD40L comprises the amino acid sequence set forth in SEQ ID NO:10.

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11. The recombinant MVA vector for use according to claim 10, wherein the nucleic acid encoding CD40L comprises the sequence set forth in SEQ ID NO:9.

12. The recombinant MVA vector for use according to any one of claims 1-11, wherein the recombinant MVA vector provides protective immunity or a protective immune response in the subject.

13. Use of the recombinant MVA vector defined in any one of claims 1-11, for manufacturing a pharmaceutical for inducing an immune response against the Marburg virus in a subject.

14. Use of the recombinant MVA vector defined in any one of claims 1-11, for inducing an immune response against the Marburg virus in a subject.

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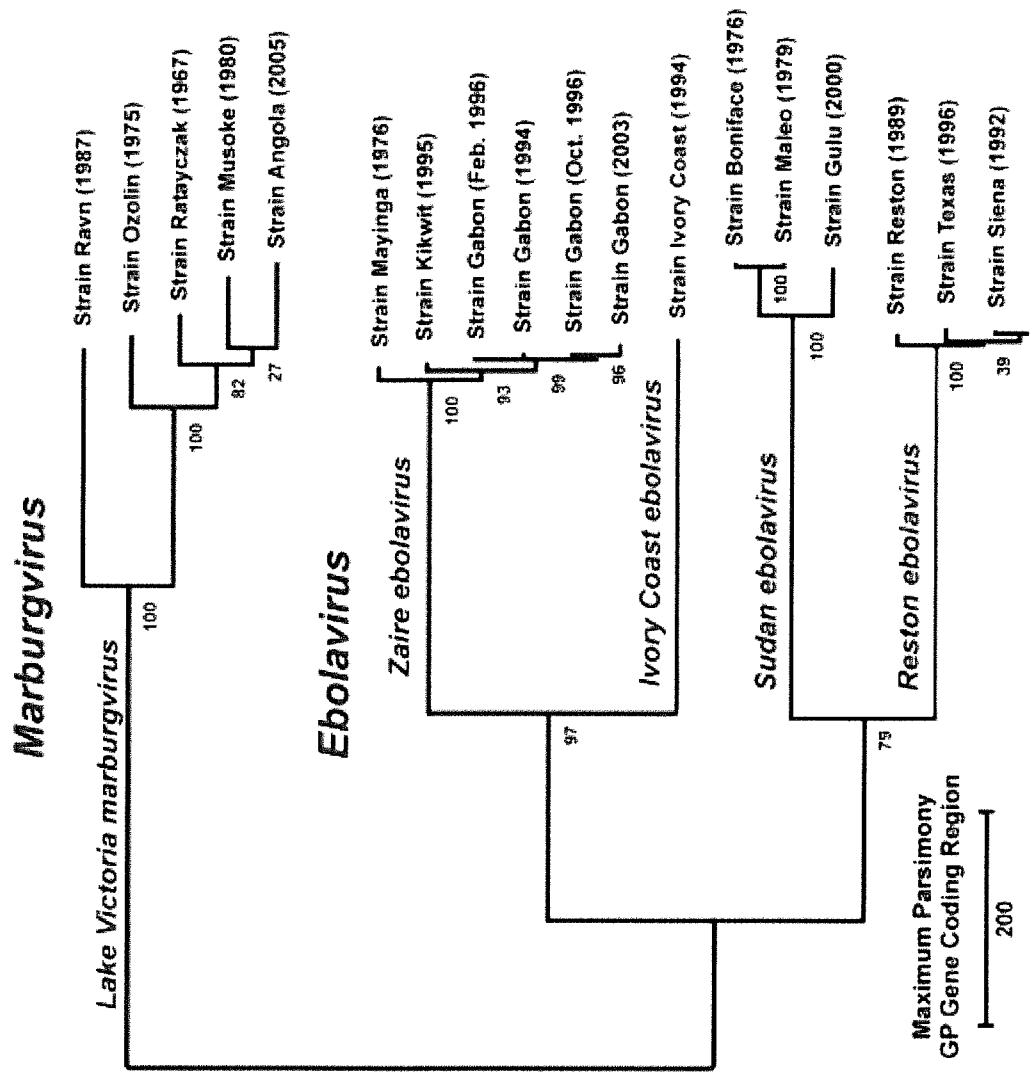
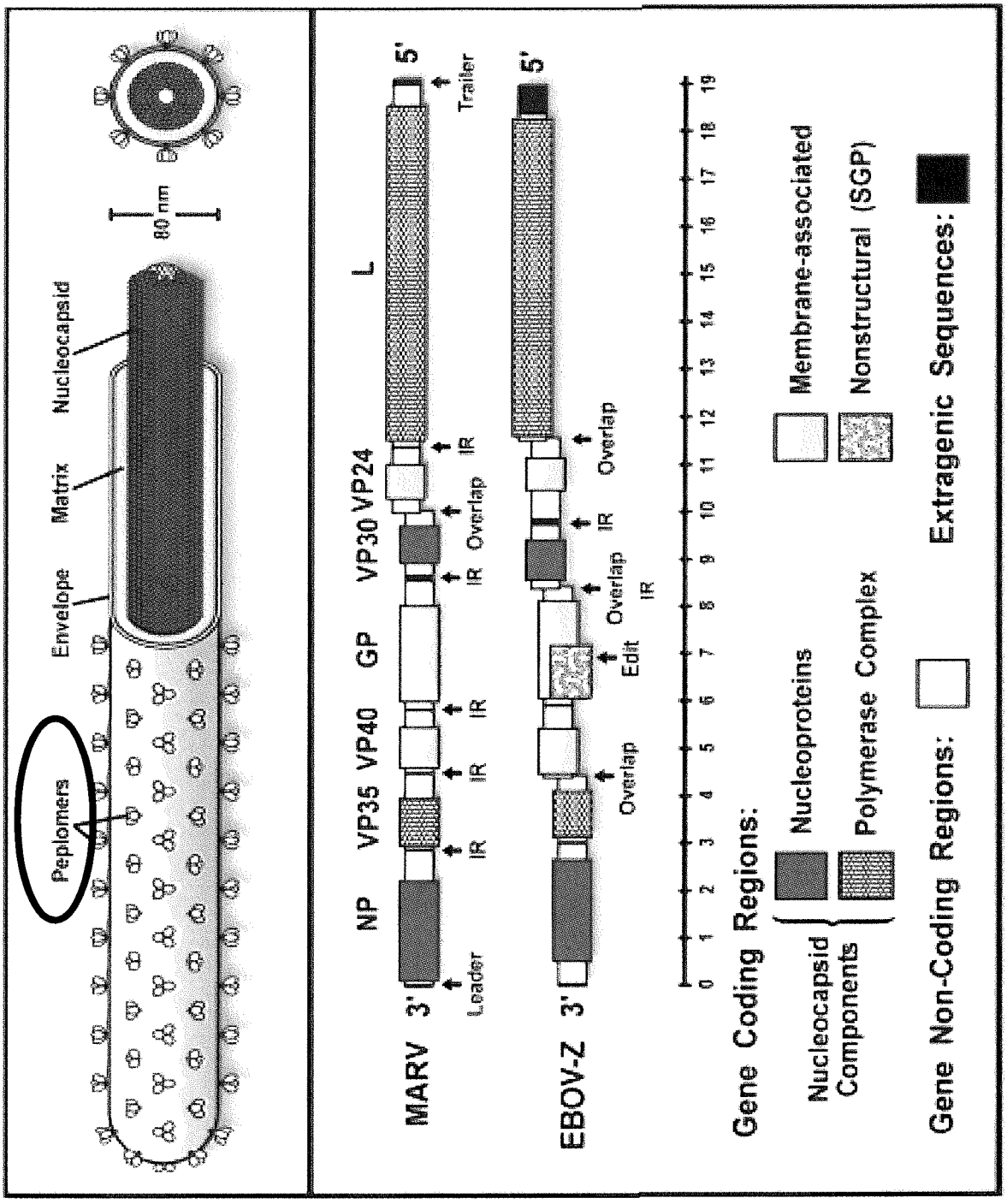

Marburgvirus
Lake Victoria marburgvirus
Strain Ravn (1987)
Strain Ozolin (1975)
Strain Ratayczak (1967)
Strain Musoke (1980)
Strain Angola (2005)
100
100
82
27
Ebolavirus
Zaire ebolavirus
Strain Mayinga (1976)
Strain Kikwit (1995)
Strain Gabon (Feb. 1996)
Strain Gabon (1994)
Strain Gabon (Oct. 1996)
Strain Gabon (2003)
100
93
99
96
Ivory Coast ebolavirus
Strain Ivory Coast (1994)
97
Sudan ebolavirus
Strain Boniface (1976)
Strain Maleo (1979)
Strain Gulu (2000)
100
100
Reston ebolavirus
Strain Reston (1989)
Strain Texas (1996)
Strain Siena (1992)
79
100
39
Maximum Parsimony
GP Gene Coding Region
200

Figure 1

Figure 2


Peplomers
Envelope
Matrix
Nucleocapsid
80 nm
NP VP35 VP40 GP VP30 VP24 L
MARV 3'
Leader
IR
IR
IR
IR
Overlap
IR
Trailer
5'
EBOV-Z 3'
Overlap
Edit
Overlap
IR
Overlap
5'
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Gene Coding Regions:
Nucleocapsid Components
Nucleoproteins
Polymerase Complex
Membrane-associated
Nonstructural (SGP)
Gene Non-Coding Regions:
Extragenic Sequences:
Adapted from Fields ed.: Virology

