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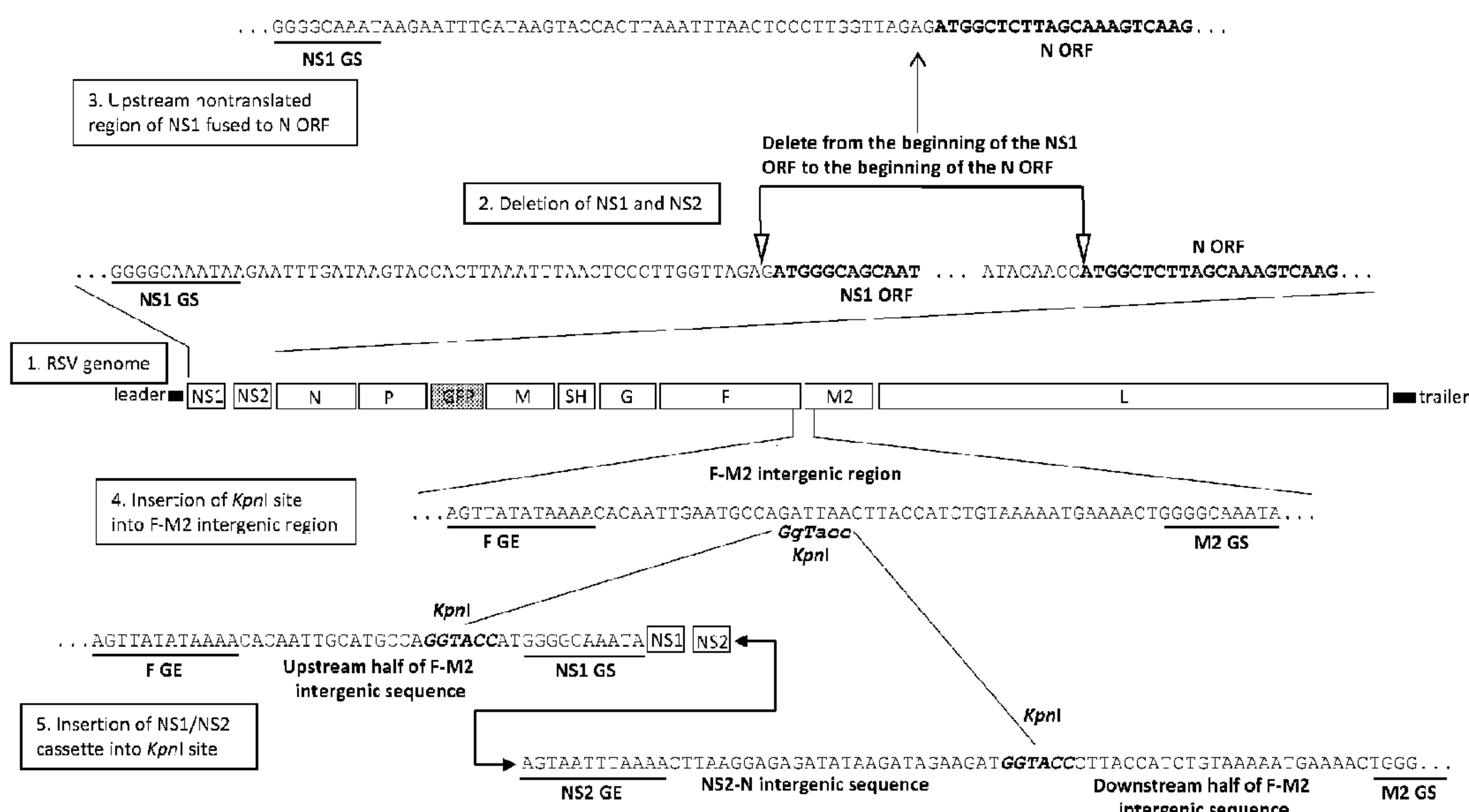
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(54) Titre : SOUCHES DE VIRUS RESPIRATOIRES SYNCYTIAUX RECOMBINES COMPRENANT DES DECALAGES
DES GENES NS1 ET NS2
(54) Title: RECOMBINANT RESPIRATORY SYNCYTIAL VIRUS STRAINS COMPRISING NS1 AND NS2 GENE SHIFTS

FIG. 1



Generation of the RSV 6120/NS12FM2/GFP virus, in which the NS1 and NS2 genes are shifted from their native positions 1 and 2 in the genome to positions 7 and 8

(57) **Abrégé/Abstract:**

Reported herein are novel recombinant respiratory syncytial viruses (RSV) having an attenuated phenotype in which the native positions of the NS1 and/or NS2 genes in the RSV genome are shifted to a higher position, that is at positions that are more distal to the promoter. The changes in the gene positions may be present in combination with mutations at other loci to achieve desired levels of attenuation and immunogenicity. The recombinant RSV strains described here are suitable for use as live- attenuated RSV vaccines. Also provided are polynucleotide sequences capable of encoding the described viruses, as well as methods for producing and using the viruses.

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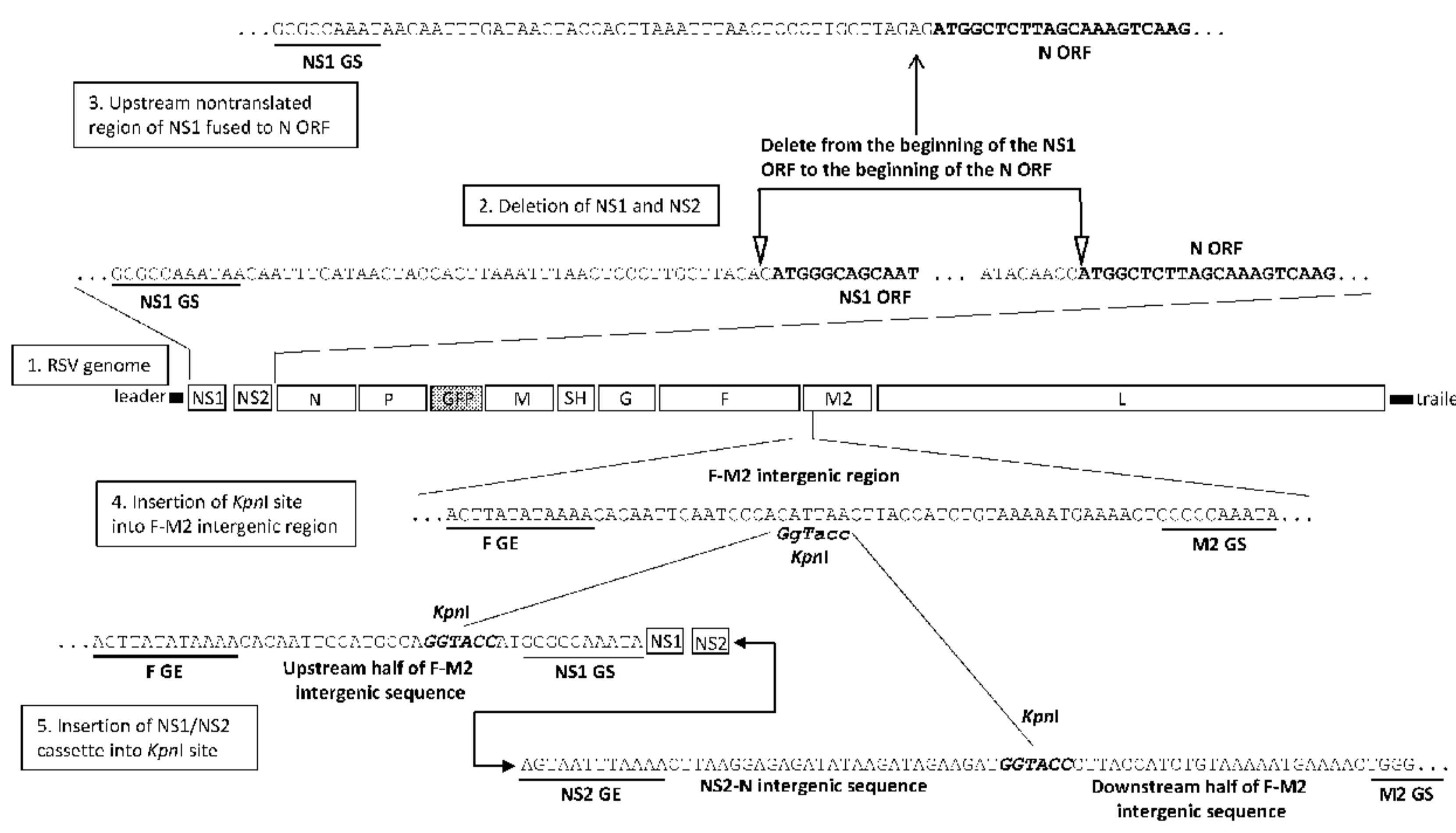
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(54) Title: RECOMBINANT RESPIRATORY SYNCYTIAL VIRUS STRAINS COMPRISING NS1 AND NS2 GENE SHIFTS

FIG. 1



Generation of the RSV 6120/NS12FM2/GFP virus, in which the NS1 and NS2 genes are shifted from their native positions 1 and 2 in the genome to positions 7 and 8

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(57) **Abstract:** Reported herein are novel recombinant respiratory syncytial viruses (RSV) having an attenuated phenotype in which the native positions of the NS1 and/or NS2 genes in the RSV genome are shifted to a higher position, that is at positions that are more distal to the promoter. The changes in the gene positions may be present in combination with mutations at other loci to achieve desired levels of attenuation and immunogenicity. The recombinant RSV strains described here are suitable for use as live-attenuated RSV vaccines. Also provided are polynucleotide sequences capable of encoding the described viruses, as well as methods for producing and using the viruses.

**RECOMBINANT RESPIRATORY SYNCYTIAL VIRUS STRAINS
COMPRISING NS1 AND NS2 GENE SHIFTS**

RELATED APPLICATION

5 This application claims priority to U.S. Provisional Application No. 62/266,206, filed December 11, 2015, which is incorporated by reference in its entirety.

FIELD

10 The subject matter disclosed herein relates to respiratory syncytial virus (RSV) and attenuated, mutant strains thereof suitable for use as vaccines.

BACKGROUND

15 Human respiratory syncytial virus (RSV) infects nearly everyone worldwide early in life and is responsible for considerable mortality and morbidity. In the United States alone, RSV is responsible for 75,000-125,000 hospitalizations yearly, and conservative estimates indicate that RSV is responsible worldwide for 64 million pediatric infections and 160,000 or more pediatric deaths each year. Another notable feature of RSV is that severe infection in infancy frequently is followed by lingering airway dysfunction, including a predisposition to airway reactivity, that in some individuals lasts for years and can extend into adolescence and beyond. RSV infection exacerbates asthma and may be involved in 20 initiating asthma.

RSV is a member of the Paramyxoviridae family and, as such, is an enveloped virus that replicates in the cytoplasm and matures by budding at the host cell plasma membrane. The genome of RSV is a single, negative-sense strand of RNA of 15.2 kilobases that is transcribed by the viral polymerase into 10 mRNAs by a sequential stop-start mechanism that initiates at a single viral promoter at the 3' end of the genome. Each mRNA encodes a single major protein, with the exception of the M2 mRNA that has two overlapping open reading frames (ORFs) encoding two separate proteins M2-1 and M2-2. The 11 RSV proteins are: the RNA-binding nucleoprotein (N), the phosphoprotein (P), the large polymerase protein (L), the attachment glycoprotein (G), the fusion protein (F), the small hydrophobic (SH) surface glycoprotein, the internal matrix protein (M), the two nonstructural proteins NS1 and NS2, and the M2-1 and M2-2 proteins. The RSV gene order is: 3'-NS1-NS2-N-P-M-SH-G-F-M2-L. Each gene is flanked by short conserved transcription signals called the gene-start (GS) signal, present on the upstream end of each gene and involved in initiating transcription of the respective gene, and the gene-end (GE) signal, present at the downstream end of each gene and involved in directing synthesis of a polyA tail followed by release of the mRNA. Transcription initiates at a single promoter at the 3' end and 25 proceeds sequentially.

30 The development of RSV vaccines has been in progress since the 1960's but has been complicated by a number of factors. For example, immunization of RSV-naïve infants with inactivated RSV has been shown to prime for enhanced disease upon subsequent natural RSV infection, and studies 35

in experimental animals indicate that disease enhancement also is associated with purified RSV subunit vaccines.

Another obstacle to immune protection is that RSV replicates and causes disease in the superficial cells of the respiratory airway lumen, where immune protection has reduced effectiveness.

5 Thus, immune control of RSV infection is inefficient and often incomplete, and it is important for an RSV vaccine to be as immunogenic as possible. Another obstacle to RSV vaccines is that the magnitude of the protective immune response is roughly proportional to the extent of virus replication (and antigen production). Thus, the attenuation of RSV necessary to make a live vaccine typically is accompanied by a reduction in replication and antigen synthesis, and a concomitant reduction in immunogenicity, and 10 therefore it is beneficial to identify a level of replication that is well tolerated yet satisfactorily immunogenic.