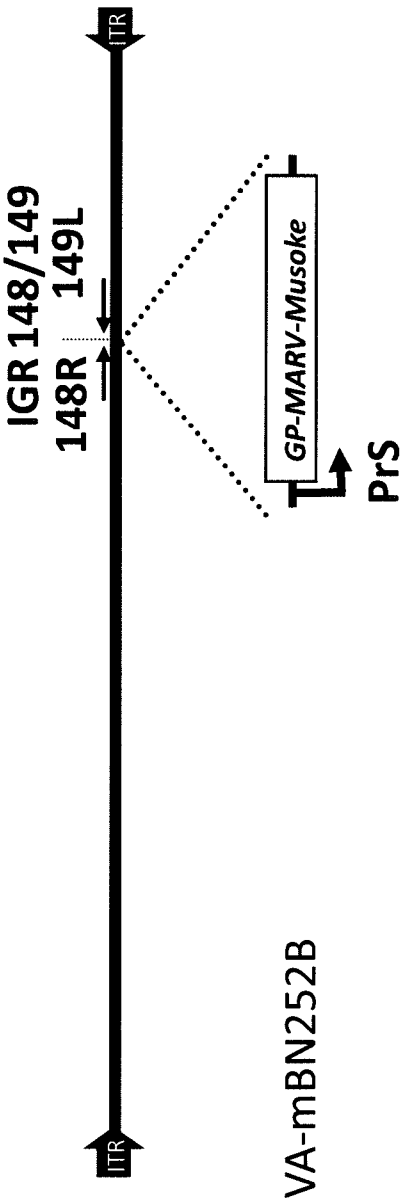


Figure 3A

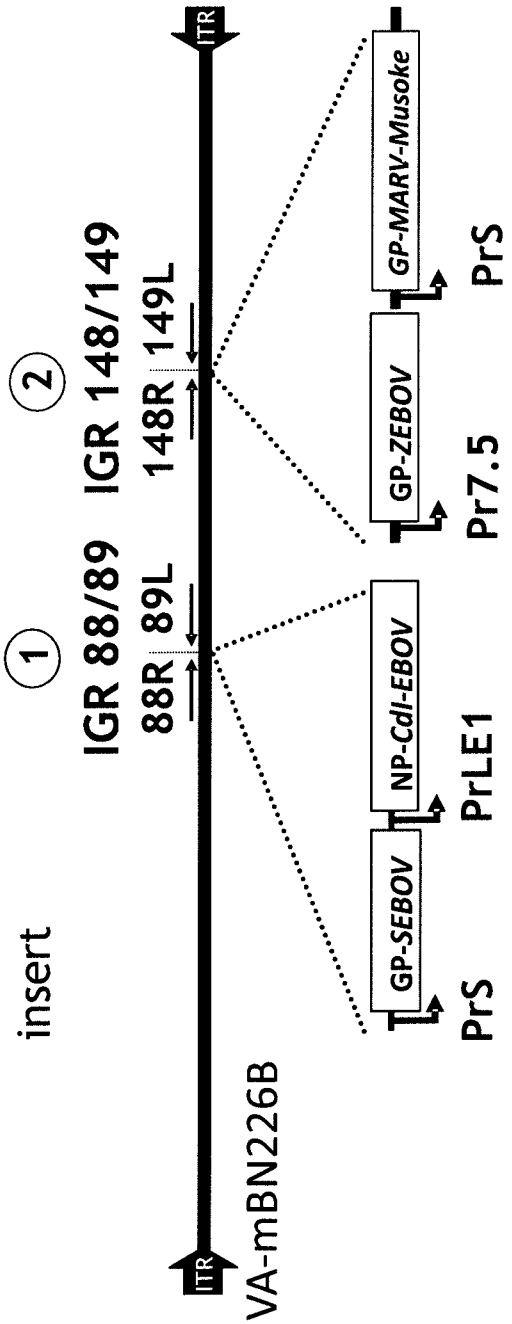


Figure 3B

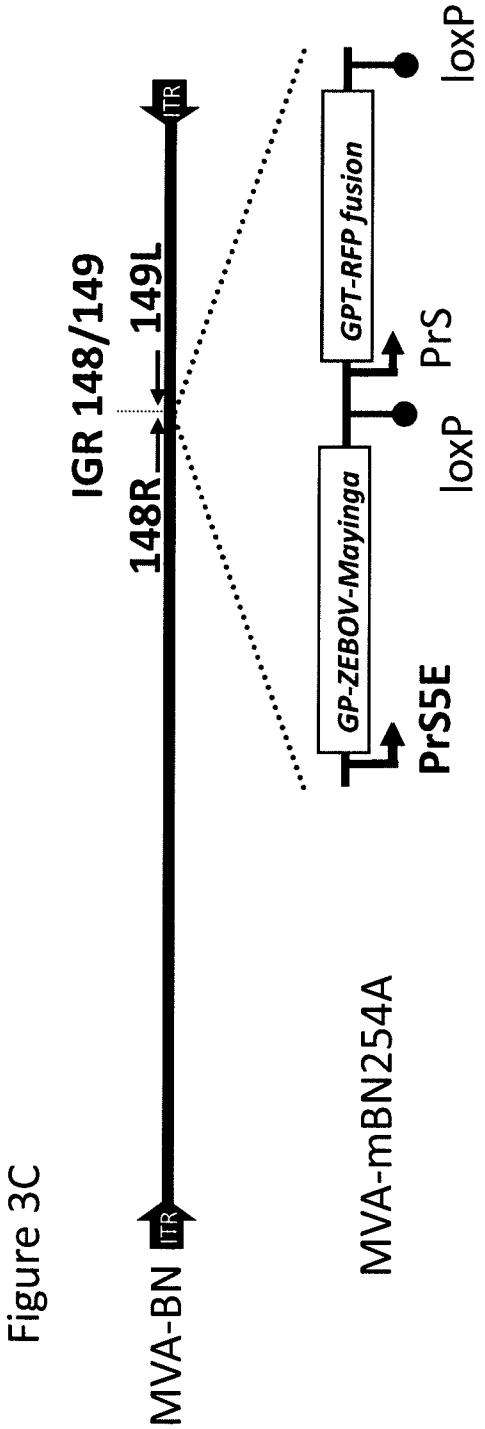


Figure 3C

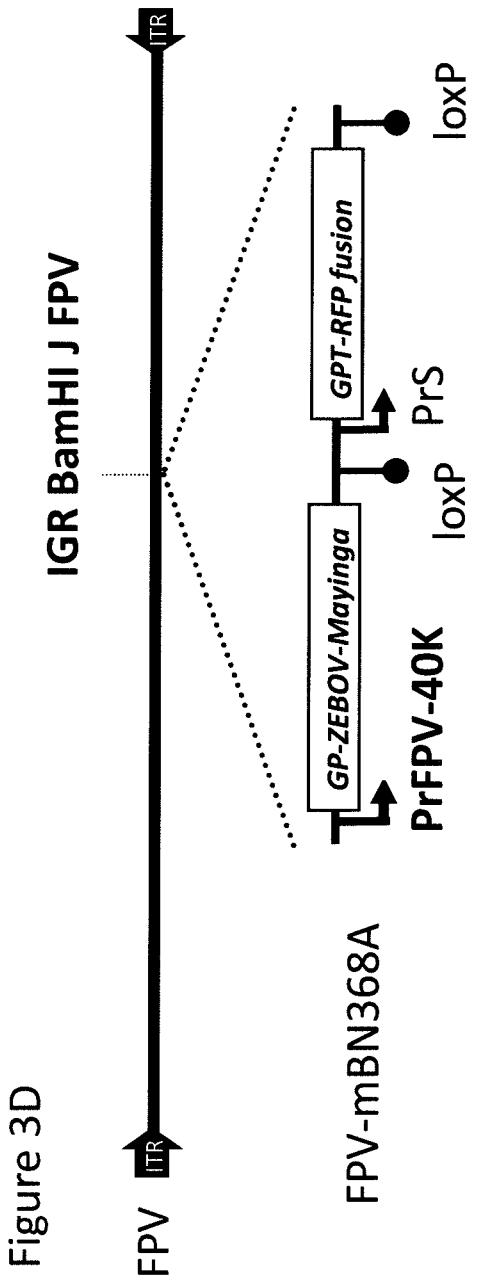


Figure 3D

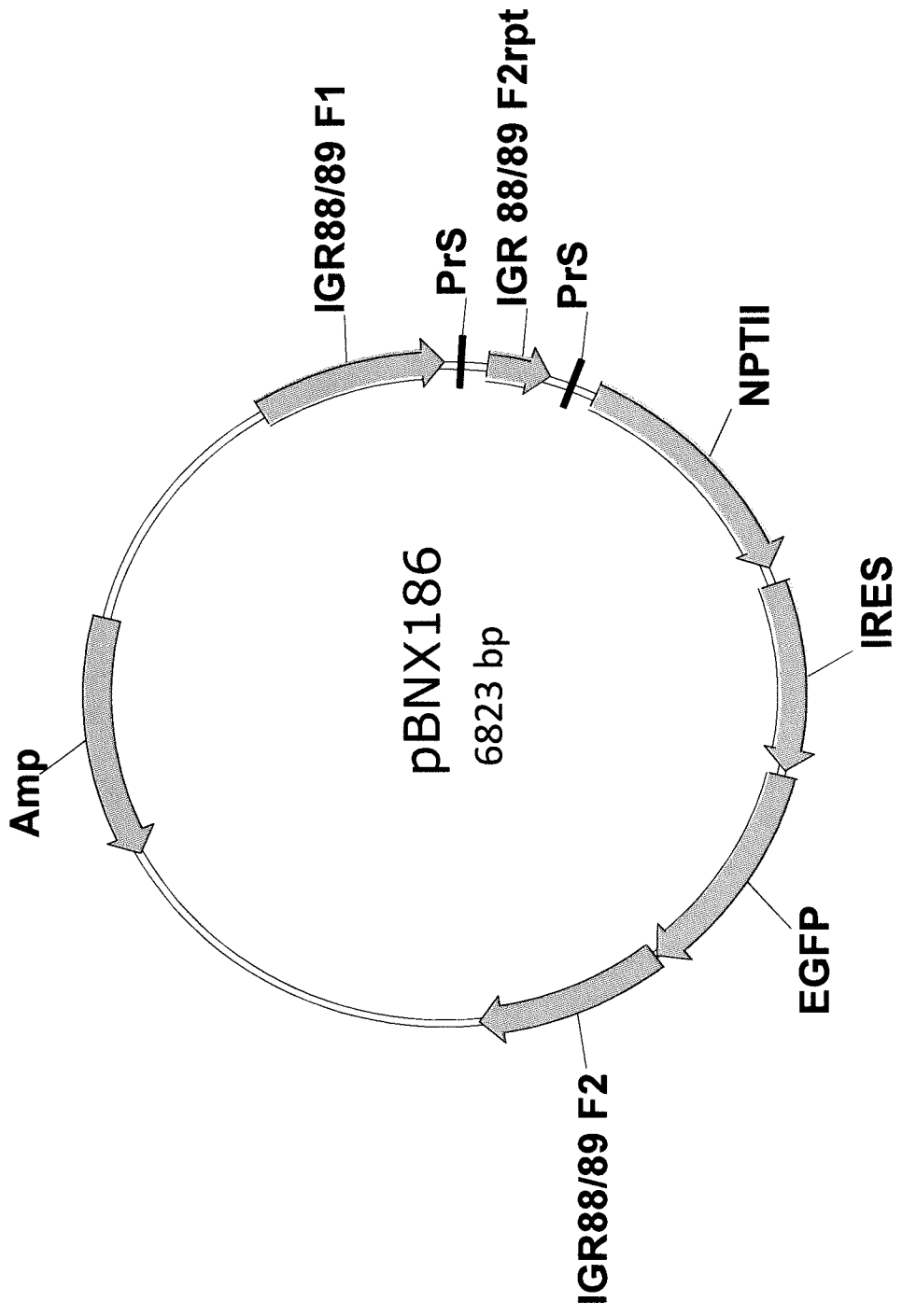

IGR88/89 F1
PrS
IGR 88/89 F2rpt
PrS
NPTII
IRES
EGFP
IGR88/89 F2
Amp
pBNX186
6823 bp

Figure 4A

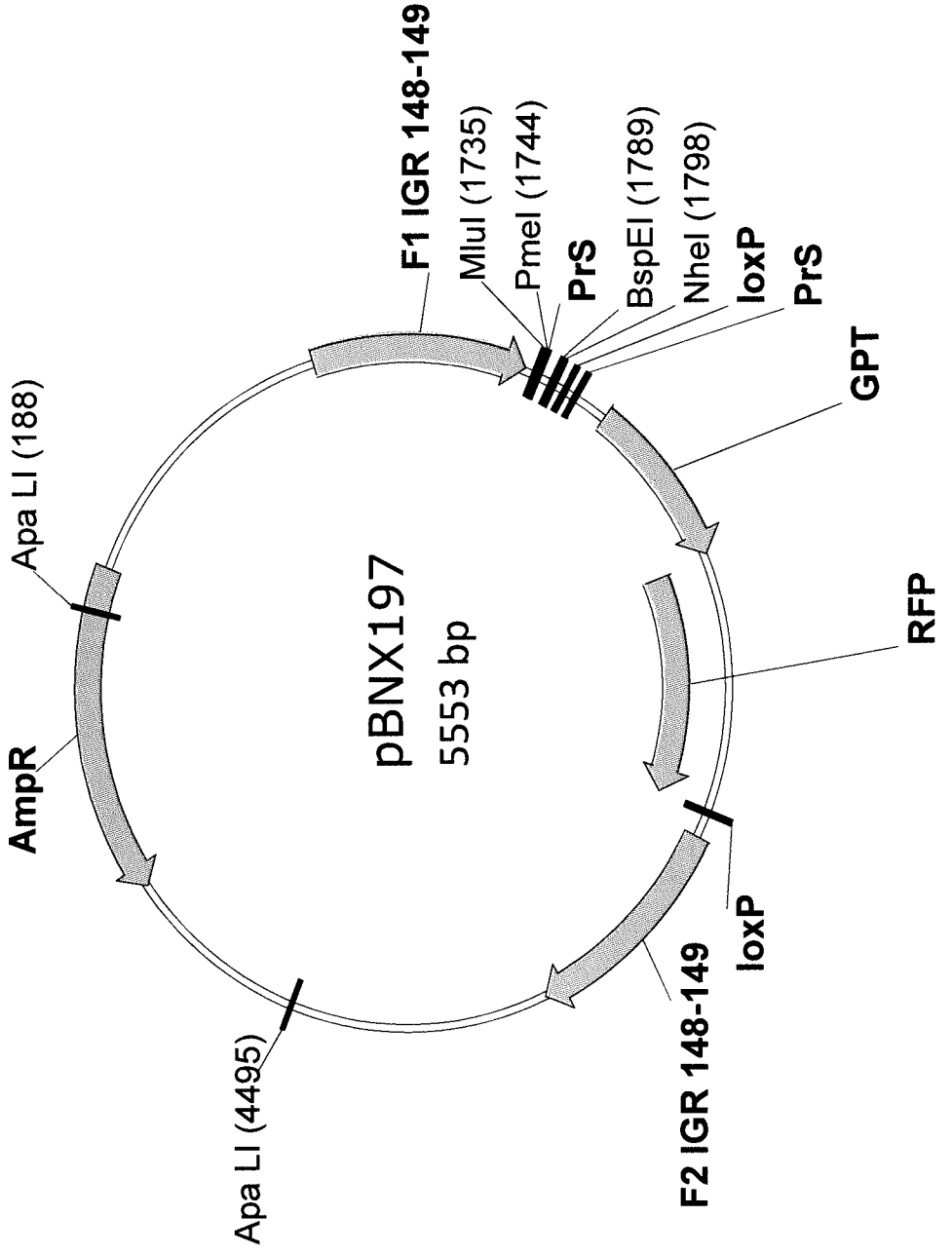

pBNX197
5553 bp
F1 IGR 148-149
MluI (1735)
PmeI (1744)
PrS
BspEI (1789)
NheI (1798)
loxP
PrS
GPT
RFP
loxP
F2 IGR 148-149
Apa LI (4495)
AmpR
Apa LI (188)

Figure 4B

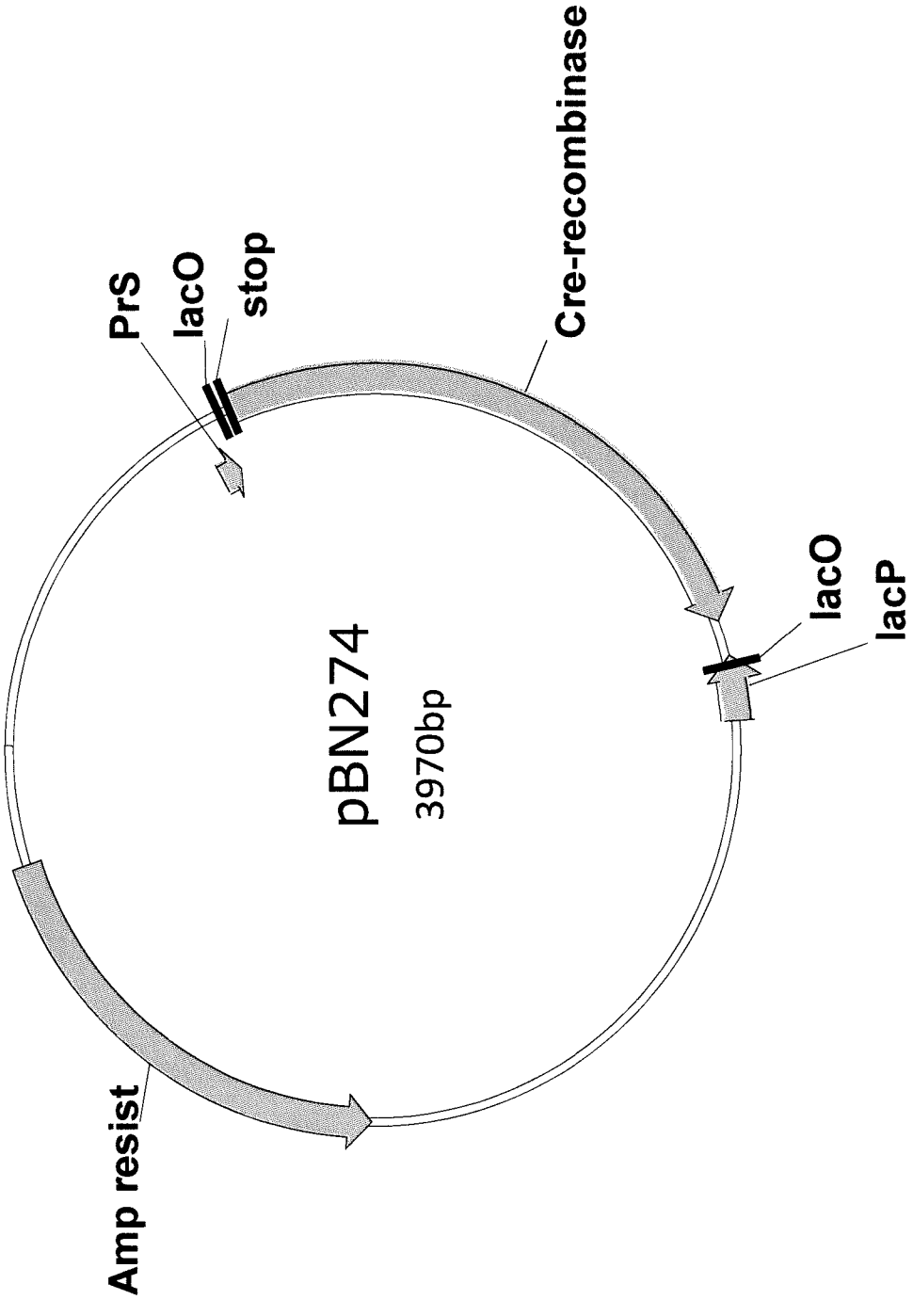

PrS
lacO
stop
Cre-recombinase
lacO
lacP
pBN274
3970bp
Amp resist

Figure 4C

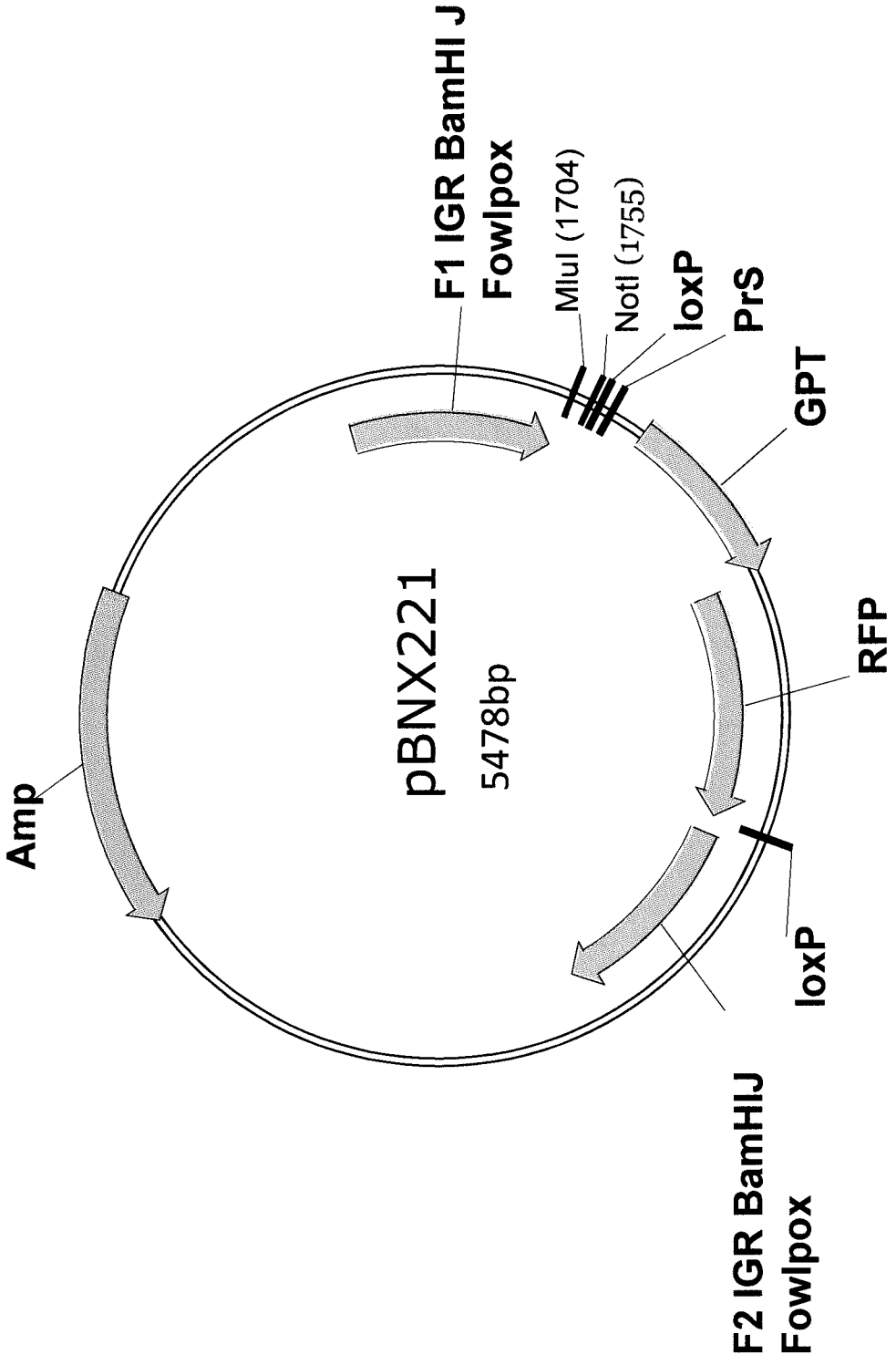

F1 IGR BamHI J
Fowlpox
MluI (1704)
NotI (1755)
loxP
PrS
GPT
RFP
loxP
F2 IGR BamHIJ
Fowlpox
Amp
pBNX221
5478bp

Figure 4D

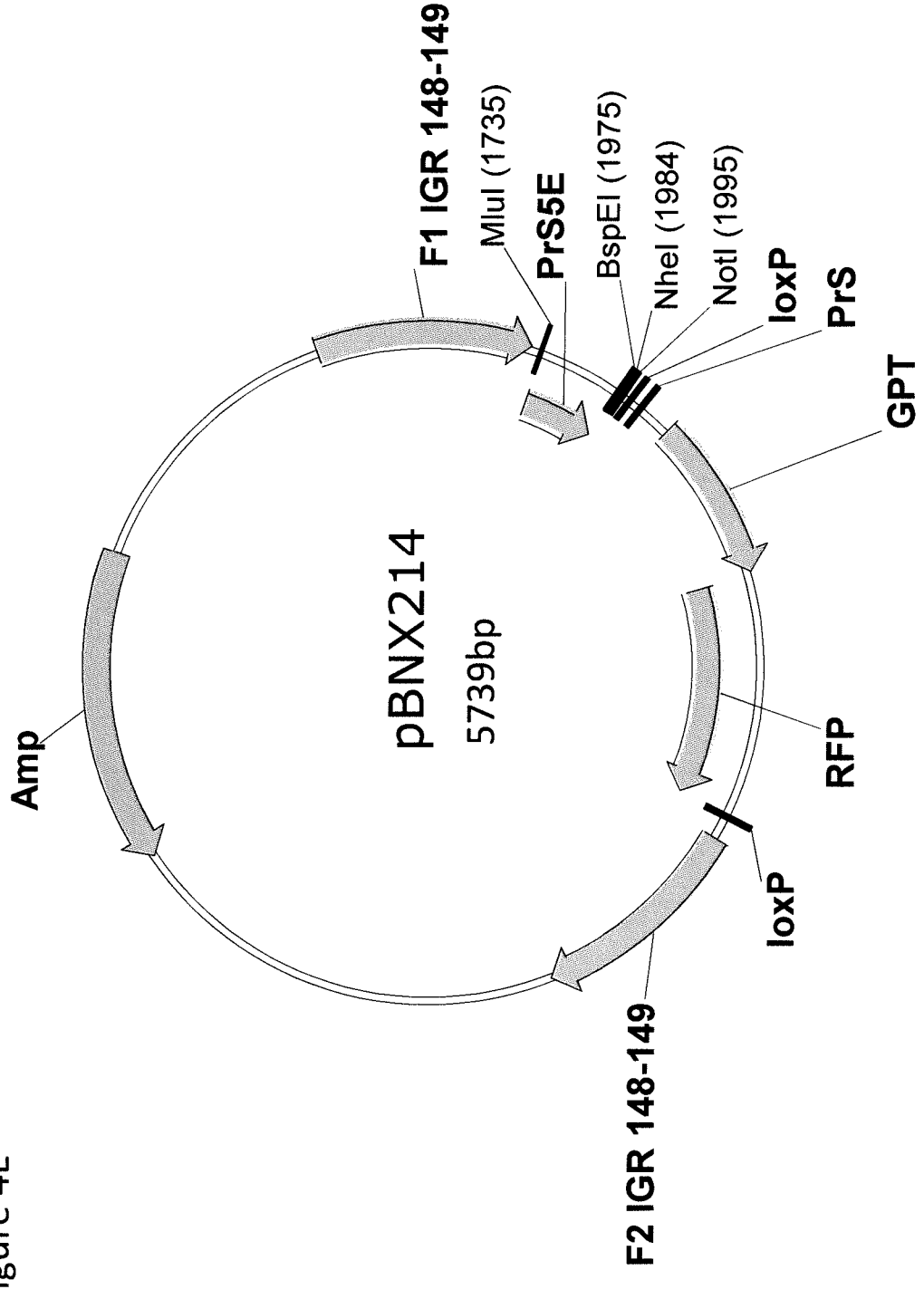

pBNX214
5739bp
F1 IGR 148-149
MluI (1735)
PrS5E
BspEI (1975)
NheI (1984)
NotI (1995)
loxP
PrS
GPT
RFP
loxP
F2 IGR 148-149
Amp