Another obstacle is that RSV grows only to moderate titers in cell culture and is often present in long filaments that are difficult to purify. RSV can readily lose infectivity during handling. Another obstacle is the difficulty in identifying and developing attenuating mutations. Appropriate mutations 15 must be attenuating *in vivo*, but should be minimally restrictive to replication *in vitro*, since this is preferred for efficient vaccine manufacture. Another obstacle is genetic instability that is characteristic of RNA viruses, whereby attenuating mutations can revert to the wild-type (wt) assignment or to an alternative assignment that confers a non-attenuated phenotype. Instability and de-attenuation are particularly problematic for point mutations.

20 Taking these factors together, there is a need for live attenuated RSV strains that replicate efficiently *in vitro*, are maximally immunogenic, are satisfactorily attenuated, and are refractory to de-attenuation.

SUMMARY

25 Reported herein are novel recombinant RSV having an attenuated phenotype in which the position of the NS1 and/or NS2 gene in the RSV genome or antigenome is shifted to a position that is more distal to the promoter. The changes in the gene positions may be present in combination with mutations at other loci to achieve desired levels of attenuation and immunogenicity. The recombinant RSV strains described here are suitable for use as live-attenuated RSV vaccines.

30 In some embodiments, a recombinant RSV is provided that is attenuated by one or more modifications to the genome of the RSV. In some embodiments, the one or more modifications comprise a shift of the NS1 gene and the NS2 gene from gene positions 1 and 2 to gene positions 7 and 8 of the RSV genome, respectively. In some embodiments, the one or more modifications comprise a shift of the NS1 and NS2 genes from gene positions 1 and 2 (of a native RSV genome) to gene positions 9 and 10 of the genome of the recombinant RSV, respectively. In some embodiments, the one or more modifications comprise a shift of the NS1 gene to a gene position higher than position 1 (for example, to gene position 7 or 9). In some embodiments, the one or more modifications comprise a shift of the NS2 gene to a gene position higher than position 2. In some embodiments, the one or more modifications comprise a shift of

the NS1 gene to a gene position higher than position 1, and the NS2 gene to a gene position higher than position 2.

In addition to the modification that shifts the gene position of the NS1 gene and/or the NS2 gene, the genome of the recombinant RSV can comprise further modifications to increase or decrease viral 5 attenuation, or other properties of the recombinant virus, such as deletion of all or part of the NS1, the NS2 gene, and/or the M2-2 gene.

In some embodiments, the RSV genome comprises the one or more modifications as discussed above, and comprises a nucleotide sequence corresponding to a positive-sense sequence at least 90%, at least 95%, and/or at least 99% identical to SEQ ID NO: 2 (6120/NS12FM2), SEQ ID NO: 4 10 (6120/NS12Ltr), SEQ ID NO: 6 (6120/NS12FM2/ΔNS2), or SEQ ID NO: 8 (6120/NS12Ltr/ΔNS2). For example, the RSV genome can comprise or consist of a nucleotide sequence corresponding to a positive-sense sequence denoted by SEQ ID NO: 2 (6120/NS12FM2), SEQ ID NO: 4 (6120/NS12Ltr), SEQ ID NO: 6 (6120/NS12FM2/ΔNS2), or SEQ ID NO: 8 (6120/NS12Ltr/ΔNS2).

In some embodiments, the RSV genome further comprises a reporter gene, such as gene 15 encoding Green Fluorescent Protein (GFP). In some embodiments, the RSV genome comprises the one or more modifications as discussed above and the reporter gene, and comprises a nucleotide sequence corresponding to a positive-sense sequence to SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, or SEQ ID NO: 7, or a nucleotide sequence corresponding to a positive-sense sequence at least 90%, at least 95%, and/or at least 99% identical to SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, or SEQ ID NO: 7.

20 In some embodiments, the recombinant RSV exhibits one or more of (a) reduced expression of the NS1 gene and/or NS2 gene compared to an RSV having the NS1 gene in gene position 1 and the NS2 gene in gene position 2, (b) reduced transcription of the NS1 gene and/or NS2 gene compared to an RSV having the NS1 gene in gene position 1 and the NS2 gene in gene position 2; and/or (c) reduced inhibition of host interferon response compared to an RSV having the NS1 gene in gene position 1 and 25 the NS2 gene in gene position 2. In some embodiments, the recombinant RSV is increasingly susceptible to restriction in cultured cells that can produce interferons in response to viral infection. In some embodiments, the recombinant RSV retains replication efficiency in cultured cells that cannot produce interferons in response to viral infection.

The embodiments of recombinant RSV disclosed herein can be subtype A RSV or a subtype B 30 RSV. The embodiments of recombinant RSV disclosed herein are infectious, attenuated, and self-replicating.

Also provided herein are methods and compositions related to the expression of the disclosed viruses. For example, isolated polynucleotide molecules that include a nucleic acid sequence encoding the genome or antigenome of the described viruses are disclosed.

35 Pharmaceutical compositions including the recombinant RSV are also provided. The compositions can further include an adjuvant. Methods of eliciting an immune response in a subject by administering an immunogenically effective amount of a disclosed recombinant RSV to the subject are also disclosed. In some embodiments, the subject is a human subject, for example, a human subject

between 1 and 6 months of age, or between 1 and 12 months of age, or between 1 and 18 months of age, or older.

The foregoing and other features and advantages of this disclosure will become more apparent from the following detailed description of several embodiments which proceeds with reference to the 5 accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows schematic diagrams illustrating the creation of the recombinant RSV 6120/NS12FM2/GFP in which NS1 and NS2 genes were shifted to gene positions 7 and 8. Note that the 10 GFP gene is not included in the gene position numbering. The sequences shown (from top to bottom) are SEQ ID NO: 9, SEQ ID NOs: 10 and 11, SEQ ID NO: 12, and SEQ ID NOs: 13 and 14, respectively.

FIG. 2 shows schematic diagrams illustrating the creation of the recombinant RSV 6120/NS12Ltr/GFP virus in which the NS1 and NS2 genes were shifted to positions 9 and 10. The sequences shown (from top to bottom) are SEQ ID NO: 9, SEQ ID NOs: 10 and 11, SEQ ID NO: 15, and SEQ ID 15 NOs: 16 and 17, respectively.

FIG. 3 shows replication of RSV 6120/NS12FM2/GFP, RSV Δ NS1/ Δ NS2/GFP and wt RSV/GFP in African green monkey Vero cells.

FIG. 4 shows replication of RSV 6120/NS12FM2/GFP, RSV Δ NS1/ Δ NS2/GFP and wt RSV/GFP in human airway A549 cells.

FIG. 5 shows replication of RSV 6120/NS12Ltr/GFP, RSV Δ NS1/ Δ NS2/GFP and wt RSV/GFP in African green monkey Vero cells.

FIG. 6 shows replication of RSV 6120/NS12Ltr/GFP, RSV Δ NS1/ Δ NS2/GFP and wt RSV/GFP in human airway A549 cells.

FIG. 7 shows schematic diagrams illustrating the deletion of the NS2 gene from RSV 6120/NS12FM2/GFP. The upper sequence line shows SEQ ID NOs: 18 and 19. The lower sequence line shows SEQ ID NOs: 20 and 21.

FIG. 8 shows schematic diagrams illustrating the deletion of the NS2 gene from RSV 6120/NS12Ltr/GFP. The upper sequence line shows SEQ ID NOs: 18 and 22. The lower sequence line shows SEQ ID NOs: 23 and 24.

FIG. 9 shows replication of RSV 6120/NS12FM2/ Δ NS2/GFP, RSV 6120/NS12FM2/GFP, RSV Δ NS1/ Δ NS2/GFP and wt RSV/GFP in African green monkey Vero cells.

FIG. 10 shows replication of RSV 6120/NS12FM2/ Δ NS2/GFP, RSV 6120/NS12FM2/GFP, RSV Δ NS1/ Δ NS2/GFP and wt RSV/GFP in human airway A549 cells.

35

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. The Sequence Listing is submitted as an ASCII text file in the form of the file

named "Sequence.txt" (~164 kb), which was created on December 9, 2016 which is incorporated by reference herein. In the accompanying sequence listing:

SEQ ID NO: 1 is the antigenomic cDNA sequence for recombinant RSV strain 6120/NS12FM2GFP.

5 **SEQ ID NO: 2** is the antigenomic cDNA sequence for recombinant RSV strain 6120/NS12FM2.

SEQ ID NO: 3 is the antigenomic cDNA sequence for recombinant RSV strain 6120/NS12LtrGFP.

SEQ ID NO: 4 is the antigenomic cDNA sequence for recombinant RSV strain 6120/NS12Ltr.

SEQ ID NO: 5 is the antigenomic cDNA sequence for recombinant RSV strain 10 6120/NS12FM2/ΔNS2/GFP.

SEQ ID NO: 6 is the antigenomic cDNA sequence for recombinant RSV strain 6120/NS12FM2/ΔNS2.

SEQ ID NO: 7 is the antigenomic cDNA sequence for recombinant RSV strain 6120/NS12Ltr/ΔNS2/GFP.

15 **SEQ ID NO: 8** is the antigenomic cDNA sequence for recombinant RSV strain 6120/NS12Ltr/ΔNS2.

SEQ ID NOs: 9-24 are fragments of recombinant RSV antigenomic cDNA sequences shown in FIGs. 1-2 and 7-8.

SEQ ID NOs: 25 and 26 are the nucleotide sequences of gene-start transcription signals.

20

DETAILED DESCRIPTION

Disclosed herein are mutations that are useful in producing recombinant strains of human RSV exhibiting a range of attenuation phenotypes. The mutations of the present invention are based on shifting of the NS1 and/or NS2 genes from their native positions in the RSV genome or antigenome to a 25 higher position i.e. a position that is more distal to the promoter. Also disclosed herein are recombinant RSV strains that include such mutations and are suitable for use as attenuated, live vaccines. Further disclosed herein are methods and compositions related to the expression of the disclosed viruses. For example, isolated polynucleotide molecules that include a nucleic acid sequence encoding the genome or antigenome of the described viruses are disclosed.

30 The recombinant RSV strains of the present invention comprise a wt RSV genome or antigenome containing modifications or mutations as described in detail below. The wt RSV genome or antigenome encodes the following 11 proteins: the RNA-binding nucleoprotein (N), the phosphoprotein (P), the large polymerase protein (L), the attachment surface glycoprotein (G), the fusion surface glycoprotein (F), the small hydrophobic surface glycoprotein (SH), the internal matrix protein (M), the two nonstructural 35 proteins NS1 and NS2, and the M2-1 and M2-2 proteins. The complete amino acid sequences of these proteins are known in the art. The genome of RSV is a single strand of negative sense RNA of 15.2 kb comprising 10 genes encoding 10 mRNAs. Each mRNA encodes a single protein, except for the M2 mRNA which encodes two separate proteins M2-1 and M2-2. The RSV gene order is: 3'-NS1-NS2-N-P-

M-SH-G-F-M2-L with a single viral promoter located at the 3' end. Thus, in the native RSV genome NS1 is at position 1, NS2 at position 2, N at position 3, P at position 4, M at position 5, SH at position 6, G at position 7, F at position 8, M2 at position 9 and L at position 10. This organization is shown schematically in FIG. 1, top panel.

5 As reported herein, moving NS1 and/or NS2 from their native positions as promoter-proximal genes to a higher gene position, that is a position further distal to the promoter, results in their decreased transcription and expression. For nonsegmented negative strand RNA viruses, the transcription gradient is an important factor in regulating viral gene expression. One recent study showed that expression of a foreign gene was four-fold higher when it was placed between the F and M2 genes in the RSV genome
10 compared to between the L gene and trailer, a difference of two gene positions (Kwilas AR et al 2010 J Virol 84:7770-7781). Another recent study with the related parainfluenza virus type 3 revealed that expression of a foreign gene from gene position 1, 2, or 3 was 30-69-fold, 15-29-fold, and 5-6-fold higher compared to gene position 6 (Liang et al 2014 J Virol 88:4237-4250). This illustrates that moving
15 one or more genes to positions that are progressively more distal to the promoter can provide incremental reductions in gene expression that, when over a range of multiple gene positions, can be substantial.

The NS1 and NS2 proteins antagonize host innate responses including interferon and apoptosis. This antagonistic effect is particularly prominent for NS1. Recombinant RSV in which NS1 and/or NS2 are deleted, in particular NS1 deletion mutant, show reduced virus replication *in vitro* due to increased apoptosis, an effect that also is observed in Vero cells used in the manufacture of live RSV vaccines
20 (Bitko et al, 2007 J Virol 81:1786-1795). For example, efforts to manufacture an RSV Δ NS2 virus as a live vaccine have been unsuccessful due to unsatisfactorily low yields (unpublished results). A Δ NS2/ Δ NS2 virus also appears to be over-attenuated in African green monkeys (Jin et al 2003 Vaccine 21:3647-3652). In contrast, the RSV recombinant viruses of the present invention comprising NS1 and/or NS2 gene shift mutations did not exhibit growth restriction in Vero cells. This indicates that the
25 levels of NS1 and NS2 that are produced by either mutant control apoptosis sufficiently to obtain efficient viral replication, a surprising result that could not have been predicted. However, these viruses were attenuated in interferon competent cells, indicating that the expected decreased expression of NS1 and/or NS2 indeed rendered the virus increasingly susceptible to restriction.