Figure 4E

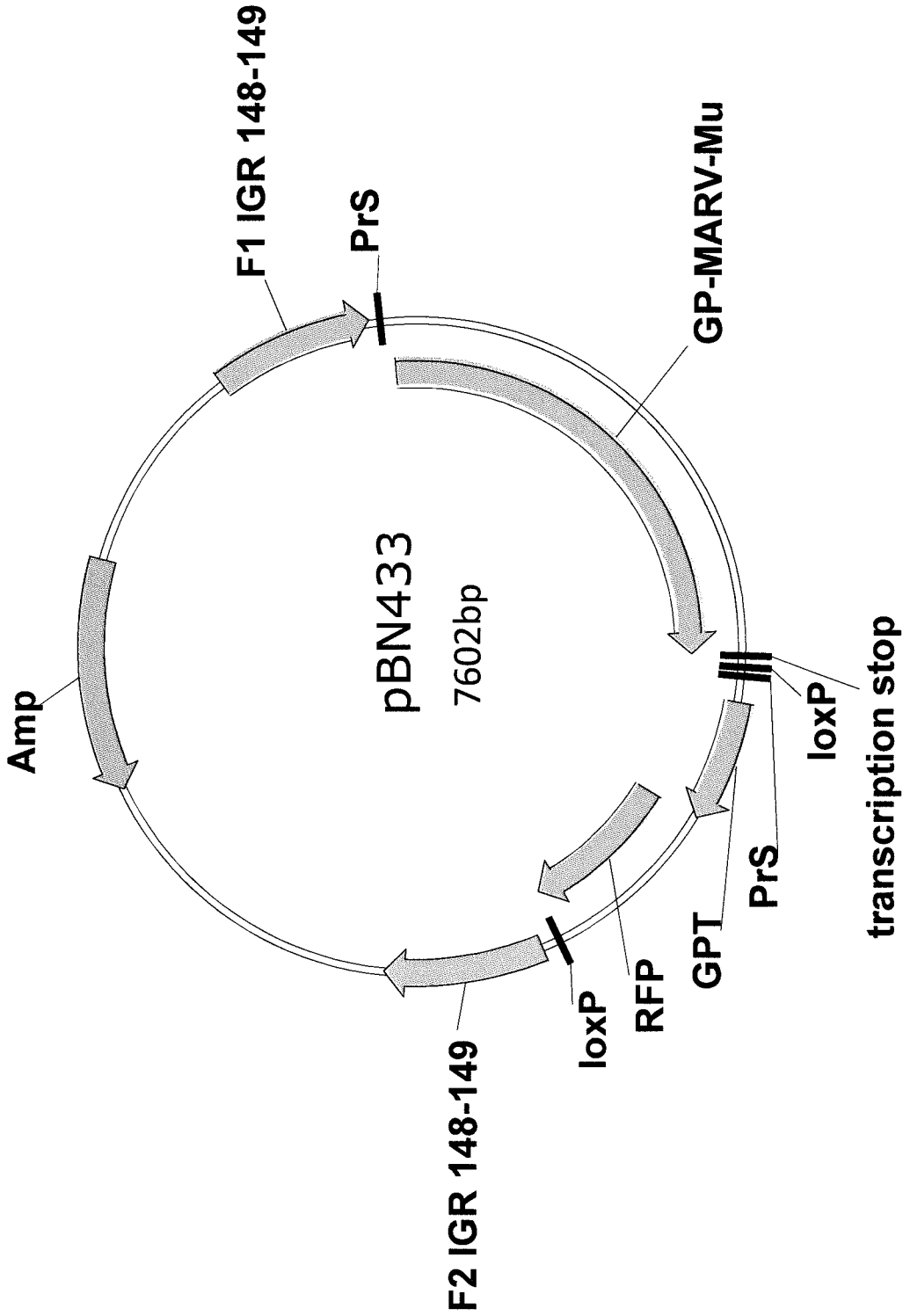

F1 IGR 148-149
PrS
GP-MARV-Mu
pBN433
7602bp
Amp
loxP
transcription stop
PrS
GPT
RFP
loxP
F2 IGR 148-149

Figure 5A

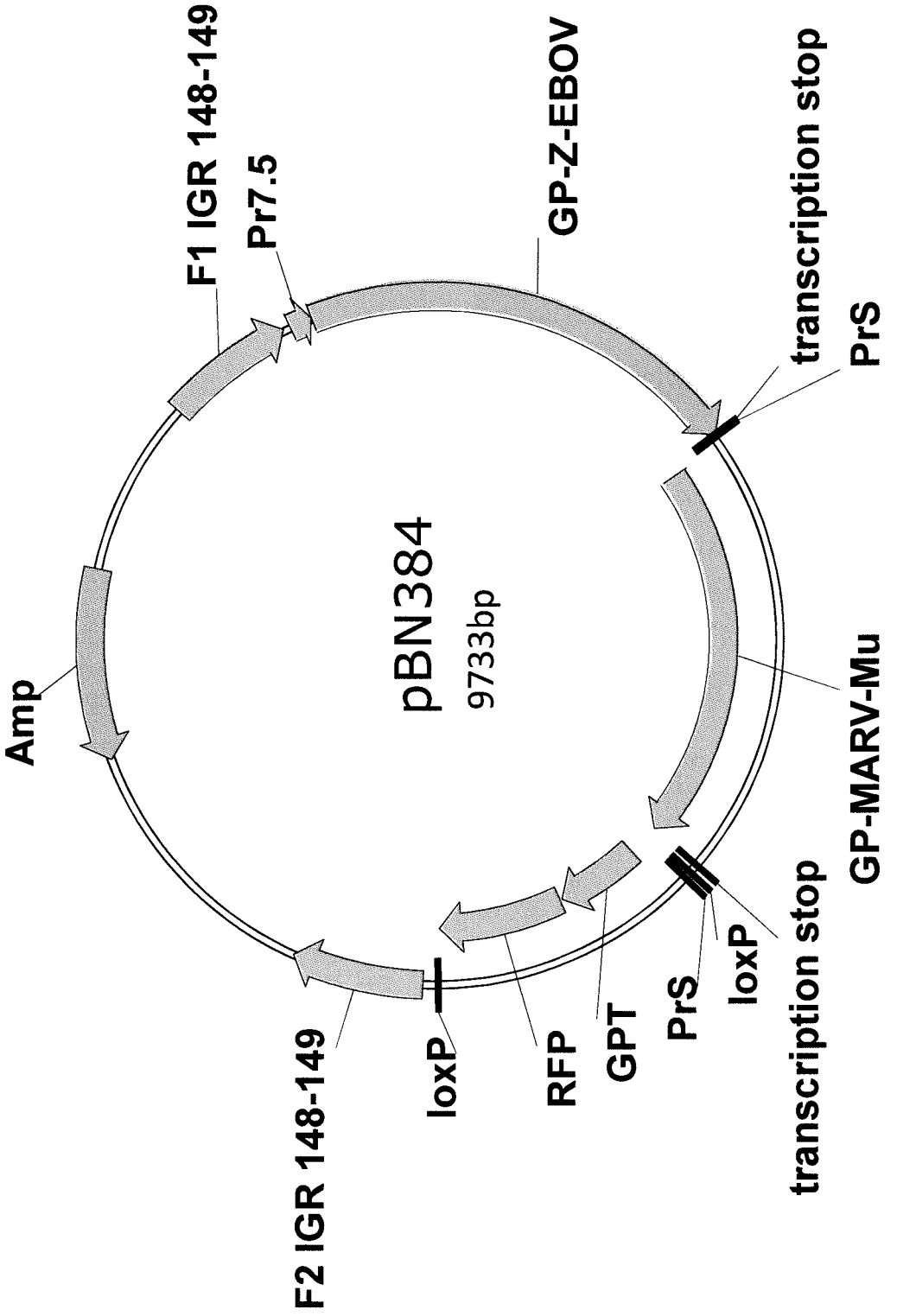
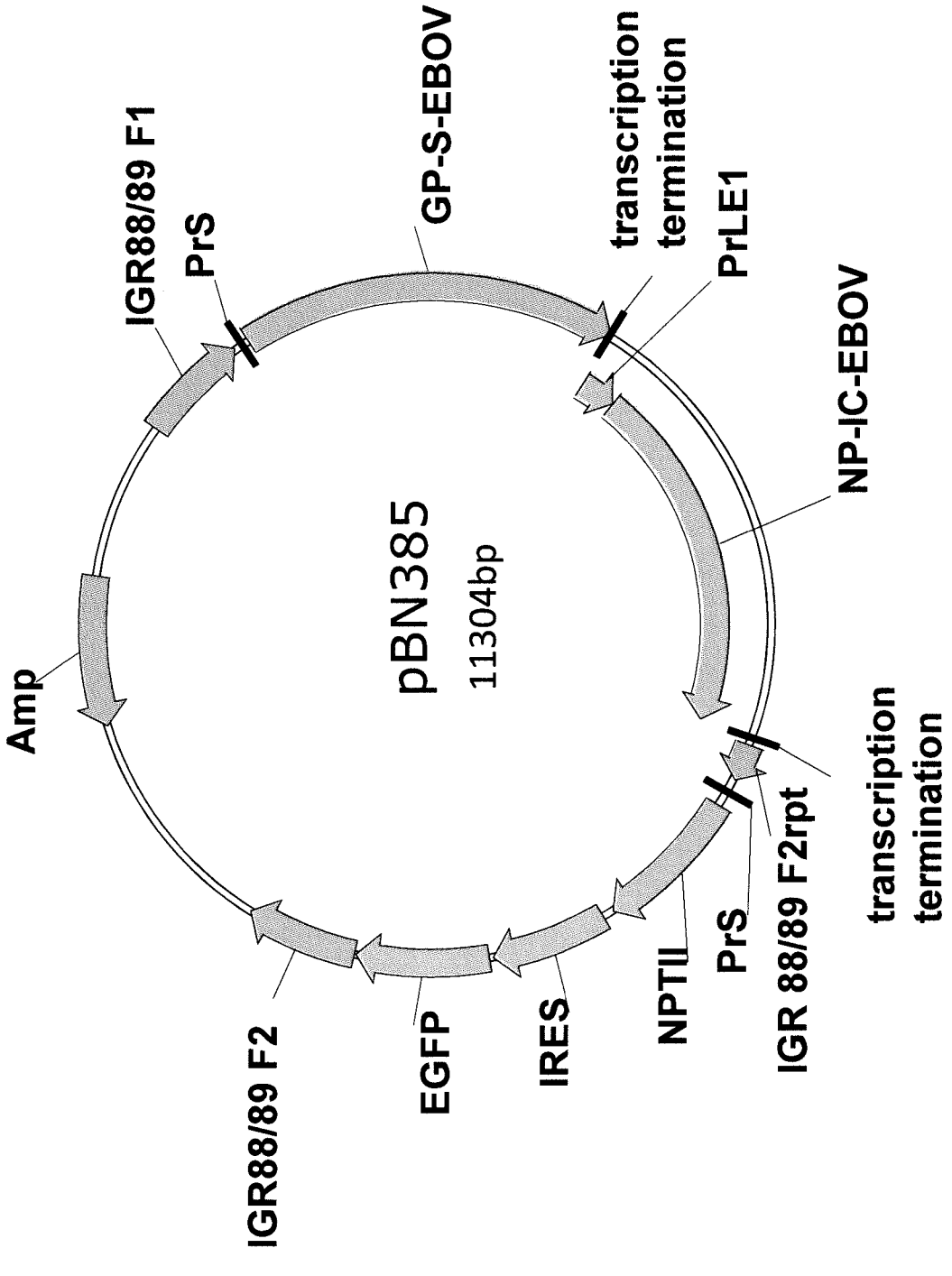

F1 IGR 148-149
Pr7.5
GP-Z-EBOV
transcription stop
PrS
pBN384
9733bp
Amp
GP-MARV-Mu
transcription stop
loxP
PrS
GPT
RFP
loxP
F2 IGR 148-149

Figure 5B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11304bp
IGR88/89 F1
PrS
GP-S-EBOV
transcription termination
PrLE1
NP-IC-EBOV
transcription termination
IGR 88/89 F2rpt
PrS
NPTII
IRES
EGFP
IGR88/89 F2
Amp

Figure 5C

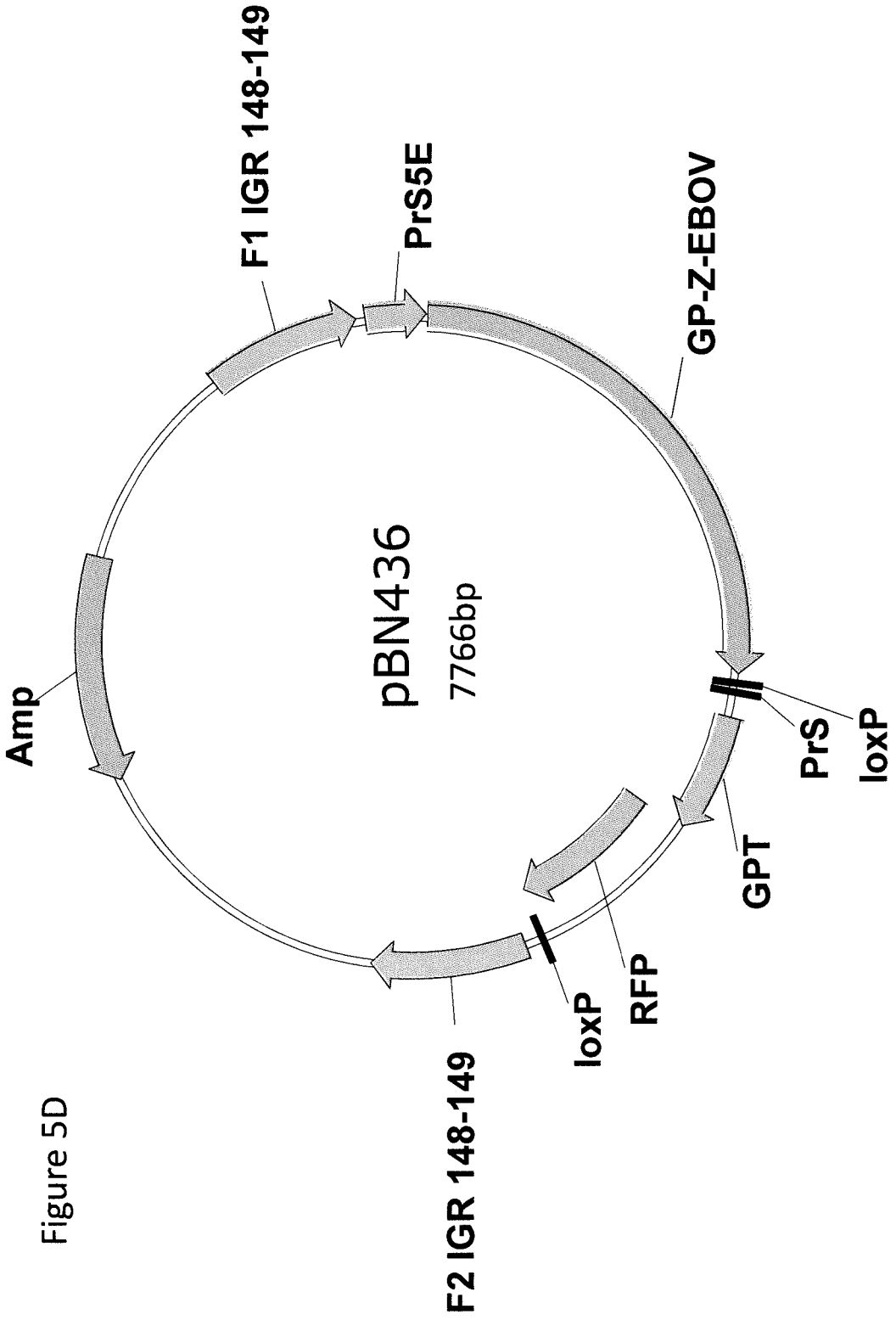

F1 IGR 148-149
PrS5E
GP-Z-EBOV
pBN436
7766bp
Amp
PrS
loxP
GPT
RFP
loxP
F2 IGR 148-149

Figure 5D

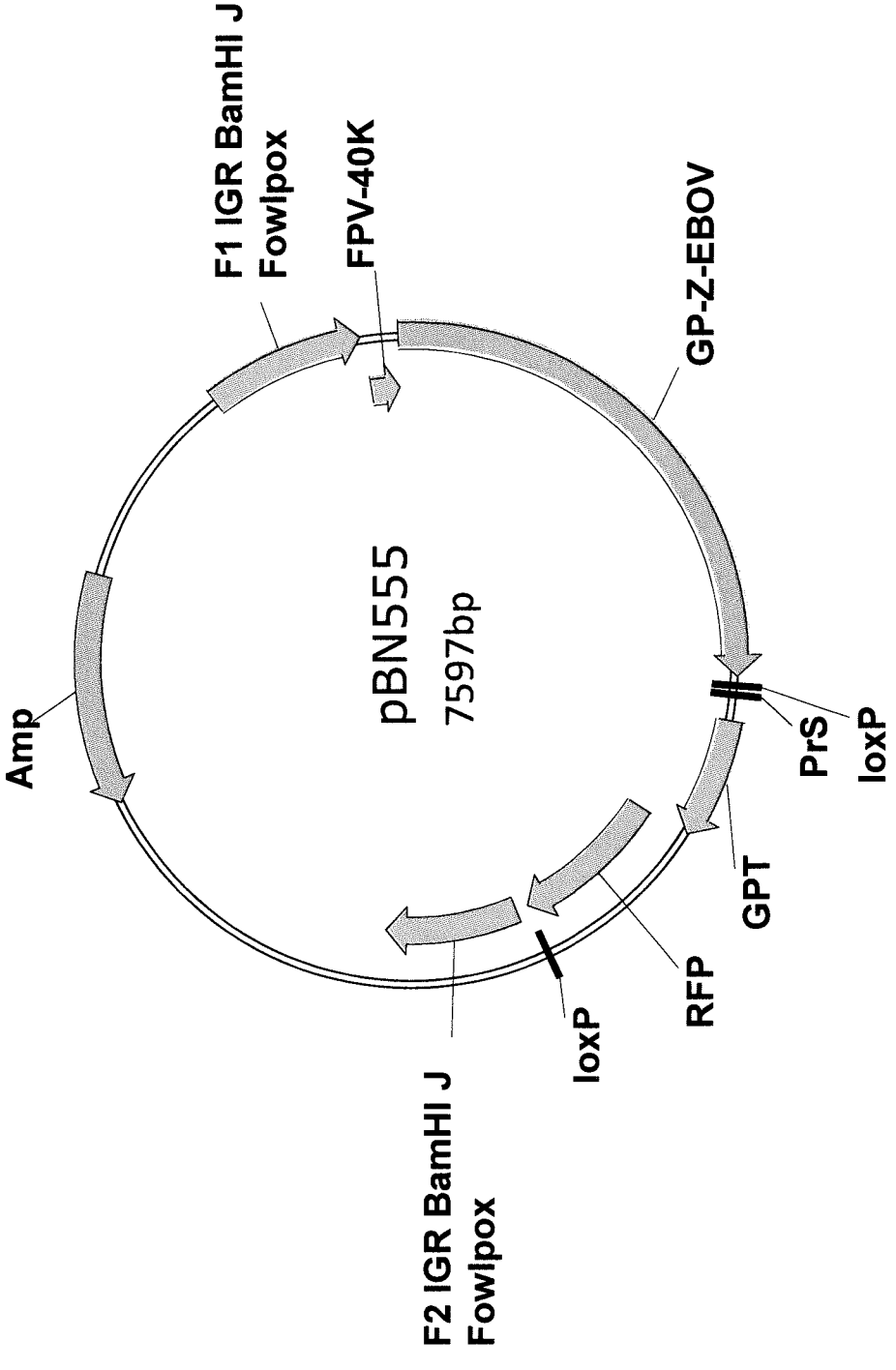
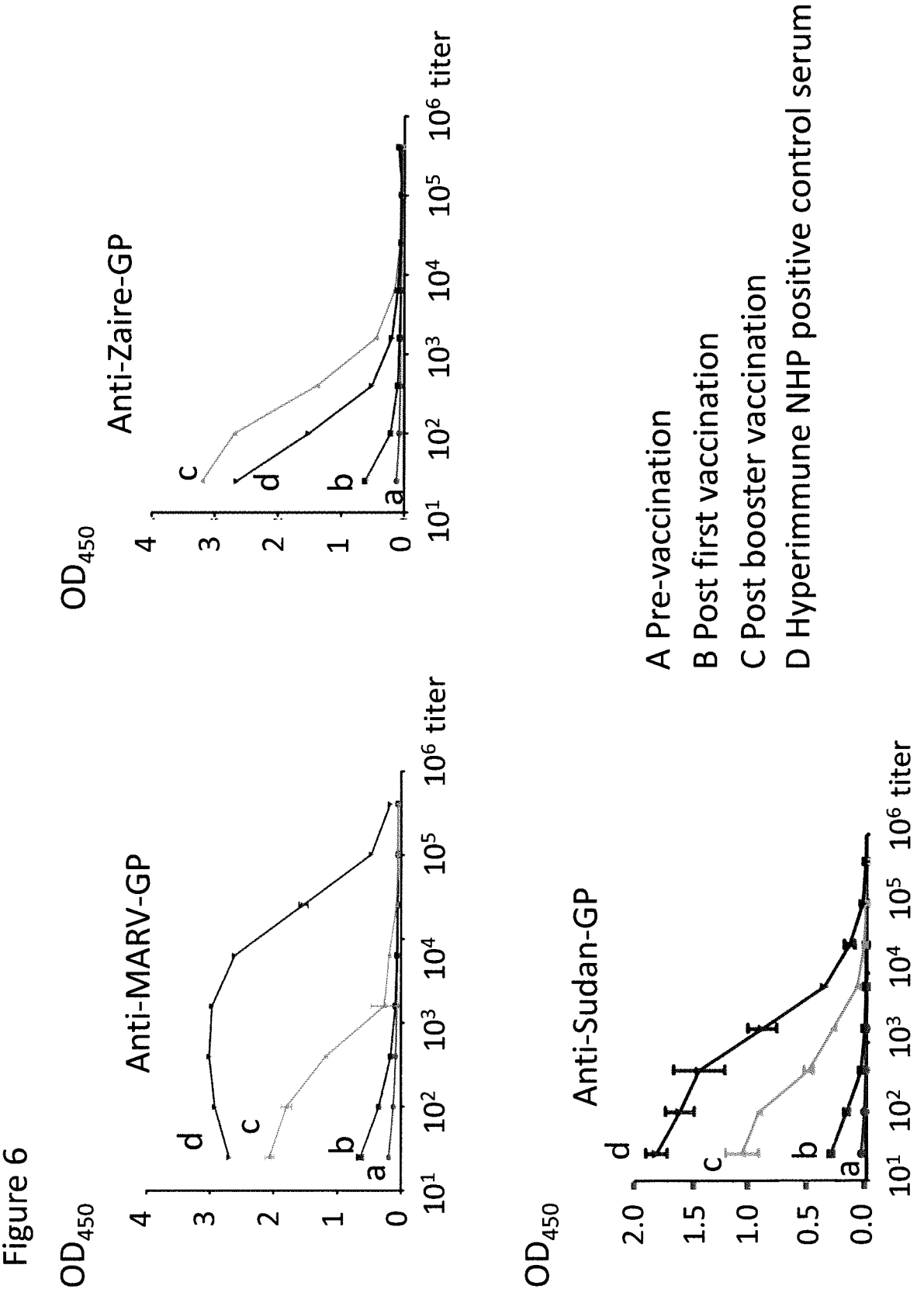