Thus, NS1 and/or NS2 gene-shift provides a novel means to avoid the over-attenuation
30 associated with gene-deletion. The ability to place the genes in incrementally distal locations relative to the promoter provides a means to incrementally change the magnitude of attenuation. Gene-shift can be combined with other previously described attenuating mutations. Additionally, since NS1 (in particular) and NS2 inhibit the host interferon response, reducing their expression may increase viral immunogenicity due to the adjuvant effects of increased interferon expression. For example, in the
35 bovine model, bovine RSV mutants with NS deletions were shown to have increased immunogenicity in the natural host (Valarcher et al 2003 J Virol 77:8426-8439). Increased apoptosis, as would result from decreased expression of the RSV NS1 and/or NS2 proteins, also has the potential to increase immunogenicity (Pulmanausahakul et al 2001 J Virol 75:10800-10807).

Since there are 8 other RSV genes, NS1 and/or NS2 may be moved to a number of different higher gene positions in different combinations to provide different levels of transcription and expression. The NS1 and/or NS2 genes may be moved to an intergenic region between other genes, or into other non-coding regions.

5 In some embodiments, the NS1 and NS2 genes may be moved in tandem to higher gene positions or progressively more distal gene positions to provide a graded set of increasing attenuated phenotypes. Thus, in some embodiments, NS1 and NS2 may be at gene positions 2 and 3, 3 and 4, 4 and 5, 5 and 6, 6 and 7, 7 and 8, 8 and 9, or 9 and 10 respectively. In some embodiments, the NS1 and NS2 genes may be moved in tandem from gene positions 1 and 2, respectively, to gene positions 7 and 8, respectively. In 10 some embodiments, the NS1 and NS2 genes may be moved in tandem from gene positions 1 and 2, respectively, to gene positions 9 and 10, respectively. The gene position numbers of genes prior to shift refer to their positions in the native RSV genome before the shift, and the gene position numbers of the genes post-shift refer to their positions in the modified RSV genome.

15 Alternatively, the NS1 and NS2 may be moved singly or independently of each other. For example, only one of the NS1 or NS2 gene may be moved to a higher gene position. Thus, in some 20 embodiments. NS1 gene may be at gene position 1 and NS2 may be at position 3, 4, 5, 6, 7, 8, 9 or 10. In some embodiments, NS2 gene may be at gene position 2 and NS1 may be at position 3, 4, 5, 6, 7, 8, 9 or 10. In some embodiments each NS1 and NS2 may be moved to a different higher position independently. For example, NS1 may be at any one of positions 2, 3, 4, 5, 6, 7, 8, 9 or 10 and NS2 may be at any one of positions 3, 4, 5, 6, 7, 8, 9 or 10.

In one exemplary embodiment described in Example 1 and shown in FIG. 1, the NS1 and NS2 genes were moved to positions 7 and 8 in the intergenic region between the F and M2 genes so that the gene order in the recombinant virus construct was 3' N-P-M-SH-G-F-NS1-NS2-M2-L. This recombinant construct is named RSV 6120/NS12FM2. The polynucleotide sequence of this construct is shown in SEQ 25 ID NO:2. Some embodiments comprise a polynucleotide sequence that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% and 100%, and any number in between, identical to SEQ ID NO: 2.

30 In some embodiments, the recombinant RSV comprises a RSV genome comprising the 6120 and NS12FM2 mutations as described herein, and a positive-sense sequence denoted by a sequence that is at least 90%, at least 95%, and/or at least 99% identical to SEQ ID NO: 2 (6120/NS12FM2).

In another exemplary embodiment described in Example 2 and shown in FIG. 2, the NS1 and NS2 genes were moved to positions 9 and 10 so that the gene order in the recombinant virus construct was 3' N-P-M-SH-G-F-M2-L-NS1-NS2. This recombinant construct is named RSV 6120/NS12LTr. The polynucleotide sequence of this construct is shown in SEQ ID NO: 4. Some embodiments comprise a 35 polynucleotide sequence that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% and 100%, and any number in between, identical to SEQ ID NO: 4.

In some embodiments, the recombinant RSV comprises a RSV genome comprising the 6120 and NS12Ltr mutations as described herein, and a positive-sense sequence denoted by a sequence that is at

least 90%, at least 95%, and/or at least 99% identical to SEQ ID NO: 4 (6120/NS12Ltr).

In some embodiments, the RSV genome or antigenome comprises one or more mutations in the NS1 and/or NS2 gene, in addition to the shift in the position of the NS1/NS2 genes (for example to positions 7 and 8, or 9 and 10, respectively). The mutation may be a point mutation, a substitution or a deletion. The deletion may be partial or complete. Some exemplary embodiments are described in Examples 5 and 6 and shown in FIGs. 7 and 8. These include the constructs 6120/NS12M2F/ΔNS2 (SEQ ID NO: 6) and 6120/NS12Ltr/ΔNS2 (SEQ ID NO: 8). Design and construction of these constructs is described in Examples 5 and 6.

In some embodiments, the recombinant RSV comprises a RSV genome comprising the 6120, 10 NS12FM2, and ΔNS2 mutations as described herein, and a positive-sense sequence denoted by a sequence that is at least 90%, at least 95%, and/or at least 99% identical to SEQ ID NO: 6 (6120/NS12FM2/ΔNS2).

In some embodiments, the recombinant RSV comprises a RSV genome comprising the 6120, NS12Ltr, and ΔNS2 mutations as described herein, and a positive-sense sequence denoted by a sequence 15 that is at least 90%, at least 95%, and/or at least 99% identical to SEQ ID NO: 8 (6120/NS12Ltr/ΔNS2).

In several embodiments, the genome of the recombinant RSV comprises the one or more mutations as discussed herein, and any remaining sequence difference of the genome of the recombinant RSV compared to the genomic sequence of D46 RSV (GenBank accession number KT992094, which is incorporated by reference herein) is biologically insignificant (for example, the remaining sequence 20 differences do not include changes to the wild-type genomic sequence that modify a known cis-acting signal or change amino acid coding, or measurably affect in vitro replication or plaque size of the virus).

In another exemplary embodiment, the antigenome cDNA may be modified to contain a reporter gene, for instance a gene encoding enhanced green fluorescent protein (GFP). The GFP gene could be inserted between the RSV P and M genes (Munir et al 2008 J Virol 82:8780-8796), or as the first gene in 25 the genome (Zhang et al 2002 J Virol 76:5654-5666), or between any pair of genes. The insertion of a GFP gene in the first gene position had little or no effect on RSV replication or pathogenesis in cell lines and in an *in vitro* human airway epithelium (HAE) culture (Zhang et al 2002 J Virol 76:5654-5666), and the same appeared to be the case for GFP inserted between the P and M genes (Munir et al 2008 J Virol 82:8780-8796). One purpose of expressing GFP from the viral genome is to facilitate monitoring 30 infection in initial experiments, because it allows visualization of infections in live cells without interfering with the infection. GFP is often used in this fashion in initial experiments. Note that, when used, GFP is not included in the gene position numbering in this disclosure. While GFP expression can be helpful in initial pre-clinical studies, it typically would not be included in products for human use. Some exemplary embodiments are described herein. These are RSV 6120/NS12FM2/GFP (SEQ ID NO: 35 1), 6120/NS12Ltr/GFP (SEQ ID NO: 3), 6120/NS12M2F/ΔNS2/GFP (SEQ ID NO: 5) and 6120/NS12Ltr/ΔNS2/GFP (SEQ ID NO: 7). Design and construction of these constructs is described in Examples 1, 2, 5 and 6 and shown in FIGs. 1, 2, 7 and 8.

Additional mutations may be introduced to construct additional viral strains with desired characteristics. For example, the added mutations may specify different magnitudes of attenuation, and thus give incremental increases in attenuation. Thus, candidate vaccine strains may be further attenuated by incorporation of at least one, and preferably two or more different attenuating mutations, for example 5 mutations identified from a panel of known, biologically derived mutant RSV strains. A number of such mutations are discussed here as examples. From this exemplary panel a large “menu” of attenuating mutations can be created, in which the NS1 and/or NS2 gene shift mutation may be combined with any other mutation(s) within the panel for calibrating the level of attenuation and other desirable phenotypes. Additional attenuating mutations may be identified in non-RSV negative stranded RNA viruses and 10 incorporated in RSV mutants of the invention by mapping the mutation to a corresponding, homologous site in the recipient RSV genome or antigenome and mutating the existing sequence in the recipient to the mutant genotype (either by an identical or conservative mutation). Additional useful mutations can be determined empirically by mutational analysis using recombinant minigenome systems and infectious 15 virus as described in the references incorporated herein. Attenuation also can be achieved by codon-pair-deoptimization, which does not depend on identification of specific attenuating lesions, but rather alters gene expression by general mechanisms such reducing the efficiency of mRNA translation, among other effects (Le Nouen et al 2014 Proc Natl Acad Sci USA 111:13169-13174). A number of exemplary additional mutations are described below. These are for exemplary purposes only and are not meant to 20 limit the scope of the present invention.

20 The recombinant RSV constructs of the present invention comprising the NS1 and/or NS2 gene shift exhibit reduced expression of the NS1 and/or NS2 gene as compared to an RSV having the NS1 and NS2 genes in their native positions 1 and 2. The term “expression” as used herein refers to is intended to encompass the entire process of protein production, including transcription, translation, post-translational modification, and physical stability required to form and accumulate a functional protein.

25 The recombinant RSV constructs exhibit reduced inhibition of host interferon response i.e., the cells carrying such viruses exhibit increased expression of host interferon mRNAs and/or proteins, and/or decreased viral inhibition of interferon-mediated effects. In interferon competent cells i.e. cultured cells that can produce interferons in response to viral infection, e.g., human airway epithelial A549 cells, ATCC CCL-185, the recombinant RSV constructs of the present invention are increasingly susceptible to 30 restriction. On the other hand, in interferon incompetent cells i.e. cultured cells that cannot produce interferons in response to viral infection, e.g., African green monkey Vero cells, ATCC CCL-81, the recombinant RSV constructs of the present invention retains replication efficiency.

35 The ability of a live RSV vaccine candidate to replicate efficiently in Vero cells is beneficial because this is a cell substrate often used for vaccine manufacture. This is relevant in the case of the NS1 and NS2 genes because deletion of either or both from RSV results in more rapid and more extensive apoptosis when cells are infected with the NS-deletion viruses, compared to wild type RSV (Bitko, et al. 2007. J Virol 81:1786-1795). Embodiments of the disclosed recombinant RSV provide the ability to reduce the expression (and interferon antagonism) of NS1 and/or NS2 without completely losing

expression, and this provides the advantage of unrestricted growth in Vero cells. In addition, shifting of the NS1 and NS2 genes to higher gen positions in the RSV genome provides a means to derive a range of incrementally-increasing attenuation phenotypes.

5 *Additional mutations*

In some embodiments, RSV genome or antigenome comprises one or more mutations in one or more of the N, P, M, SH, G, F, M2 (M2-1 ORF or M2-2 ORF) and L genes, in addition to the shift in the position of the NS1/NS2 genes (for example to positions 7 and 8, or 9 and 10, respectively). For example, the RSV genome or antigenome may comprise a mutation in the M2-2 ORF of the M2 gene which 10 ablates or reduces the expression of the M2-2 protein, in addition to the shift in the position of the NS1/NS2 genes (for example to positions 7 and 8, or 9 and 10, respectively). Such mutation may comprise one or more point mutations, a partial deletion of the M2-2 ORF, or a complete deletion of the M2-2 protein.

In some embodiments, the recombinant RSV strains of the present invention comprises a deletion 15 of the non-translated sequences in genes, in the intergenic regions, and in the trailer region, in addition to the shift in the position of the NS1/NS2 genes (for example to positions 7 and 8, or 9 and 10, respectively). In one embodiment, such deletion occurs in the downstream end of the SH gene, resulting in a mutation called the “6120 Mutation” herein. It involves deletion of 112 nucleotides of the downstream non-translated region of the SH gene and the introduction of five translationally-silent point 20 mutations in the last three codons and the termination codon of the SH gene (Bukreyev, *et al.* 2001. J Virol 75:12128-12140). The 6120 mutation stabilizes the antigenomic cDNA in bacteria so that it can be more easily manipulated and prepared. In wt RSV, this mutation was previously found to confer a 5-fold increase in replication efficiency *in vitro* (Bukreyev, *et al.* 2001. J Virol 75:12128-12140), whereas it was not thought to increase replication efficiency *in vivo*.

25 In some embodiments the recombinant RSV strains may comprise the “cp” mutation, in addition to the shift in the position of the NS1/NS2 genes (for example to positions 7 and 8, or 9 and 10, respectively). This mutation refers to a set of five amino acid substitutions in three proteins (N (V267I), F (E218A and T523I), and L (C319Y and H1690Y)) that together (on their own) confer an approximate 10-fold reduction in replication in seronegative chimpanzees, and a reduction in illness (Whitehead, *et al.* 1998. J Virol 72:4467-4471). The cp mutation was previously shown to be associated with a moderate 30 attenuation phenotype (Whitehead, *et al.* 1999. J Virol 72:4467-4471).