F1 IGR BamHI J
Fowlpox
FPV-40K
GP-Z-EBOV
pBN555
7597bp
Amp
PrS
loxP
GPT
RFP
loxP
F2 IGR BamHI J
Fowlpox

Figure 5E

Figure 6


Anti-Zaire-GP
Anti-MARV-GP
Anti-Sudan-GP
OD450
titer
A Pre-vaccination
B Post first vaccination
C Post booster vaccination
D Hyperimmune NHP positive control serum

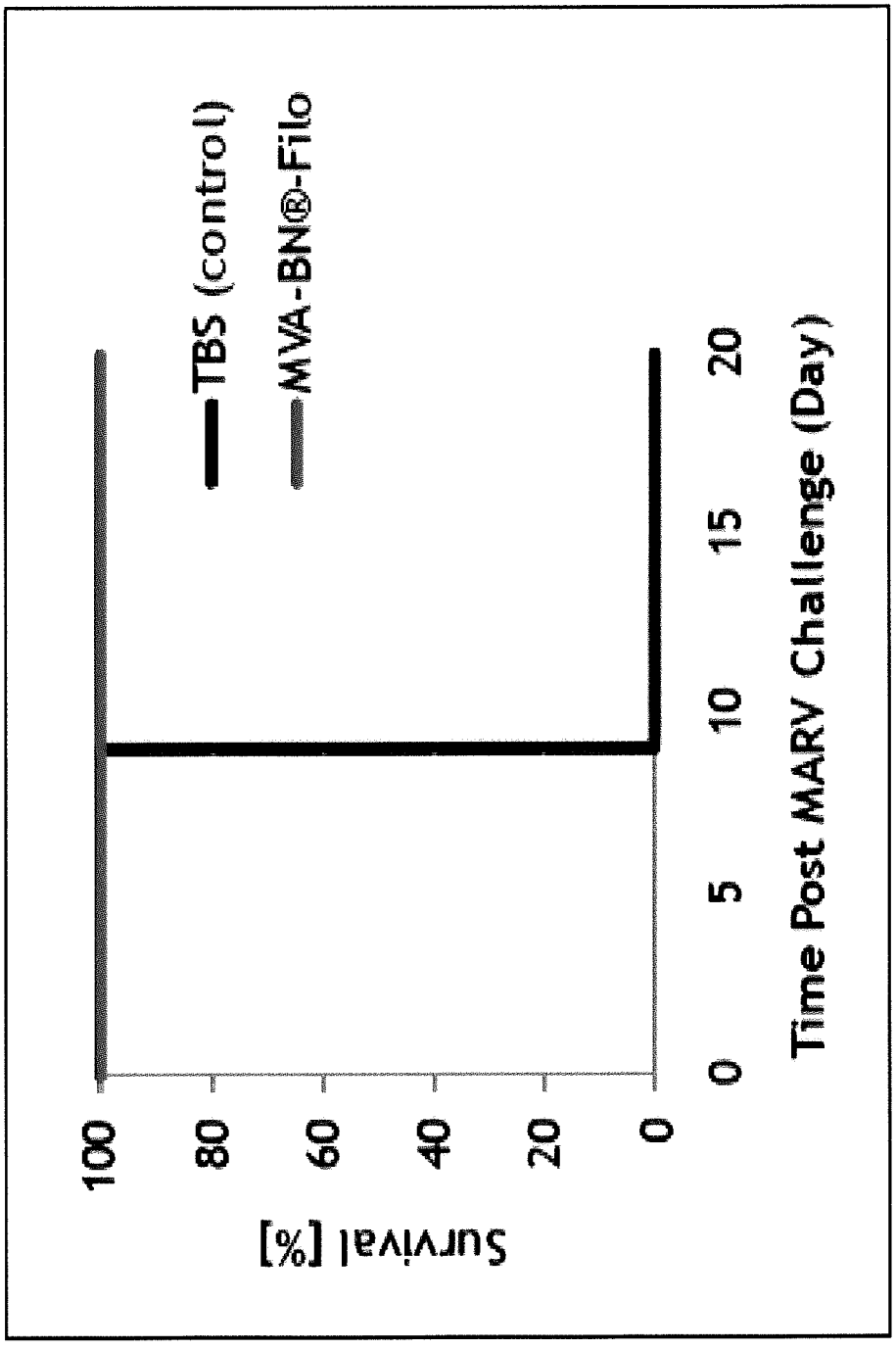

TBS (control)
MVA-BN®-Filo
Survival [%]
100
80
60
40
20
0
0
5
10
15
20
Time Post MARV Challenge (Day)

Figure 7A

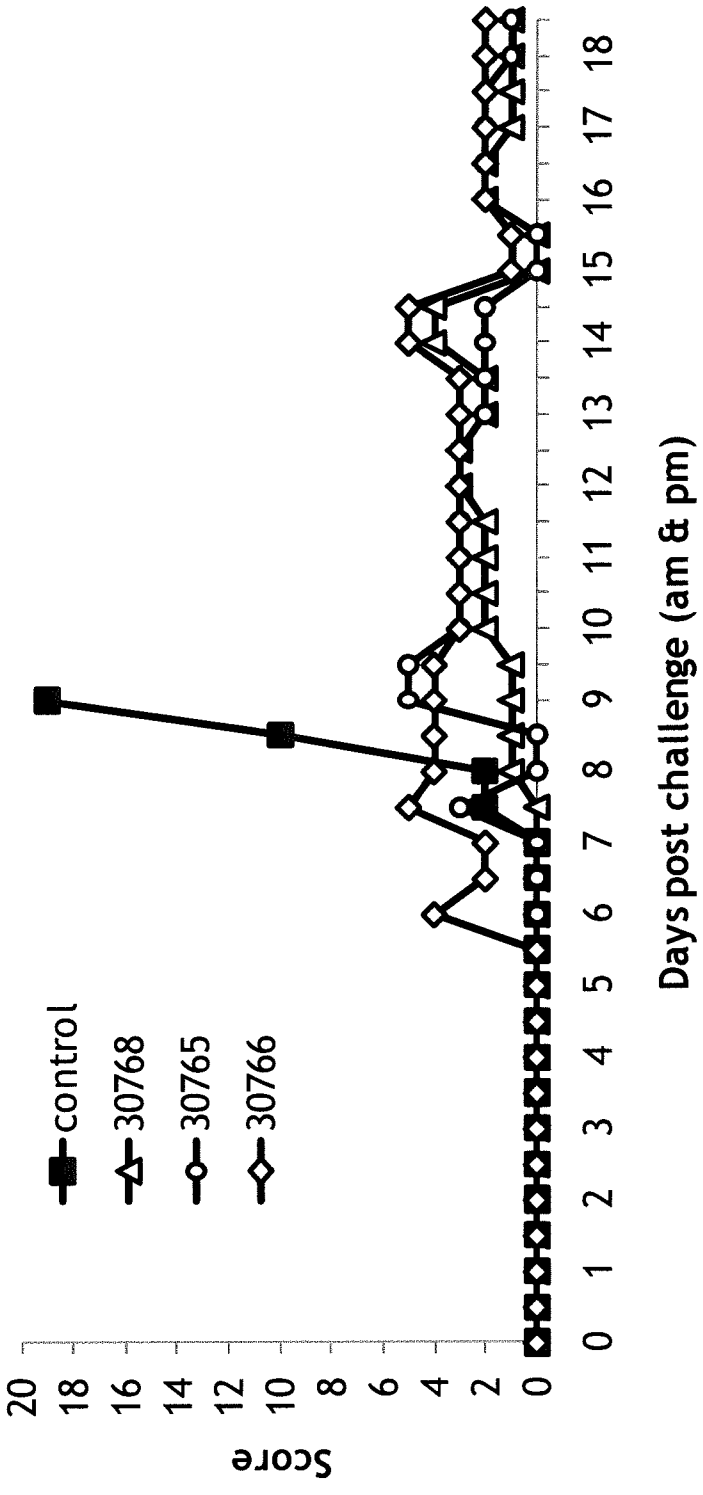

Clinical scores post MARV challenge
control
30768
30765
30766
Score
0
2
4
6
8
10
12
14
16
18
20
Days post challenge (am & pm)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 7B