In addition, previous analysis of 6 biological viruses that had been derived by chemical 35 mutagenesis of cpRSV and selected for the temperature-sensitive (ts) phenotype yielded a total of 6 independent mutations that each conferred a ts attenuation phenotype and could be introduced in the recombinant viruses of the present invention, in addition to the shift in the position of the NS1/NS2 genes (for example to positions 7 and 8, or 9 and 10, respectively). Five of these were amino acid substitutions in the L protein, which were named based on virus number rather than sequence position: “955” (N43I), “530” (F521L), “248” (Q831L), “1009” (M1169V), and “1030” (Y1321N) (Juhasz, *et al.* 1999. Vaccine

17:1416-1424; Collins, *et al.* 1999. *Adv Virus Res* 54:423-451; Firestone, *et al.* 1996. *Virology* 225:419-422; Whitehead, *et al.* 1999. *J Virol* 73:871-877). The sixth mutation (called "404") was a single nucleotide change in the gene-start transcription signal of the M2 gene (GGGGCAAATA, SEQ ID NO: 25 to GGGGCAAACA, SEQ ID NO: 26, mRNA-sense) (Whitehead, *et al.* 1998. *Virology* 247:232-239).

5 Reverse genetics was recently used to increase the genetic stability of the 248 and 1030 mutations (Luongo, *et al.* 2009. *Vaccine* 27:5667-5676; Luongo, *et al.* 2012. *J Virol* 86:10792-10804). Another attenuating mutation comprises a deletion of codon 1313 in the L protein and combining it with an I1314L substitution to confer increased genetic stability (Luongo, *et al.* 2013. *J Virol* 87:1985-1996).

In some embodiments, the recombinant RSV strains may comprise one or more changes in the F protein, e.g. the "HEK" mutation, which comprises two amino acid substitutions in the F protein namely K66E and Q101P (described in Connors, *et al.* 1995. *Virology* 208:478-484; Whitehead, *et al.* 1998. *J Virol* 72:4467-4471), in addition to the shift in the position of the NS1/NS2 genes (for example to positions 7 and 8, or 9 and 10, respectively). The introduction of the HEK amino acid assignments into the strain A2 F sequence of this disclosure results in an F protein amino acid sequence that is identical to that of an early-passage (human embryonic kidney cell passage 7, HEK-7) of the original clinical isolate of strain A2 (Connors, *et al.* 1995. *Virology* 208:478-484; Whitehead, *et al.* 1998. *J Virol* 72:4467-4471). It results in an F protein that is much less fusogenic and is thought to represent the phenotype of the original A2 strain clinical isolate (Liang *et al.* *J Virol* 2015 89:9499-9510). The HEK F protein also forms a more stable trimer (Liang *et al.* *J Virol* 2015 89:9499-9510). This may provide a more authentic and immunogenic form of the RSV F protein, possibly enriched for the highly immunogenic pre-fusion conformation (McLellan *et al.* *Science* 2013 340(6136):1113-7; *Science* 2013 342(6158):592-8.). Thus, mutations can be introduced with effects additional to effects on the magnitude of virus replication.

In some embodiments the recombinant strains may comprise one or more changes in the L protein, e.g. the stabilized 1030 or the "1030s" mutation which comprises 1321K(AAA)/S1313(TCA) (Luongo, *et al.* 2012. *J Virol* 86:10792-10804), in addition to the shift in the position of the NS1/NS2 genes (for example to positions 7 and 8, or 9 and 10, respectively).

In some embodiments the recombinant strains may comprise one or more changes in the N protein, e.g. an amino substitution such as T24A, or in the NS protein, e.g. an amino acid substitution such as K51R, in addition to the shift in the position of the NS1/NS2 genes (for example to positions 7 and 8, or 9 and 10, respectively).

In some embodiments, the viral strains comprise a deletion in the SH gene, in addition to the shift in the position of the NS1/NS2 genes (for example to positions 7 and 8, or 9 and 10, respectively). For example, in some embodiments, the viral strains comprise a 419 nucleotide deletion at position 4197-4615 (4198-4616 of SEQ ID NO: 1), denoted herein as the "ΔSH" mutation. This deletion results in the deletion of M gene-end, M/S_H intergenic region, and deletion of the SH ORF.

The F and/or G protein amino acid sequences of the disclosed recombinant RSV strains can be modified to represent currently-circulating strains (in addition to the shift in the position of the NS1/NS2 genes, for example to positions 7 and 8, or 9 and 10, respectively), which can be particularly relevant in

the case of the divergent G protein, or to represent early-passage clinical isolates. Deletions or substitutions may be introduced into the G protein to obtain improved immunogenicity or other desired properties. For example, the CX3C fractalkine motif in the G protein might be ablated to improve immunogenicity (Chirkova *et al.* J Virol 2013 87:13466-13479). In some embodiments, the nucleotide sequence encoding the G protein of the RSV may be replaced with nucleotide sequence G001 from the clinical isolate A/Maryland/001/11 (“G001”). In some embodiments, the nucleotide sequence encoding the F protein of the RSV may be replaced with the nucleotide sequence F001 from the clinical isolate A/Maryland/001/11 (“F001”).

In some embodiments, a native or naturally occurring nucleotide sequence encoding a protein of the RSV may be replaced with a codon optimized sequence designed for increased expression in a selected host, in particular the human, in addition to the shift in the position of the NS1/NS2 genes (for example to positions 7 and 8, or 9 and 10, respectively). Alternatively, a sequence can be designed to be suboptimal on the codon or codon-pair level.

In addition to the above described mutations, recombinant RSV according to the invention can incorporate heterologous, coding or non-coding nucleotide sequences from any RSV or RSV-like virus, e.g., human, bovine, ovine, murine (pneumonia virus of mice), or avian (turkey rhinotracheitis virus) pneumovirus, or from another enveloped virus, e. g., parainfluenza virus (PIV). Exemplary heterologous sequences include RSV sequences from one human RSV strain combined with sequences from a different human RSV strain. In yet additional aspects, one or more human RSV coding or non-coding polynucleotides are substituted with a counterpart sequence from a heterologous RSV or non-RSV virus to yield novel attenuated vaccine strains. Alternatively, the recombinant RSV may incorporate sequences from two or more, wild-type or mutant human RSV subgroups, for example a combination of human RSV subgroup A and subgroup B sequences. RSV exists as two antigenic subgroups, A and B, which have substantial sequence and antigenic differences, in particular for the G protein. It is common for A and B strains to alternate predominance in 1- to 2-year cycles, suggesting that the antigenic differences are sufficient to facilitate re-infection by a heterologous subgroup strain (Hall *et al* 1990 J Infect 162:1283-1290; Wattis 1991 J Infect Dis 163:464-469; Peret *et al* 1998 J Gen Virol 79:2221-2229). Therefore, the recombinant RSV may incorporate sequences from the heterologous subgroup in order to increase the breadth of protection. For example, the F and/or G proteins of an attenuated RSV of one subgroup might be swapped with those of the second subgroup in order to make a new vaccine matched to the heterologous subgroup (Whitehead *et al* 1999 J Virol 73:9773-9780). As another example, the G protein of the heterologous subgroup can be expressed as an additional gene. In this way, an RSV vaccine could be designed with one or more components that represent both antigenic subgroups.

In addition to the recombinant RSVs having the particular mutations described herein, the disclosed viruses may be modified further as would be appreciated by those skilled in the art. For example, the recombinant RSVs may have one or more of its proteins deleted or otherwise mutated or a heterologous gene from a different organism may be added to the genome or antigenome so that the recombinant RSV expresses or incorporates that protein upon infecting a cell and replicating.

Furthermore, those skilled in the art will appreciate that other previously defined mutations known to have an effect on RSV may be combined with one or more of any of the mutations described herein to produce a recombinant RSV with desirable attenuation or stability characteristics.

In some embodiments, the disclosed recombinant RSV vaccine strains can be produced using a recombinant DNA-based technique called reverse genetics (Collins, *et al.* 1995. Proc Natl Acad Sci USA 92:11563-11567). This system allows *de novo* recovery of infectious virus entirely from cDNA in a qualified cell substrate under defined conditions. Reverse genetics provides a means to introduce predetermined mutations into the RSV genome via the cDNA intermediate. Specific attenuating mutations were characterized in preclinical studies and combined to achieve the desired level of attenuation. Derivation of vaccine viruses from cDNA minimizes the risk of contamination with adventitious agents and helps to keep the passage history brief and well documented. Once recovered, the engineered virus strains propagate in the same manner as a biologically derived virus. As a result of passage and amplification, the vaccine viruses do not contain recombinant DNA from the original recovery.

The Examples in the present disclosure utilized RSV strain A2 of antigenic subgroup A, which is the most widely used experimental strain and also is the parent of numerous live attenuated RSV vaccine candidates that have been evaluated in clinical studies. Given that a variety of additional RSV strains exist (e.g., RSV B1, RSV Long, RSV Line 19), those skilled in the art will appreciate that certain strains of RSV may have nucleotide or amino acid insertions or deletions that alter the position of a given residue. For example, if a protein of another RSV strain had, in comparison with strain A2, two additional amino acids in the upstream end of the protein, this would cause the amino acid numbering of downstream residues relative to strain A2 to increase by an increment of two. However, because these strains share a large degree of sequence identity, those skilled in the art would be able to determine the location of corresponding sequences by simply aligning the nucleotide or amino acid sequence of the A2 reference strain with that of the strain in question. Therefore, it should be understood that the amino acid and nucleotide positions described herein, though specifically enumerated in the context of this disclosure, can correspond to other positions when a sequence shift has occurred or due to sequence variation between virus strains. In the comparison of a protein, or protein segment, or gene, or genome, or genome segment between two or more related viruses, a “corresponding” amino acid or nucleotide residue is one that is thought to be exactly or approximately equivalent in function in the different species.

Unless context indicates otherwise, the numbering used in this disclosure is based on the sequence of the wild-type RSV A2 strain (GenBank accession number M74568) and viral genomic sequences described are in positive-sense.

In some embodiments of the present invention, the recombinant RSV strains were derived from the recombinant version of strain A2 that is called D46. The complete sequence of D46 is shown in US patent 6,790,449 and is being made available as GenBank accession number KT992094. (In some instances and publications, the parent virus and sequence is called D53 rather than D46, a book-keeping

difference that refers to the strain of bacteria used to propagate the antigenomic cDNA and has no other known significance or effect. For the purposes of this disclosure, D46 and D53 are interchangeable.) The nucleotide sequence of D46 differs from the sequence of RSV A2 strain M74568 in 25 nucleotide positions, which includes a 1-nucleotide insert at position 1099.

5 With regard to sequence numbering of nucleotide and amino acid sequence positions for the described viruses, a convention was used whereby each nucleotide or amino acid residue in a given viral sequence retained the sequence position number that it has in the original 15,222-nucleotide biological wt strain A2 virus (Genbank accession number M74568), irrespective of any modifications. Thus, although a number of genomes contain deletions and/or insertions that cause changes in nucleotide length, and in 10 some cases amino acid length, the numbering of all of the other residues (nucleotide or amino acid) in the genome and encoded proteins remains unchanged. It also is recognized that, even without the expedient of this convention, one skilled in the art can readily identify corresponding sequence positions between viral genomes or proteins that might differ in length, guided by sequence alignments as well as the 15 positions of open reading frames, well-known RNA features such as gene-start and gene-end signals, and amino acid sequence features.

Recombinant viruses may be evaluated in cell culture, rodents and non-human primates for 20 infectivity, replication kinetics, yield, efficiency of protein expression, and genetic stability using the methods known in the art. While these semi-permissive systems may not reliably detect every difference in replication, substantial differences in particular may be detected. Also recombinant strains may be evaluated successively in adults, seropositive children, and seronegative children. In some cases, where a 25 previous similar strain has already been shown to be well-tolerated in seronegative children, a new strain may be evaluated directly in seronegative children. Evaluation may be done, for example, in groups of 10 vaccine recipients and 5 placebo recipients, which is a small number that allows simultaneous evaluation of multiple candidates. Candidates may be evaluated in the period immediately post-immunization for 30 vaccine virus infectivity, replication kinetics, shedding, tolerability, immunogenicity, and genetic stability, and the vaccinees may be subjected to surveillance during the following RSV season for safety, RSV disease, and changes in RSV-specific serum antibodies, as described in Karron, *et al.* 2015, *Science Transl Med* 2015 7(312):312ra175, which is incorporated herein in its entirety. Thus, analysis of selected representative viruses may provide for relatively rapid triage to narrow down candidates to identify the most optimal.

Reference to a protein or a peptide includes its naturally occurring form, as well as any fragment, 35 domain, or homolog of such protein. As used herein, the term "homolog" is used to refer to a protein or peptide which differs from a naturally occurring protein or peptide (*i.e.*, the "prototype" or "wild-type" protein) by minor modifications to the naturally occurring protein or peptide, but which maintains the basic protein and side chain structure of the naturally occurring form. Such changes include, but are not limited to: changes in one or a few amino acid side chains; changes in one or a few amino acids, including deletions (e.g., a truncated version of the protein or peptide) insertions and/or substitutions; changes in stereochemistry of one or a few atoms; and/or minor derivatizations, including but not limited

to: methylation, glycosylation, phosphorylation, acetylation, myristylation, prenylation, palmitation, amidation. A homolog can have either enhanced, decreased, or substantially similar properties as compared to the naturally occurring protein or peptide. A homolog of a given protein may comprise, consist essentially of, or consist of, an amino acid sequence that is at least about 50%, or at least about 55%, or at least about 60%, or at least about 65%, or at least about 70%, or at least about 75%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% identical (or any percent identity between 45% and 99%, in whole integer increments), to the amino acid sequence of the reference protein.

In one aspect of the invention, a selected gene segment, such as one encoding a selected protein or protein region (e.g., a cytoplasmic tail, transmembrane domain or ectodomain, an epitopic site or region, a binding site or region, an active site or region containing an active site, etc.) from one RSV, can be substituted for a counterpart gene segment from the same or different RSV or other source, to yield novel recombinants having desired phenotypic changes compared to wild-type or parent RSV strains. For example, recombinants of this type may express a chimeric protein having a cytoplasmic tail and/or transmembrane domain of one RSV fused to an ectodomain of another RSV. Other exemplary recombinants of this type express duplicate protein regions, such as duplicate immunogenic regions. As used herein, “counterpart” genes, gene segments, proteins or protein regions, are typically from heterologous sources (e.g., from different RSV genes, or representing the same (*i.e.*, homologous or allelic) gene or gene segment in different RSV strains). Typical counterparts selected in this context share gross structural features, e.g., each counterpart may encode a comparable structural “domain,” such as a cytoplasmic domain, transmembrane domain, ectodomain, binding site or region, epitopic site or region, etc. Counterpart domains and their encoding gene segments embrace an assemblage of species having a range of size and amino acid (or nucleotide) sequence variations, which range is defined by a common biological activity among the domain or gene segment variants. For example, two selected protein domains encoded by counterpart gene segments within the invention may share substantially the same qualitative activity, such as providing a membrane spanning function, a specific binding activity, an immunological recognition site, etc. More typically, a specific biological activity shared between counterparts, e.g., between selected protein segments or proteins, will be substantially similar in quantitative terms, *i.e.*, they will not vary in respective quantitative activity profiles by more than 30%, preferably by no more than 20%, more preferably by no more than 5-10%.

In alternative aspects of the invention, the infectious RSV produced from a cDNA-expressed genome or antigenome can be any of the RSV or RSV-like strains, e.g., human, bovine, murine, etc., or of any pneumovirus or metapneumovirus, e.g., pneumonia virus of mice or avian metapneumovirus. To engender a protective immune response, the RSV strain may be one which is endogenous to the subject being immunized, such as human RSV being used to immunize humans. The genome or antigenome of endogenous RSV can be modified, however, to express RSV genes or gene segments from a combination of different sources, e.g., a combination of genes or gene segments from different RSV species, subgroups, or strains, or from an RSV and another respiratory pathogen such as human parainfluenza

virus (PIV) (see, e.g., Hoffman *et al.* *J. Virol.* 71:4272-4277 (1997); Durbin *et al.* *Virology* 235(2):323-32 (1997); Murphy *et al.* U.S. Patent Application Ser. No. 60/047,575, filed May 23, 1997, and the following plasmids for producing infectious PIV clones: p3/7(131) (ATCC 97990); p3/7(131)2G(ATCC 97889); and p218(131) (ATCC 97991); each deposited Apr. 18, 1997 under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC) of 10801 University Blvd., Manassas, Va. 20110-2209, USA., and granted the above identified accession numbers.

In certain embodiments of the invention, recombinant RSV are provided wherein individual internal genes of a human RSV are replaced with, e.g., a bovine or other RSV counterpart, or with a counterpart or foreign gene from another respiratory pathogen such as PIV. Also, human RSV cis-acting sequences, such as promoter or transcription signals, can be replaced with, e.g., their bovine RSV counterpart. Reciprocally, means are provided to generate live attenuated bovine RSV by inserting human attenuating genes or cis-acting sequences into a bovine RSV genome or antigenome background.

Thus, infectious recombinant RSV intended for administration to humans can be a human RSV that has been modified to contain genes from, e.g., a bovine RSV or a PIV, such as for the purpose of attenuation. For example, by inserting a gene or gene segment from PIV, a bivalent vaccine to both PIV and RSV is provided. Alternatively, a heterologous RSV species, subgroup or strain, or a distinct respiratory pathogen such as PIV, may be modified, e.g., to contain genes that encode epitopes or proteins which elicit protection against human RSV infection. For example, the human RSV glycoprotein genes can be substituted for the bovine glycoprotein genes such that the resulting bovine RSV, which now bears the human RSV surface glycoproteins and would retain a restricted ability to replicate in a human host due to the remaining bovine genetic background, elicits a protective immune response in humans against human RSV strains.

The ability to analyze and incorporate other types of attenuating mutations into infectious RSV for vaccine development extends to a broad assemblage of targeted changes in RSV clones. For example, any RSV gene which is not essential for growth may be ablated or otherwise modified to yield desired effects on virulence, pathogenesis, immunogenicity and other phenotypic characters.

As used herein, “heterologous genes” refers to genes taken from different RSV strains or types or non-RSV sources. These heterologous genes can be inserted in whole or in part, the order of genes changed, gene overlap removed, the RSV genome promoter replaced with its antigenome counterpart, portions of genes removed or substituted, and even entire genes deleted. Different or additional modifications in the sequence can be made to facilitate manipulations, such as the insertion of unique restriction sites in various intergenic regions or elsewhere. Nontranslated gene sequences can be removed to increase capacity for inserting foreign sequences.

Deletions, insertions, substitutions and other mutations involving changes of whole viral genes or gene segments in recombinant RSV of the invention yield highly stable vaccine candidates, which may be relevant in the case of immunosuppressed individuals. Many of these mutations will result in attenuation of resultant vaccine strains, whereas others will specify different types of desired phenotypic changes. For example, certain viral genes are known which encode proteins that specifically interfere

with host immunity (see, e.g., Kato *et al.*, EMBO J. 16:578-87 (1997). Ablation of such genes in vaccine viruses is expected to reduce virulence and pathogenesis and/or improve immunogenicity.

Other mutations within RSV of the present invention involve replacement of the 3' end of genome with its counterpart from antigenome, which is associated with changes in RNA replication and transcription. In addition, the intergenic regions (Collins *et al.*, Proc. Natl. Acad. Sci. USA 83:4594-4598 (1986)) can be shortened or lengthened or changed in sequence content, and the naturally-occurring gene overlap (Collins *et al.*, Proc. Natl. Acad. Sci. USA 84:5134-5138 (1987)) can be removed or changed to a different intergenic region by the methods described herein.

In another embodiment, a sequence surrounding a translational start site (preferably including a nucleotide in the -3 position) of a selected RSV gene is modified, alone or in combination with introduction of an upstream start codon, to modulate RSV gene expression by specifying up- or down-regulation of translation.

Alternatively, or in combination with other RSV modifications disclosed herein, RSV gene expression can be modulated by altering a transcriptional GS signal of a selected gene(s) of the virus. In one exemplary embodiment, the GS signal of M2 is modified to include a defined mutation to superimpose a ts restriction on viral replication.

Yet additional RSV clones within the invention incorporate modifications to a transcriptional GE signal. For example, RSV clones are provided which substitute or mutate the GE signal of the NS1 and NS2 genes for that of the N gene, resulting in decreased levels of readthrough mRNAs and increased expression of proteins from downstream genes. The resulting recombinant virus exhibits increased growth kinetics and increased plaque size, providing but one example of alteration of RSV growth properties by modification of a cis-acting regulatory element in the RSV genome.

In another aspect, expression of the G protein may be increased by modification of the G mRNA. The G protein is expressed as both a membrane bound and a secreted form, the latter form being expressed by translational initiation at a start site within the G gene translational open reading frame. The secreted form may account for as much as one-half of the expressed G protein. Ablation of the internal start site (e.g., by sequence alteration, deletion, etc.), alone or together with altering the sequence context of the upstream start site yields desired changes in G protein expression. Ablation of the secreted form of the G protein also will improve the quality of the host immune response to exemplary, recombinant RSV, because the soluble form of the G protein is thought to act as a "decoy" to trap neutralizing antibodies. Also, soluble G protein has been implicated in enhanced immunopathology due to its preferential stimulation of a Th2-biased response.

In yet other embodiments, RSV useful in a vaccine formulation may be conveniently modified to accommodate antigenic drift in circulating virus. Typically the modification will be in the G and/or F proteins. The entire G or F gene, or the segments encoding particular immunogenic regions thereof, is incorporated into the RSV genome or antigenome cDNA by replacement of the corresponding region in the infectious clone or by adding one or more copies of the gene such that several antigenic forms are represented.

In addition to the above described modifications to recombinant RSV, different or additional modifications in RSV clones can be made to facilitate manipulations, such as the insertion of unique restriction sites in various intergenic regions or elsewhere. Nontranslated gene sequences can be removed to increase capacity for inserting foreign sequences.

5 Introduction of the foregoing, defined mutations into an infectious RSV clone can be achieved by a variety of well-known methods. By “infectious clone” is meant cDNA or its product, synthetic or otherwise, which can be transcribed into genomic or antigenomic RNA capable of producing an infectious virus. The term “infectious” refers to a virus or viral structure that is capable of replicating in a cultured cell or animal or human host to produce progeny virus or viral structures capable of the same 10 activity. Thus, defined mutations can be introduced by conventional techniques (e.g., site-directed mutagenesis) into a cDNA copy of the genome or antigenome. The use of antigenome or genome cDNA subfragments to assemble a complete antigenome or genome cDNA is well-known by those of ordinary skill in the art and has the advantage that each region can be manipulated separately (smaller cDNAs are easier to manipulate than large ones) and then readily assembled into a complete cDNA. Thus, the 15 complete antigenome or genome cDNA, or any subfragment thereof, can be used as template for oligonucleotide-directed mutagenesis. A mutated subfragment can then be assembled into the complete antigenome or genome cDNA. Mutations can vary from single nucleotide changes to replacement of large cDNA pieces containing one or more genes or genome regions.

Recombinant RSV may be produced by the intracellular coexpression of a cDNA that encodes 20 the RSV genomic RNA, together with those viral proteins necessary to generate a transcribing, replicating nucleocapsid. Plasmids encoding other RSV proteins may also be included with these essential proteins. Alternatively, RNA may be synthesized in *in vitro* transcription reactions and transfected into cultured cells.

Accordingly, also described herein are isolated polynucleotides that encode the described 25 mutated viruses, make up the described genomes or antigenomes, express the described genomes or antigenomes, or encode various proteins useful for making recombinant RSV *in vitro*. The nucleic acid sequences of a number of exemplary polynucleotides are also provided. Included within the scope of the invention are polynucleotides comprising sequences that consist or consist essentially of any of the aforementioned nucleic acid sequences. Further included are polynucleotides that possess at least about 30 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 percent or more identity, or any number in between, to any of the aforementioned sequences or SEQ ID NOs, as well as polynucleotides that hybridize to, or are the complements of the aforementioned molecules.

These polynucleotides can be included within or expressed by vectors in order to produce a 35 recombinant RSV. Accordingly, cells transfected with the isolated polynucleotides or vectors are also within the scope of the invention and are exemplified herein.

In related aspects of the invention, compositions (e.g., isolated polynucleotides and vectors incorporating an RSV-encoding cDNA) and methods are provided for producing an recombinant RSV.

Included within these aspects of the invention are novel, isolated polynucleotide molecules and vectors incorporating such molecules that comprise a RSV genome or antigenome which is modified as described herein. Also provided is the same or different expression vector comprising one or more isolated polynucleotide molecules encoding the RSV proteins. These proteins also can be expressed 5 directly from the genome or antigenome cDNA. The vector(s) are preferably expressed or coexpressed in a cell or cell-free lysate, thereby producing an infectious mutant RSV particle or subviral particle.

In one aspect, the invention includes a method for producing one or more purified RSV protein(s) which involves infecting a host cell permissive of RSV infection with a recombinant RSV strain under conditions that allow for RSV propagation in the infected cell. After a period of replication 10 in culture, the cells are lysed and recombinant RSV is isolated therefrom. One or more desired RSV protein(s) is purified after isolation of the virus, yielding one or more RSV protein(s) for vaccine, diagnostic and other uses.

The above methods and compositions yield infectious viral or subviral particles, or derivatives thereof. An infectious virus is comparable to the authentic RSV virus particle and is infectious as is. It 15 can directly infect fresh cells. An infectious subviral particle typically is a subcomponent of the virus particle which can initiate an infection under appropriate conditions. For example, a nucleocapsid containing the genomic or antigenomic RNA and the N, P, L and M2-1 proteins is an example of a subviral particle which can initiate an infection if introduced into the cytoplasm of cells. Subviral particles provided within the invention include viral particles which lack one or more protein(s), protein 20 segment(s), or other viral component(s) not essential for infectivity.

In other embodiments the invention provides a cell or cell free lysate containing an expression vector which comprises an isolated polynucleotide molecule encoding mutant RSV genome or antigenome as described above, and an expression vector (the same or different vector) which comprises one or more isolated polynucleotide molecules encoding the N, P, L and RNA polymerase elongation 25 factor proteins of RSV. One or more of these proteins also can be expressed from the genome or antigenome cDNA. Upon expression the genome or antigenome and N, P, L, and RNA polymerase elongation factor proteins combine to produce an infectious RSV viral or sub-viral particle.

The recombinant RSV of the invention are useful in various compositions to generate a desired immune response against RSV in a host susceptible to RSV infection. Attenuated mutant RSV strains of 30 the invention are capable of eliciting a protective immune response in an infected human host, yet are sufficiently attenuated so as to not cause unacceptable symptoms of severe respiratory disease in the immunized host. The attenuated virus or subviral particle may be present in a cell culture supernatant, isolated from the culture, or partially or completely purified. The virus may also be lyophilized, and can be combined with a variety of other components for storage or delivery to a host, as desired.