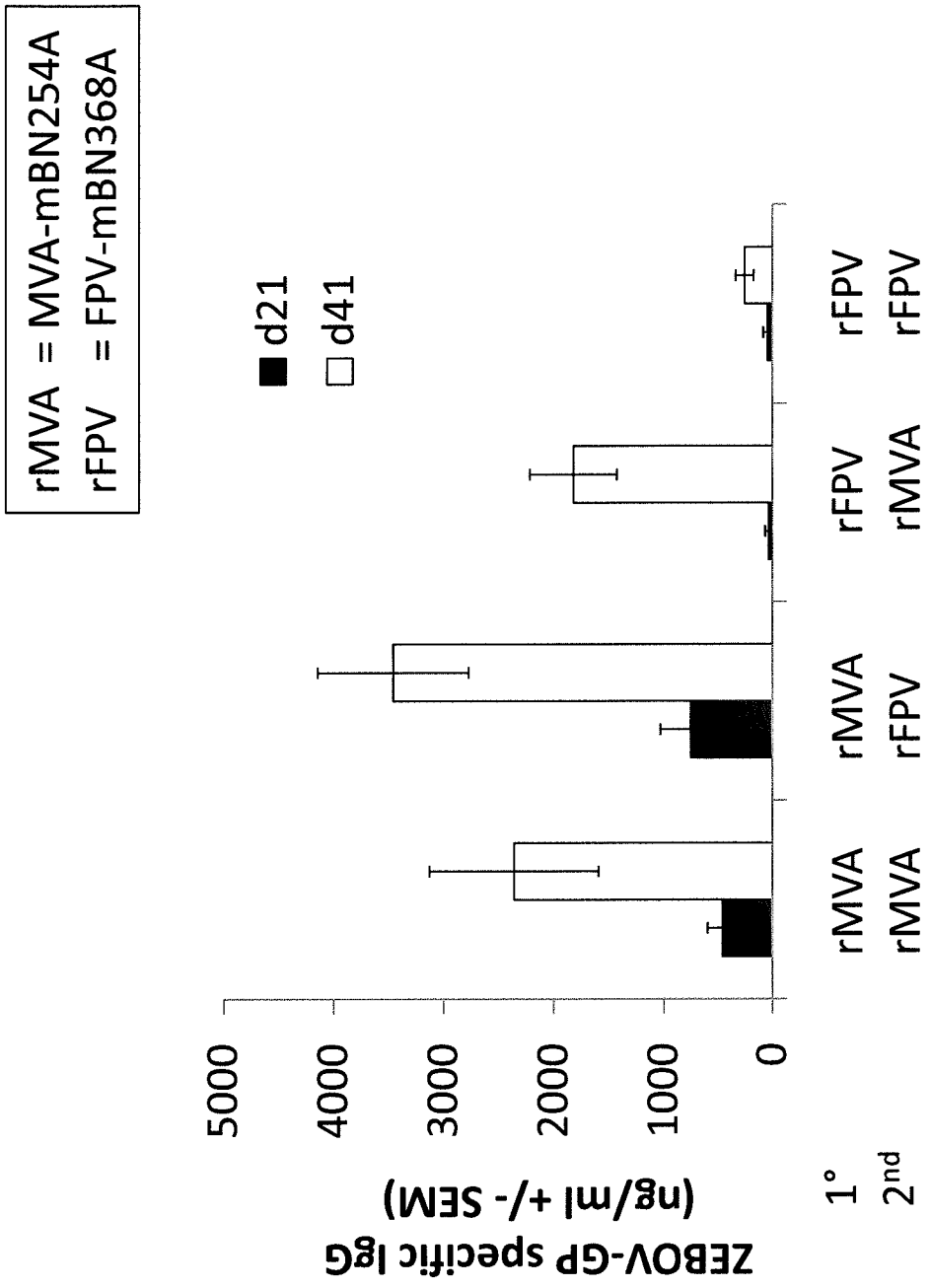
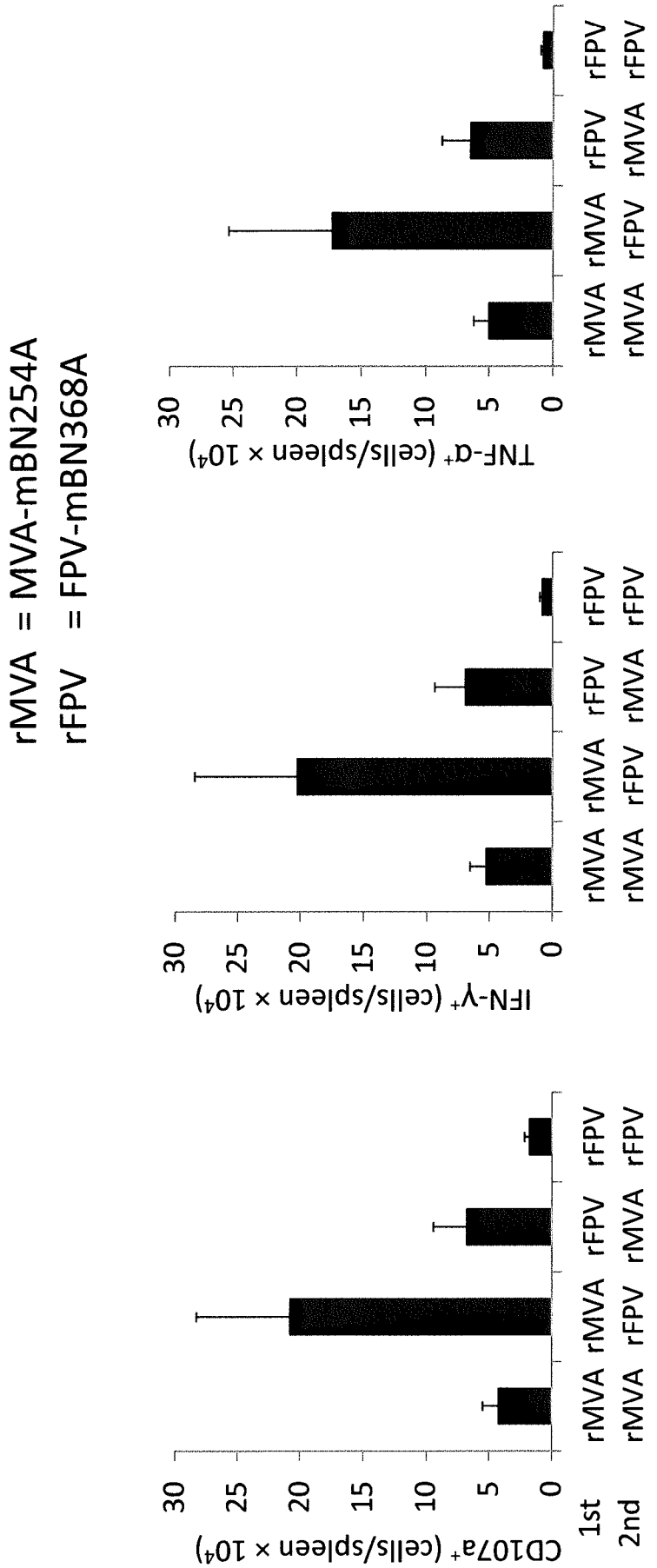

rMVA = MVA-mBN254A
rFPV = FPV-mBN368A
d21
d41
ZEBOV-GP specific IgG
(ng/ml +/- SEM)
5000
4000
3000
2000
1000
0
1°
2nd
rMVA
rMVA
rMVA
rFPV
rFPV
rMVA
rFPV
rFPV

Figure 8A

Figure 8B


rMVA = MVA-mBN254A
rFPV = FPV-mBN368A
CD107a+ (cells/spleen x 10⁴)
IFN-γ+ (cells/spleen x 10⁴)
TNF-α+ (cells/spleen x 10⁴)
0
5
10
15
20
25
30
1st rMVA rMVA rFPV rFPV
2nd rMVA rFPV rMVA rFPV

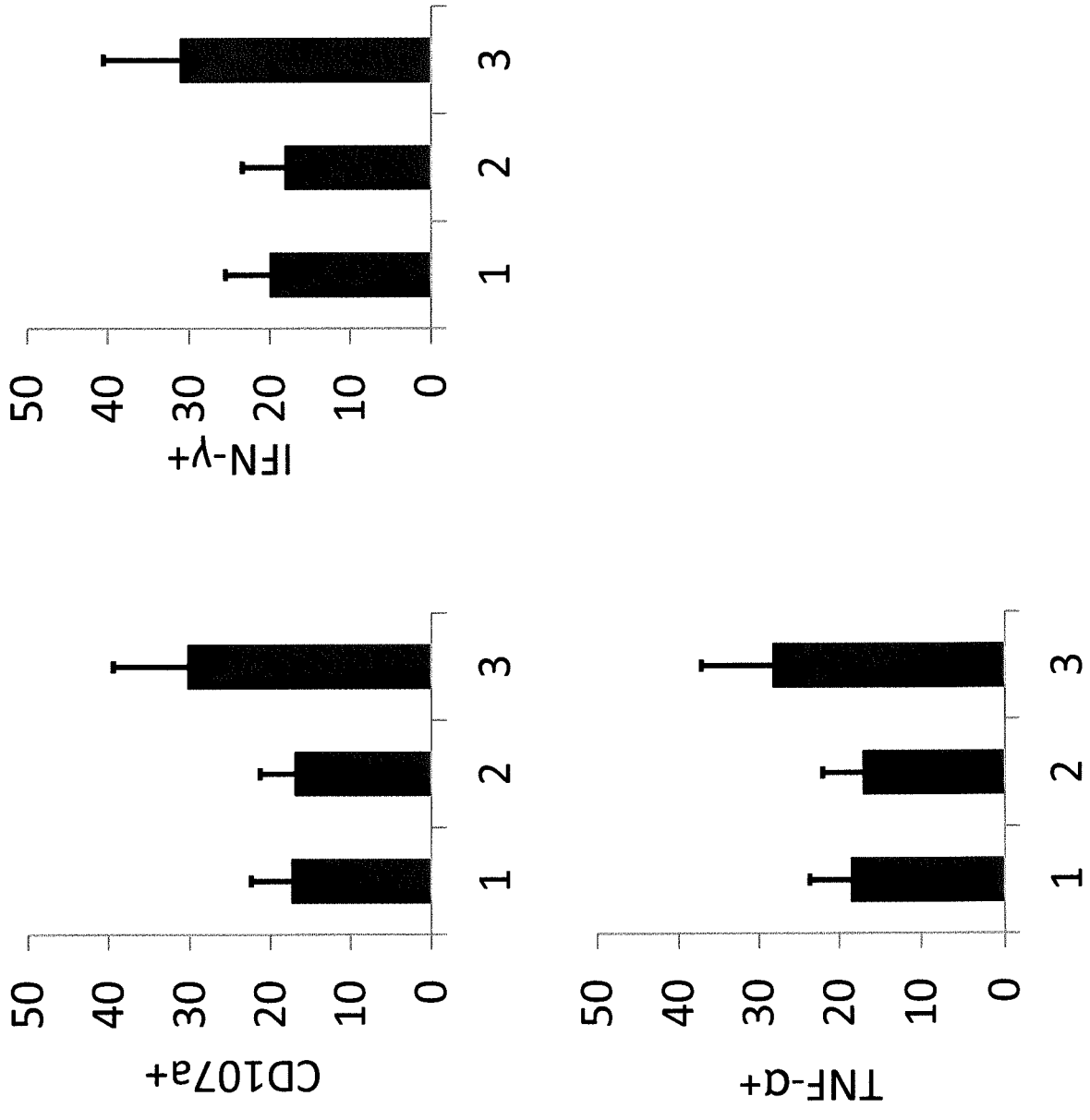


Figure 9

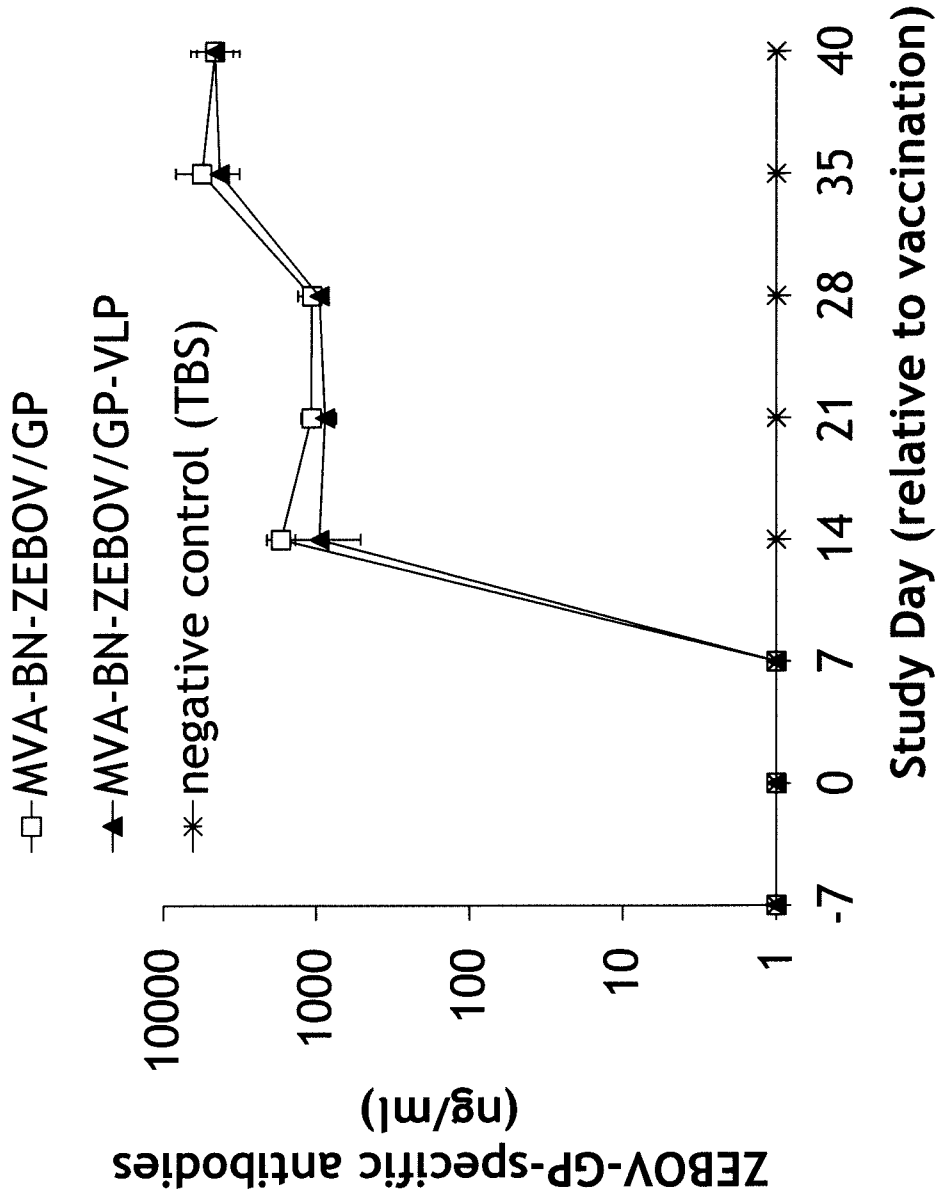

MVA-BN-ZEBOV/GP
MVA-BN-ZEBOV/GP-VLP
negative control (TBS)
ZEBOV-GP-specific antibodies (ng/ml)
10000
1000
100
10
1
-7
0
7
14
21
28
35
40
Study Day (relative to vaccination)