35 In another aspect of the invention, the recombinant mutants may be employed as “vectors” for protective antigens of other pathogens, particularly respiratory tract pathogens such as parainfluenza virus (PIV). For example, recombinant RSV may be engineered which incorporate, sequences that encode protective antigens from PIV to produce infectious, attenuated vaccine virus.

In related aspects, the invention provides a method for stimulating the immune system of an individual to elicit an immune response against RSV in a mammalian subject. The method comprises administering an immunogenic formulation of an immunologically sufficient amount of an attenuated, recombinant mutant RSV in a physiologically acceptable carrier and/or adjuvant.

5 The invention further provides novel vaccines comprising a physiologically acceptable carrier and/or adjuvant and an isolated attenuated mutant RSV particle or subviral particle.

To select candidate vaccine viruses from the host of recombinant RSV strains provided herein, the criteria of viability, efficient replication *in vitro*, attenuation *in vivo*, immunogenicity, and phenotypic stability are determined according to well-known methods. Viruses which will be most desired in 10 vaccines of the invention should maintain viability, should replicate sufficiently *in vitro* well under permissive conditions to make vaccine manufacture possible, should have a stable attenuation phenotype, should be well-tolerated, should exhibit replication in an immunized host (albeit at lower levels), and should effectively elicit production of an immune response in a vaccine sufficient to confer protection against serious disease caused by subsequent infection from wild-type virus.

15 To propagate a RSV virus for vaccine use and other purposes, a number of cell lines which allow for RSV growth may be used. RSV grows in a variety of human and animal cells. Preferred cell lines for propagating attenuated RS virus for vaccine use include DBSFRhL-2, MRC-5, and Vero cells. Highest virus yields are usually achieved with epithelial cell lines such as Vero cells. Cells are typically inoculated with virus at a multiplicity of infection ranging from about 0.001 to 1.0, or more, and are 20 cultivated under conditions permissive for replication of the virus, e.g., at about 30-37°C and for about 3-10 days, or as long as necessary for virus to reach an adequate titer. Temperature-sensitive viruses often are grown using 32°C as the “permissive temperature.” Virus is removed from cell culture and separated from cellular components, typically by well-known clarification procedures, e.g., centrifugation, and may be further purified as desired using procedures well known to those skilled in the art.

25 RSV which has been attenuated as described herein can be tested in various well known and generally accepted *in vitro* and *in vivo* models to confirm adequate attenuation, resistance to phenotypic reversion, and immunogenicity for vaccine use. In *in vitro* assays, the modified virus, which can be a multiply attenuated, biologically derived or recombinant RSV, is tested for temperature sensitivity of virus replication or “ts phenotype,” and for the small plaque phenotype. Modified virus also may be 30 evaluated in an *in vitro* human airway epithelium (HAE) model, which appears to provide a means of ranking viruses in the order of their relative attenuation in non-human primates and humans (Zhang et al 2002 J Virol 76:5654-5666; Schaap-Nutt et al 2010 Vaccine 28:2788-2798; Ilyushina et al 2012 J Virol 86:11725-11734). Modified viruses are further tested in animal models of RSV infection. A variety of animal models (e.g., murine, cotton rat, and primate) have been described and are known to those skilled 35 in the art.

In accordance with the foregoing description and based on the Examples below, the invention also provides isolated, infectious RSV compositions for vaccine use. The attenuated virus which is a component of a vaccine is in an isolated and typically purified form. By isolated is meant to refer to RSV

which is in other than a native environment of a wild-type virus, such as the nasopharynx of an infected individual. More generally, isolated is meant to include the attenuated virus as a component of a cell culture or other artificial medium. For example, attenuated RSV of the invention may be produced by an infected cell culture, separated from the cell culture and added to a stabilizer.

5 RSV vaccines of the invention contain as an active ingredient an immunogenically effective amount of RSV produced as described herein. Biologically derived or recombinant RSV can be used directly in vaccine formulations. The biologically derived or recombinantly modified virus may be introduced into a host with a physiologically acceptable carrier and/or adjuvant. Useful carriers are well known in the art, and include, e.g., water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and 10 the like. The resulting aqueous solutions may be packaged for use as is, or in frozen form that is thawed prior to use, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration, as mentioned above. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, which include, but are not limited to, pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for 15 example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sucrose, magnesium sulfate, phosphate buffers, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, sorbitan monolaurate, and triethanolamine oleate. Acceptable adjuvants include incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum, which are materials well known in the art. Preferred adjuvants also include Stimulon™ QS-21 (Aquila Biopharmaceuticals, Inc., 20 Worcester, Mass.), MPL™ (3-O-deacylated monophosphoryl lipid A; RIBI ImmunoChem Research, Inc., Hamilton, Mont.), and interleukin-12 (Genetics Institute, Cambridge, Mass.).

Upon immunization with a RSV vaccine composition, the host responds to the vaccine by producing antibodies specific for RSV virus proteins, e.g., F and G glycoproteins. In addition, innate and cell-mediated immune responses are induced, which can provide antiviral effectors as well as regulating 25 the immune response. As a result of the vaccination the host becomes at least partially or completely immune to RSV infection, or resistant to developing moderate or severe RSV disease, particularly of the lower respiratory tract.

The vaccine compositions containing the attenuated RSV of the invention are administered to a subject susceptible to or otherwise at risk of RSV infection in an "immunogenically effective dose" 30 which is sufficient to induce or enhance the individual's immune response capabilities against RSV. An RSV vaccine composition may be administered by any suitable method, including but not limited to, via injection, aerosol delivery, nasal spray, nasal droplets, oral inoculation, or topical application. In the case of human subjects, the attenuated virus of the invention is administered according to well established human RSV vaccine protocols (Karron *et al.* JID 191:1093-104, 2005). Briefly, adults or children are 35 inoculated intranasally via droplet with an immunogenically effective dose of RSV vaccine, typically in a volume of 0.5 ml of a physiologically acceptable diluent or carrier. This has the advantage of simplicity and safety compared to parenteral immunization with a non-replicating vaccine. It also provides direct stimulation of local respiratory tract immunity, which plays a major role in resistance to RSV. Further,

this mode of vaccination effectively bypasses the immunosuppressive effects of RSV-specific maternally-derived serum antibodies, which typically are found in the very young. Also, while the parenteral administration of RSV antigens can sometimes be associated with immunopathologic complications, this has not been observed with a live virus.

5 In some embodiments, the vaccine may be administered intranasally or subcutaneously or intramuscularly. In some embodiments, it may be administered to the upper respiratory tract. This may be performed by any suitable method, including but not limited to, by spray, droplet or aerosol delivery. Often, the composition will be administered to an individual seronegative for antibodies to RSV or possessing transplacentally acquired maternal antibodies to RSV.

10 In all subjects, the precise amount of RSV vaccine administered and the timing and repetition of administration will be determined by various factors, including the patient's state of health and weight, the mode of administration, the nature of the formulation, etc. Dosages will generally range from about 3.0 \log_{10} to about 6.0 \log_{10} plaque forming units ("PFU") or more of virus per patient, more commonly from about 4.0 \log_{10} to 5.0 \log_{10} PFU virus per patient. In one embodiment, about 5.0 \log_{10} to 6.0 \log_{10} PFU per patient may be administered during infancy, such as between 1 and 6 months of age, and one or more additional booster doses could be given 2-6 months or more following the first dose. In another embodiment, young infants could be given a dose of about 5.0 \log_{10} to 6.0 \log_{10} PFU per patient at approximately 2, 4, and 6 months of age, which is the recommended time of administration of a number of other childhood vaccines. In yet another embodiment, an additional booster dose could be 15 administered at approximately 10-15 months of age. In any event, the vaccine formulations should provide a quantity of attenuated RSV of the invention sufficient to effectively stimulate or induce an anti-RSV immune response (an "effective amount").

20 In some embodiments, the vaccine may comprise attenuated recombinant virus that elicits an immune response against a single RSV strain or antigenic subgroup, e.g. A or B, or against multiple RSV strains or subgroups. In this regard, the recombinant mutant RSV can be combined in vaccine 25 formulations with other RSV vaccine strains or subgroups having different immunogenic characteristics for more effective protection against one or multiple RSV strains or subgroups. They may be administered in a vaccine mixture, or administered separately in a coordinated treatment protocol to elicit 30 more effective protection against one RSV strain, or against multiple RSV strains or subgroups.

35 The resulting immune response can be characterized by a variety of methods. These include taking samples of nasal washes or sera for analysis of RSV-specific antibodies, which can be detected by tests including, but not limited to, complement fixation, plaque neutralization, enzyme-linked immunosorbent assay, luciferase-immunoprecipitation assay, and flow cytometry. In addition, immune responses can be detected by assay of cytokines in nasal washes or sera, ELISPOT of immune cells from either source, quantitative RT-PCR or microarray analysis of nasal wash or serum samples, and restimulation of immune cells from nasal washes or serum by re-exposure to viral antigen *in vitro* and analysis for the production or display of cytokines, surface markers, or other immune correlates measures

by flow cytometry or for cytotoxic activity against indicator target cells displaying RSV antigens. In this regard, individuals are also monitored for signs and symptoms of upper respiratory illness.

The level of attenuation of vaccine virus may be determined by, for example, quantifying the amount of virus present in the respiratory tract of an immunized host and comparing the amount to that produced by wild-type RSV or other attenuated RS viruses which have been evaluated as candidate vaccine strains. For example, the attenuated virus of the invention will have a greater degree of restriction of replication in the upper respiratory tract of a highly susceptible host, such as a chimpanzee, compared to the levels of replication of wild-type virus, e.g., 10- to 1000-fold less. In order to further reduce the development of rhinorrhea, which is associated with the replication of virus in the upper respiratory tract, an ideal vaccine candidate virus should exhibit a restricted level of replication in both the upper and lower respiratory tract. However, the attenuated viruses of the invention must be sufficiently infectious and immunogenic in humans to confer protection in vaccinated individuals. Methods for determining levels of RSV in the nasopharynx of an infected host are well known in the literature. Specimens are obtained by aspiration or washing out of nasopharyngeal secretions and virus quantified in tissue culture or other by laboratory procedure. See, for example, Belshe *et al.*, J. Med. Virology 1:157-162 (1977), Friedewald *et al.*, J. Amer. Med. Assoc. 204:690-694 (1968); Gharpure *et al.*, J. Virol. 3:414-421 (1969); and Wright *et al.*, Arch. Ges. Virusforsch. 41:238-247 (1973). The virus can conveniently be measured in the nasopharynx of host animals, such as chimpanzees.

In summary, the materials, information, and methods described in this disclosure provide an array of attenuated strains with graded attenuation phenotypes, and provide guidance in selecting suitable vaccine candidate strains based on clinical benchmarks. The following examples are provided by way of illustration, not limitation.

Additional embodiments

Clause 1. An isolated polynucleotide molecule encoding a recombinant respiratory syncytial virus (RSV) variant having an attenuated phenotype comprising a RSV genome or antigenome sequence, wherein the RSV genome or antigenome comprises a modification selected from the group consisting of:

- (a) the NS1 gene is in a gene position higher than position 1;
- (b) the NS2 gene is in a gene position higher than position 2; and
- (c) a combination of (a) and (b),
optionally, further comprising a reporter gene.

Clause 2. The isolated polynucleotide molecule of clause 1, wherein the modification is a combination of (a) and (b).

Clause 3. The isolated polynucleotide molecule of clause 2, wherein the NS1 gene is in gene position 7 and the NS2 gene is in gene position 8.

Clause 4. The isolated polynucleotide molecule of clause 2, wherein the NS1 gene is in gene position 9 and the NS2 gene is in gene position 10.

Clause 5. The isolated polynucleotide molecule of clause 1, wherein the modification is (a).

Clause 6. The isolated polynucleotide molecule of clause 5, wherein the NS1 gene is in gene position 7 or 9.

Clause 7. The isolated polynucleotide molecule of clause 1, wherein the modification is (b).

Clause 8. The isolated polynucleotide molecule of clause 5 or 6 or 7, wherein the RSV genome or antigenome further comprises a deletion of all or part of the NS1 or NS2 gene.

Clause 9. The isolated polynucleotide molecule of any one of clause 1-8, further comprising a deletion of all or part of the M2-2 gene.

Clause 10. The isolated polynucleotide molecule of clause 1, wherein the RSV genome or antigenome has a positive-sense sequence denoted by a sequence that is at least 90% identical to SEQ ID 10 NO:2.

Clause 11. The isolated polynucleotide molecule of clause 1, wherein the RSV genome or antigenome has a positive-sense sequence denoted by SEQ ID NO:2.

Clause 12. The isolated polynucleotide molecule of clause 1, wherein the RSV genome or antigenome has a positive-sense sequence denoted by a sequence that is at least 90% identical to SEQ ID 15 NO:4.

Clause 13. The isolated polynucleotide molecule of clause 1, wherein the RSV genome or antigenome has a positive-sense sequence denoted by SEQ ID NO:4.

Clause 14. The isolated polynucleotide molecule of clause 8, wherein the RSV genome or antigenome has a positive-sense sequence denoted by a sequence that is at least 90% identical to a 20 sequence selected from the group consisting of SEQ ID NO:6 and SEQ ID NO:8.

Clause 15. The isolated polynucleotide molecule of clause 1, wherein the reporter gene encodes Green Fluorescent Protein (GFP).

Clause 16. The isolated polynucleotide molecule of clause 15, wherein the RSV genome or antigenome has a positive-sense sequence denoted by a sequence that is at least 90% identical to a 25 sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7.

Clause 17. The isolated polynucleotide molecule of any one of clauses 1-16, which exhibits reduced expression of the NS1 gene and/or NS2 gene compared to an RSV having the NS1 gene in gene position 1 and the NS2 gene in gene position 2.

Clause 18. The isolated polynucleotide molecule of any one of clauses 1-17, which exhibits reduced transcription of the NS1 gene and/or NS2 gene compared to an RSV having the NS1 gene in gene position 1 and the NS2 gene in gene position 2.

Clause 19. The isolated polynucleotide molecule of any one of clauses 1-16, which exhibits reduced inhibition of host interferon response compared to an RSV having the NS1 gene in gene position 35 1 and the NS2 gene in gene position 2.

Clause 20. The isolated polynucleotide molecule of any one of clauses 1-16, wherein the RSV variant is increasingly susceptible to restriction in cultured cells that can produce interferons in response to viral infection.

Clause 21. The isolated polynucleotide molecule of any one of clauses 1-16, wherein the RSV variant retains replication efficiency in cultured cells that cannot produce interferons in response to viral infection

Clause 22. A vector comprising the isolated polynucleotide molecule of any one of clauses 1 - 5 21.

Clause 23. A cell comprising the isolated polynucleotide of any one of clauses 1 - 21.

Clause 24. A pharmaceutical composition comprising an immunologically effective amount of the recombinant RSV variant encoded by the isolated polynucleotide molecule of any one of clauses 1- 21.

10 Clause 25. The pharmaceutical composition of clause 24 further comprising an adjuvant.

Clause 26. A method of vaccinating a subject against RSV comprising administering the pharmaceutical composition of clause 24.

Clause 27. The method of clause 26, wherein the pharmaceutical composition is administered intranasally.

15 Clause 28. The method of clause 26 or 27, wherein the pharmaceutical composition is administered via injection, aerosol delivery, nasal spray or nasal droplets.

Exemplary Sequences

Antigenomic cDNA sequence of RSV 6120/NS12FM2GFP (SEQ ID NO: 1)

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Antigenomic cDNA sequence of RSV 6120/NS12FM2 (SEQ ID NO: 2)

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Antigenomic cDNA sequence of RSV 6120/NS12LtrGFP (SEQ ID NO: 3)

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Antigenomic cDNA sequence of RSV 6120/NS12Ltr (SEQ ID NO: 4)

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Antigenomic cDNA sequence of RSV 6120/NS12FM2/ΔNS2GFP (SEQ ID NO: 5)

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Antigenomic cDNA sequence of RSV 6120/NS12Ltr/ΔNS2/GFP (SEQ ID NO: 7)

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Antigenomic cDNA sequence of RSV 6120/NS12Ltr/ΔNS2 (SEQ ID NO: 8):

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EXAMPLES

In summary, the materials, information, and methods described in this disclosure provide an array of attenuated strains with graded attenuation phenotypes, and provide guidance in selecting suitable 5 vaccine candidate strains based on clinical benchmarks. The following examples are provided by way of illustration, not limitation.

Example 1

This example illustrates design, construction, and recovery of recombinant RSV 6120/NS12FM2/GFP and 6120/NS12FM2.

5 The RSV antigenome that was used for constructing 6120/NS12FM2/GFP was the “6120” derivative of the unmodified WT RSV strain A2 antigenomic cDNA called D46 (or D53). D46/D53 is the basis for the present reverse genetics system (Collins, et al. 1995. Proc Natl Acad Sci USA 92:11563-11567), and its complete sequence is shown in US patent 6,790,449 and in GenBank KT992094, with a single difference at position 1938 (in the N gene ORF) compared to the constructs in the present 10 invention. Specifically, the nucleotide assignment at position 1938 in US patent 6,790,449 and in GenBank KT992094 is A, but in the sequences provided herein it is G. This difference does not change amino acid coding and is understood to be inconsequential. The 6120 derivative contained a 112-nucleotide deletion of the downstream non-translated region of the SH gene together with 5 nucleotide 15 substitutions that involve the last three codons and stop codon of the SH ORF and do not change amino acid coding (Bukreyev, et al. 2001. J Virol 75:12128-12140). In addition, the antigenome cDNA had previously been modified to contain a gene encoding enhanced green fluorescent protein (GFP) inserted between the RSV P and M genes as the third gene (Munir et al 2008 J Virol 82:8780-8796). Insertion of a GFP gene in the first gene position was previously shown to have little or no effect on RSV replication or pathogenesis in cell lines and in an *in vitro* human airway epithelium (HAE) culture (Zhang et al 2002 20 J Virol 76:5654-5666), and the same appeared to be the case for GFP inserted between the P and M genes (Munir et al 2008 J Virol 82:8780-8796). The purpose of expressing GFP from the viral genome was to facilitate monitoring infection in initial experiments, because it allows visualization of infections in live cells without interfering with the infection. GFP is often used in this fashion in initial experiments. Note that the GFP gene was not included in the gene position numbering.

25 The top part of FIG. 1 (above the “RSV genome” diagram) illustrates the deletion of the NS1 and NS2 ORFs along with most of their flanking gene sequences from their native positions 1 and 2 in the genome. This deletion has the result of fusing the upstream nontranslated region of the NS1 gene to the N gene ORF. The bottom part of the FIG. 1 (below the “RSV genome” diagram) illustrates the creation 30 of a *Kpn*I site in the intergenic region between the F and M2 genes and the insertion of an NS1/NS2 gene cassette at this site. In 6120/NS12FM2/GFP, the NS1 and NS2 genes were shifted by recombinant DNA methods from their native positions 1 and 2 in the RSV antigenomic cDNA to positions 7 and 8. Note that the GFP gene was not included in the gene position numbering. The shifts are shown in FIG. 1. The recombinant RSV 6120/NS12FM2/GFP virus was recovered by reverse genetics.

35 For a final vaccine product, GFP preferably would not be present. Therefore, site-directed mutagenesis was used to remove the GFP gene from the RSV 6120/NS12FM2/GFP cDNA, creating RSV 6120/NS12FM2. This construct otherwise is identical to that shown in FIG. 1. RSV 6120/NS12FM2 was recovered by reverse genetics and was found to replicate similarly to RSV 6120/NS12FM2/GFP in Vero cells.

With regard to nomenclature, note that of necessity there is some flexibility in usage. For example, 6120/NS12FM2/ΔNS2 also can be referred to as 6120_NS12FM2_ΔNS2 and to 6120_NS12FM2_DNS2 (reflecting that some symbols can be altered *in silico*), or 6120/NS12FM2/ΔNS2, or 6120/NS12FM2/ΔNS2, or 6120NS12FM2/ΔNS2, etc. As another example, 5 RSV 6120/NS12FM2 is equivalent to 6120/NS12FM2 (some descriptors are not essential to the meaning). As another example, various names can be abbreviated for simplicity, as will be noted in the text; for example, 6120/NS12FM2 can be abbreviated as F-M2.

Example 2

10 This example illustrates design and construction of rRSV 6120/NS12Ltr/GFP and 6120/NS12Ltr. The RSV antigenome that was used for constructing 6120/NS12Ltr/GFP was the “6120” derivative of the WT RSV antigenomic cDNA, which also contained the GFP gene between the viral M and P genes, as described in Example 1.

15 In RSV 6120/NS12Ltr/GFP, the NS1 and NS2 genes were shifted by recombinant DNA methods from their native positions 1 and 2 in the antigenomic cDNA to positions 9 and 10. Note that the GFP gene is not included in gene position numbering. The top part of the FIG. 2 (above the “RSV genome” diagram) illustrates the deletion of the NS1 and NS2 ORFs as in FIG. 1. The bottom part of FIG. 2 (below the “RSV genome” diagram) illustrates the creation of a *Kpn*I site in the trailer region shortly after the L gene, and the insertion of an NS1/NS2 gene cassette at this site. The RSV 6120/NS12Ltr/GFP 20 virus was recovered by reverse genetics.

In addition, a subsequent version called RSV 6120/NS12Ltr was constructed, in which the GFP gene was deleted by site-directed mutagenesis. This construct otherwise is identical to that shown in FIG. 2.

Example 3

25 This example describes the replication characteristics of the recombinant RSV 6120/NS12FM2/GFP.

The kinetics and yield of multi-cycle replication of recombinant RSV 6120/NS12FM2/GFP (F-M2) virus was compared to that of wt RSV/GFP (wt RSV) and RSV ΔNS1/ΔNS2/GFP (delNS1_NS2) in 30 African green monkey Vero cells, which are unable to produce type I interferons in response to virus infection. Note that the wt RSV and delNS1_NS2 viruses also are in the 6120 backbone and contain the GFP gene between viral genes P and M. Thus, the F-M2 virus and the control viruses are based on the same viral backbone and can be compared directly.

35 Two independent cultures (01 and 02) were evaluated per virus, using virus stocks that were prepared in Vero cells and infected at an MOI of 0.01. Following infection, cell supernatant samples were taken daily and subsequently evaluated in parallel by plaque titration in Vero cells. These results showed that the F-M2 virus replicated as efficiently as wt RSV in Vero cells, which is the substrate for vaccine virus manufacture, whereas delNS1_NS2 virus was restricted. See FIG. 3. Thus, the F-M2 virus

retains the capacity for efficient vaccine manufacture. This result was not predictable because deletion of NS1 or NS2 has been shown to substantially reduce the efficiency of RSV replication in cell culture, including Vero cells used in vaccine manufacture, because deletion of either or both of these viral proteins results in increased apoptosis resulting in deterioration of the cell monolayer (Bitko et al 2007 J

5 Virol 81:1786-1795). This is evidenced in FIG. 3 by the substantially decreased replication of the delNS1_NS2 mutant. Further, attempts to produce clinical trial material with a Δ NS1 mutant of RSV were unsuccessful because of insufficient titer. The ability of the F-M2 virus to replicate with an efficiency indistinguishable from that of wt RSV indicates that the reduced levels of expression of NS1 and NS2 were sufficient to maintain inhibition of apoptosis sufficient for efficient viral replication.

10 A similar comparison of virus replication was performed in parallel in human airway A549 cells, which are competent for interferon responses to viral infection. Infections were performed with the same Vero-grown virus as in FIG. 3, infected at an MOI of 0.01, with virus replication quantified by plaque assay on Vero cells. These results showed that the F-M2 virus replicated less efficiently than wt RSV, but more efficiently than the delNS1_NS2 virus. See FIG. 4. Growth efficiency in A549 cells by viruses 15 with mutation/deletion in the NS1 and NS2 accessory proteins is a marker for attenuation *in vivo*. A similar Δ NS1 Δ NS2 virus has been shown to be over-attenuated *in vivo* (Jin et al 203 Vaccine 21:3647-3652). The intermediate level of restriction of the F-M2 virus indicates that this gene shift is a useful attenuating mutation in a vaccine virus, either alone or combined with another attenuating mutation.

20

Example 4

This example describes the replication characteristics of the recombinant RSV 6120/NS12Ltr/GFP.

The RSV 6120/NS12Ltr/GFP (L-tr) virus was compared to wt RSV/GFP (wt RSV) and RSV Δ NS1/ Δ NS2GFP (delNS1_NS2) for multi-cycle replication in Vero cells, following the experimental 25 design in Example 3. These results showed that the L-tr virus replicated as efficiently as wt RSV in Vero cells, and thus retains the capacity for efficient vaccine manufacture. See FIG. 5.

A similar comparison of virus replication was done in parallel in human airway A549 cells. These results showed that the L-tr virus replicated less efficiently than wt RSV, but more efficiently than the delNS1_NS2 virus. See FIG. 6. Furthermore, the L-tr virus replicated less efficiently than the F-M2 30 virus (see FIG. 4), consistent with the interpretation that moving the NS1 and NS2 genes to increasingly promoter-distal positions increased the level of restriction. This gene shift provided a more attenuated alternative to the F-M2 virus, and is a useful vaccine virus as-is (preferably with the GFP gene deleted) or further modified by the addition of another attenuating mutation.

35

Example 5

This example describes the deletion of the NS2 gene from RSV 6120/ NS12M2F/GFP and RSV 6120/NS12FM2

To exemplify how gene-shift of NS1 and/or NS2 could be combined with another attenuating

mutation, the NS2 gene was deleted from RSV 6120/NS12FM2/GFP and RSV 6120/NS12FM2, resulting in RSV 6120/NS12FM2/ΔNS2/GFP and RSV 6120/NS12FM2/ΔNS2, respectively. The creation of RSV 6120/NS12FM2/ΔNS2/GFP is illustrated in FIG. 7. RSV 6120/NS12FM2/GFP (top), identical to that in FIG. 1, was modified by site-directed mutagenesis to delete the region from the first nucleotide of the GS 5 signal of the NS2 gene to the first nucleotide of the M2-2 gene, inclusive. This deleted the complete NS2 gene as well as the long NS2-M2 intergenic region in 6120/NS12FM2/GFP. Deletion of the NS2 gene on its own has previously been shown to provide a modest amount of attenuation (Whitehead et al 1999 J Virol 73:3438-3442), compared to the much higher level of attenuation associated with deletion of the NS1 gene (Teng et al 2000 J Virol 74:9317-9321). Both viruses were readily recovered by reverse 10 genetics.