Figure 10

Figure 11

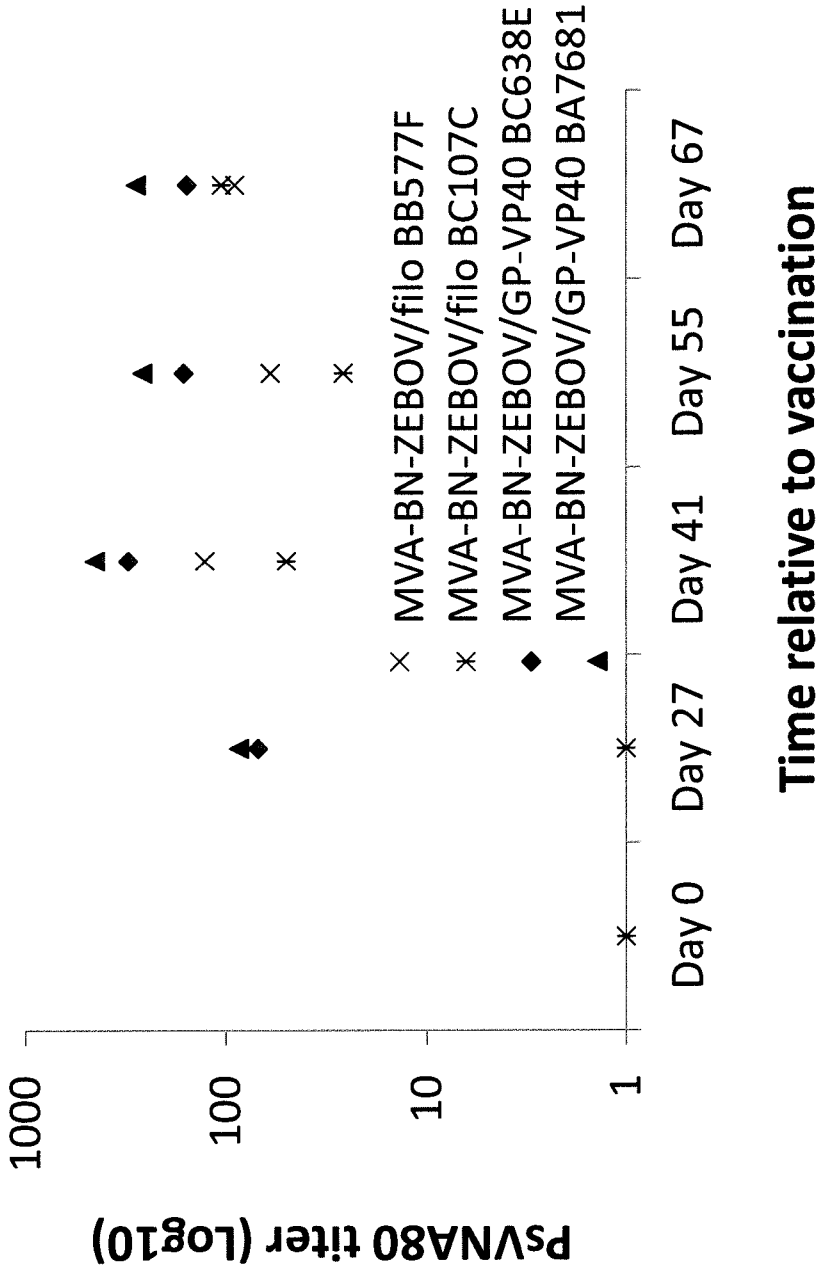

PsVNA80 titer (Log10)
1000
100
10
1
Day 0
Day 27
Day 41
Day 55
Day 67
Time relative to vaccination
MVA-BN-ZEBOV/filo BB577F
MVA-BN-ZEBOV/filo BC107C
MVA-BN-ZEBOV/GP-VP40 BC638E
MVA-BN-ZEBOV/GP-VP40 BA7681

Figure 12

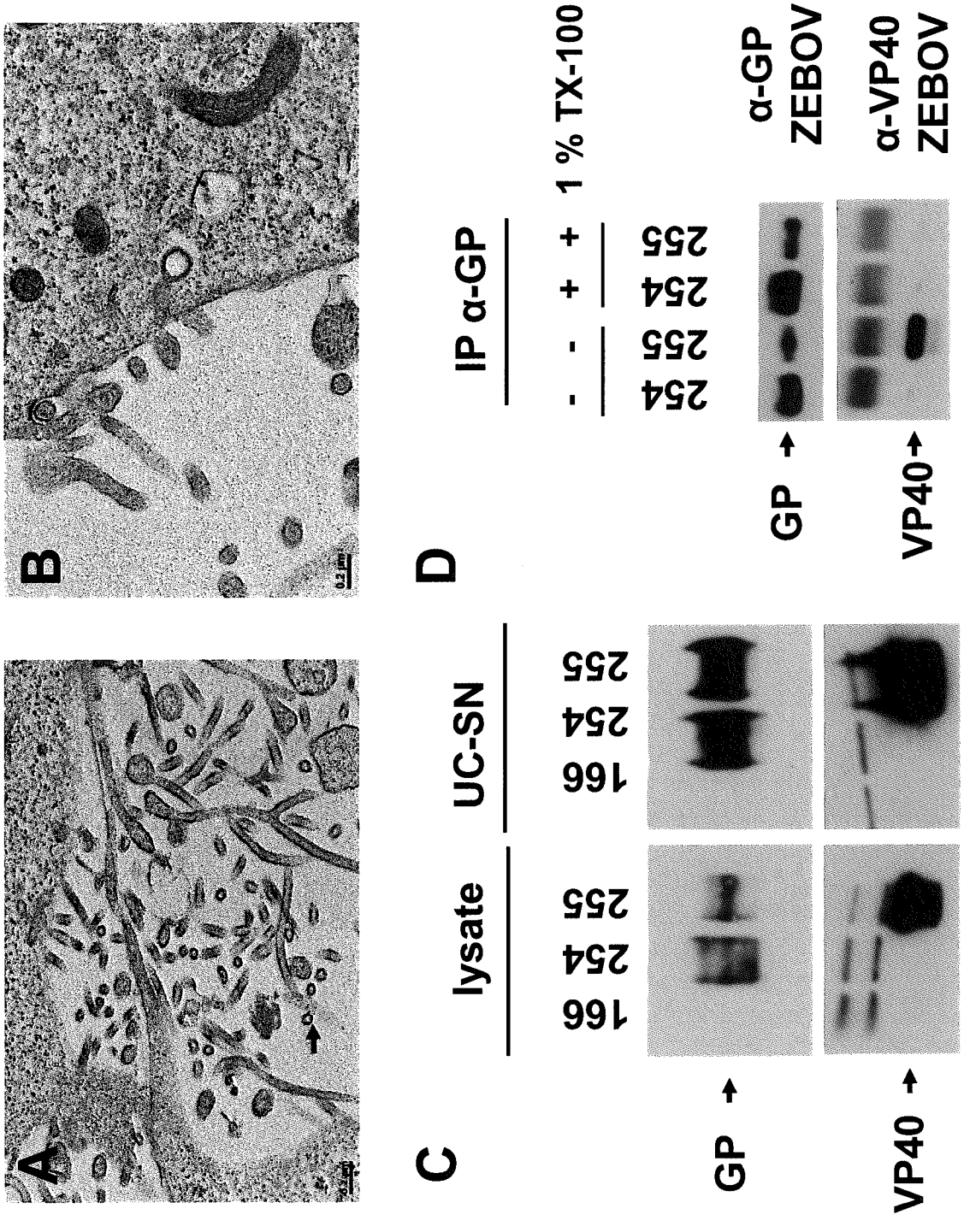
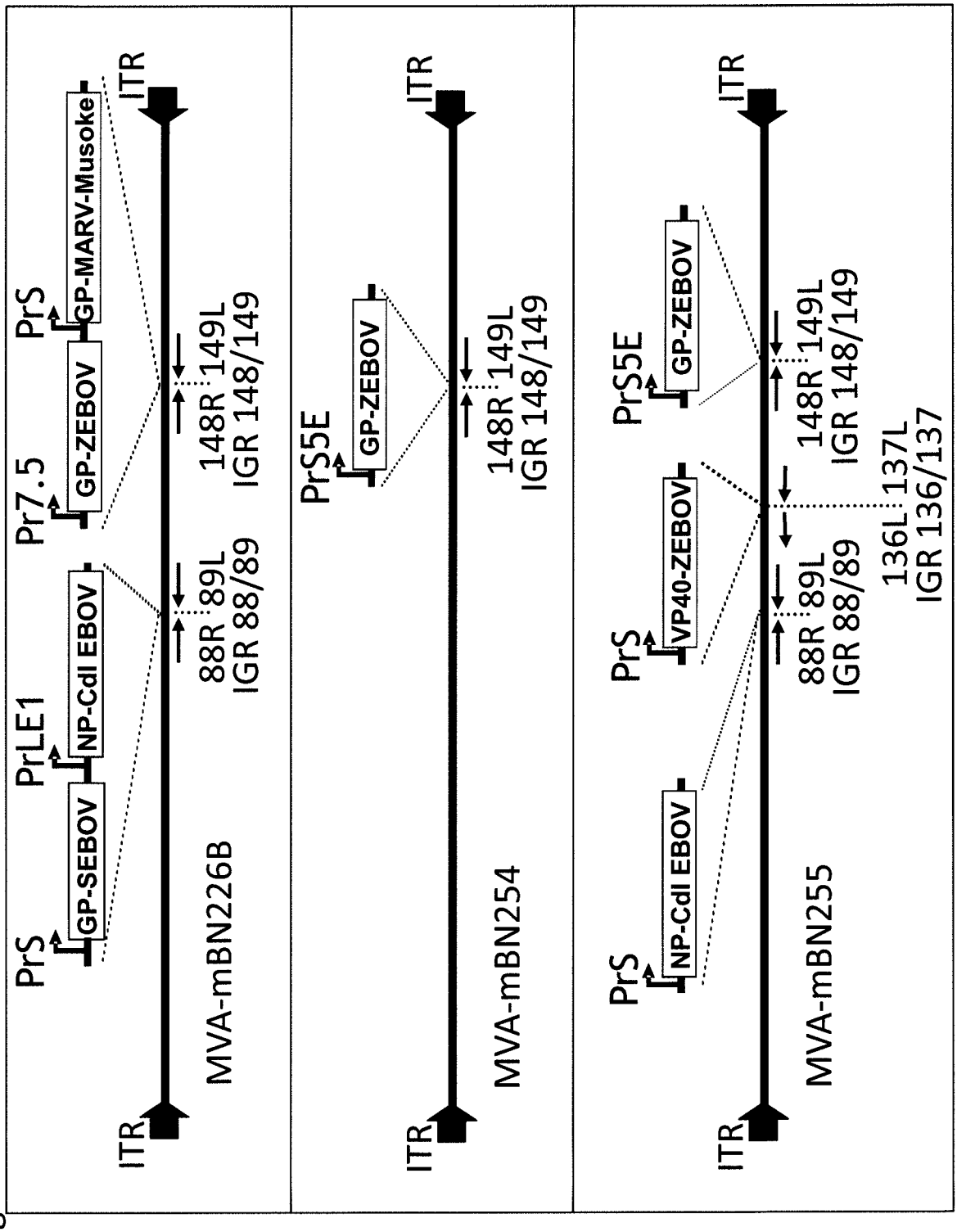

A
B
C
lysate
UC-SN
166
254
255
GP
VP40
D
IP α-GP
254
255
254
255
- - + +
1 % TX-100
α-GP ZEBOV
α-VP40 ZEBOV
GP
VP40

Figure 13


PrS
GP-SEBOV
PrLE1
NP-Cdl EBOV
Pr7.5
GP-ZEBOV
PrS
GP-MARV-Musoke
ITR
88R 89L
IGR 88/89
148R 149L
IGR 148/149
MVA-mBN226B
ITR
PrS5E
GP-ZEBOV
148R 149L
IGR 148/149
MVA-mBN254
ITR
PrS
NP-Cdl EBOV
PrS
VP40-ZEBOV
PrS5E
GP-ZEBOV
ITR
88R 89L
IGR 88/89
136L 137L
IGR 136/137
148R 149L
IGR 148/149
MVA-mBN255
ITR

rMVA = MVA-mBN254A
rFPV = FPV-mBN368A