Example 6

This example describes the deletion of the NS2 gene from RSV 6120/ NS12Ltr/GFP and RSV 6120/NS12Ltr.

15 The NS2 gene was also deleted from RSV 6120/NS12Ltr/GFP and RSV 6120/NS12Ltr, resulting in RSV 6120/NS12Ltr/ΔNS2/GFP and RSV 6120/NS12Ltr/ΔNS2. The creation of RSV 6120/NS12Ltr/ΔNS2/GFP is shown in FIG. 8. RSV 6120/NS12Ltr/GFP (top), identical to that in Fig. 2, was modified by site-directed mutagenesis to delete the region from the first nucleotide of the GS signal 20 of the NS2 gene to the last nucleotide of the GE signal of the NS2 gene, inclusive. This deleted the complete NS2 gene.

Example 7

This example describes the replication characteristics of the recombinant virus 6120/NS12FM2/ΔNS2/GFP

25 The kinetics and yield of multi-cycle replication of recombinant RSV 6120/NS12FM2/ΔNS2/GFP (F-M2/delNS2) virus were compared to those of its immediate parent RSV 6120/NS12FM2/GFP (F-M2), wt RSV/GFP (wt RSV), and RSV ΔNS1/ΔNS2/GFP (delNS1_NS2) in African green monkey Vero cells following the general experimental design of Example 3. These results (FIG. 9) showed that the F-M2/delNS2 virus replicated approximately as efficiently as F-M2 and wt RSV 30 in Vero cells, which is the substrate for vaccine virus manufacture, whereas delNS1_NS2 was restricted, as previously shown. Thus, the F-M2/delNS2 virus retains the capacity for efficient vaccine manufacture even though it now contains two attenuating elements: F-M2 and ΔNS2.

A similar comparison of virus replication was performed in parallel in human airway A549 cells, following the general experimental design of Example 3. These results (FIG. 10) showed that these 35 viruses had a range of increasing restriction, in the order: wt RSV < F-M2 < F-M2/delNS2 < delNS1_NS2. As noted, growth restriction in A549 cells by viruses with mutation/deletion in the NS1 and/or NS2 accessory proteins is a marker for attenuation *in vivo*. The observation that F-M2/delNS2 is more restricted than F-M2 shows that the combination of these mutations indeed yielded a further-

restricted derivative. A Δ NS1 Δ NS2 virus similar to the delNS1_NS2 virus in FIG. 10 was previously shown to be over-attenuated in African green monkeys (Jin et al 203 Vaccine 21:3647-3652). Therefore, the availability of the F-M2 and F-M2/delNS2 viruses provides two alternatives that are less restricted and exhibit a range of restriction.

5 These can now be evaluated in HAE cultures, rodents, and African green monkeys in parallel with previous vaccine candidates as benchmarks (Karron et al 2013 Curr Top Microbiol Immunol 372:259-284). Preferably, this analysis would involve the versions of F-M2 and F-M2/delNS2 that do not contain the GFP gene, as described. In addition, the RSV 6120/NS12Ltr/ Δ NS2/GFP (L-tr/delNS2) construct described in FIG. 8, and its derivative lacking GFP, also can be evaluated in parallel. Since the 10 L-tr shift is more attenuating than the F-M2 shift (FIG. 6 versus FIG. 4), this will provide a further range of restriction. One or more appropriate candidates can be evaluated in human volunteers as described (e.g. Karron, *et al.* 2015, Science Transl Med 2015 7(312):312ra175).

15 It will be apparent that the precise details of the methods or compositions described may be varied or modified without departing from the spirit of the described embodiments. We claim all such modifications and variations that fall within the scope and spirit of the claims below.

Claims:

1. A recombinant respiratory syncytial virus (RSV) attenuated by one or more modifications to a RSV genome, wherein the one or more modifications comprise:
 - (a) a NS1 gene and a NS2 gene shifted from gene positions 1 and 2 to gene positions 7 and 8 of the RSV genome, respectively;
 - (b) the NS1 gene and the NS2 gene shifted from gene positions 1 and 2 to gene positions 9 and 10 of the RSV genome, respectively;
 - (c) the NS1 gene shifted to a gene position higher than position 1;
 - (d) the NS2 gene shifted to a gene position higher than position 2; or
 - (e) a combination of (c) and (d).
2. The recombinant RSV of claim 1, wherein the one or more modifications comprise (a).
3. The recombinant RSV claim 1, wherein the one or more modifications comprise (b).
4. The recombinant RSV claim 1, wherein the one or more modifications comprise (c).
5. The recombinant RSV claim 1, wherein the one or more modifications comprise (d).
6. The recombinant RSV claim 1, wherein the one or more modifications comprise (e).
7. The recombinant RSV of any of claims 4-6, wherein the NS1 gene is shifted to gene position 7 or 9.
8. The recombinant RSV of any of the prior claims, wherein the RSV genome further comprises a modification comprising a deletion of all or part of the NS1 gene or the NS2 gene.
9. The recombinant RSV any of the prior claims, wherein the RSV genome further comprises a modification comprising deletion of all or part of an M2-2 gene.
10. The recombinant RSV of any of claims 1-2, or 4-9, wherein the RSV genome comprises the one or more modifications, and a nucleotide sequence corresponding to a positive-sense sequence at least 90%, at least 95%, and/or at least 99% identical to SEQ ID NO: 2 (6120/NS12FM2).
11. The recombinant RSV of claim 10, wherein the RSV genome comprises a positive-sense sequence denoted by SEQ ID NO: 2 (6120/NS12FM2).
12. The recombinant RSV of any of claims 1 or 3-9, wherein the RSV genome comprises the

one or more modifications, and a nucleotide sequence corresponding to a positive-sense sequence at least 90%, at least 95%, and/or at least 99% identical to SEQ ID NO: 4 (6120/NS12Ltr).

13. The recombinant RSV of claim 12, wherein the RSV genome comprises a positive-sense sequence denoted by SEQ ID NO: 4 (6120/NS12Ltr).

14. The recombinant RSV of claim 8, wherein the RSV genome comprises the one or more modifications, and a nucleotide sequence corresponding to a positive-sense sequence at least 90%, at least 95%, and/or at least 99% identical to SEQ ID NO: 6 (6120/NS12FM2/ΔNS2) or SEQ ID NO: 8 (6120/NS12Ltr/ΔNS2).

15. The recombinant RSV of claim 14, wherein the RSV genome comprises a positive-sense sequence denoted by SEQ ID NO: 6 (6120/NS12FM2/ΔNS2) or SEQ ID NO: 8 (6120/NS12Ltr/ΔNS2).

16. The recombinant RSV of claim 1, wherein the RSV genome further comprises a reporter gene, optionally wherein the reporter gene encodes a Green Fluorescent Protein (GFP).

17. The recombinant RSV of claim 16, wherein the RSV genome comprises:
the one or more modifications and the reporter gene, and a nucleotide sequence corresponding to a positive-sense sequence at least 90%, at least 95%, and/or at least 99% identical to SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, or SEQ ID NO: 7; and/or
a nucleotide sequence corresponding to a positive-sense sequence set forth as SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7.

18. The recombinant RSV of any one of claims 1-17, which exhibits:
reduced expression of the NS1 gene and/or NS2 gene compared to an RSV having the NS1 gene in gene position 1 and the NS2 gene in gene position 2;
reduced transcription of the NS1 gene and/or NS2 gene compared to an RSV having the NS1 gene in gene position 1 and the NS2 gene in gene position 2; and/or
reduced inhibition of host interferon response compared to an RSV having the NS1 gene in gene position 1 and the NS2 gene in gene position 2.

19. The recombinant RSV of any one of claims 1-18, wherein the recombinant RSV is increasingly susceptible to restriction in cultured cells that can produce interferons in response to viral infection.

20. The recombinant RSV of any one of claims 1-18, wherein the recombinant RSV retains replication efficiency in cultured cells that cannot produce interferons in response to viral infection.

21. The recombinant RSV of any of the prior claims, wherein the recombinant RSV is a subtype A RSV or a subtype B RSV.

22. The recombinant RSV of any of the prior claims, wherein the recombinant RSV is infectious, attenuated, and self-replicating.

23. An isolated polynucleotide molecule comprising the nucleotide sequence of the genome of the recombinant RSV genome of any of claims 1-22, or an antigenomic cDNA or RNA sequence of the RSV genome.

24. A vector comprising the isolated polynucleotide molecule of claim 23.

25. A cell comprising the isolated polynucleotide or vector of claim 23 or claim 24.

26. A method of producing a recombinant RSV, comprising: transfecting a permissive cell culture with the vector of claim 24; incubating the cell culture for a sufficient period of time to allow for viral replication; and purifying the replicated recombinant RSV.

27. A recombinant RSV produced by the method of claim 26.

28. A pharmaceutical composition comprising the recombinant RSV of any of claims 1-22 or 27.

29. A method of eliciting an immune response to RSV in a subject comprising administering an immunogenically effective amount of the pharmaceutical composition of claim 28 to the subject.

30. The method of claim 29, wherein the pharmaceutical composition is administered intranasally.

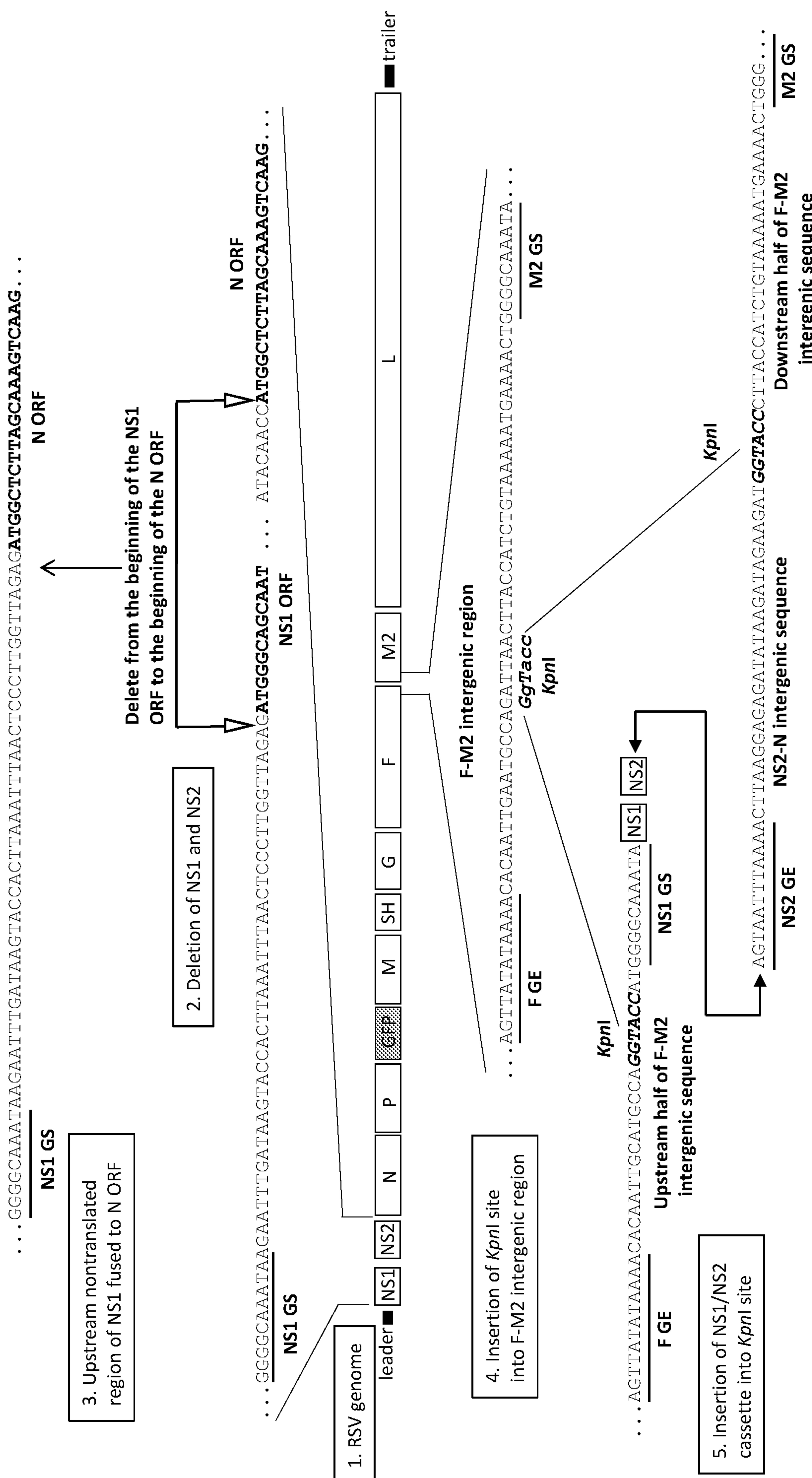
31. The method of claim 29 or claim 30, wherein the RSV is administered via injection, aerosol delivery, nasal spray or nasal droplets.

32. The method of any of claims 29-31, wherein the subject is a human.

33. The method of any of claims 29-32, wherein the subject is between 1 and 6 months of age.

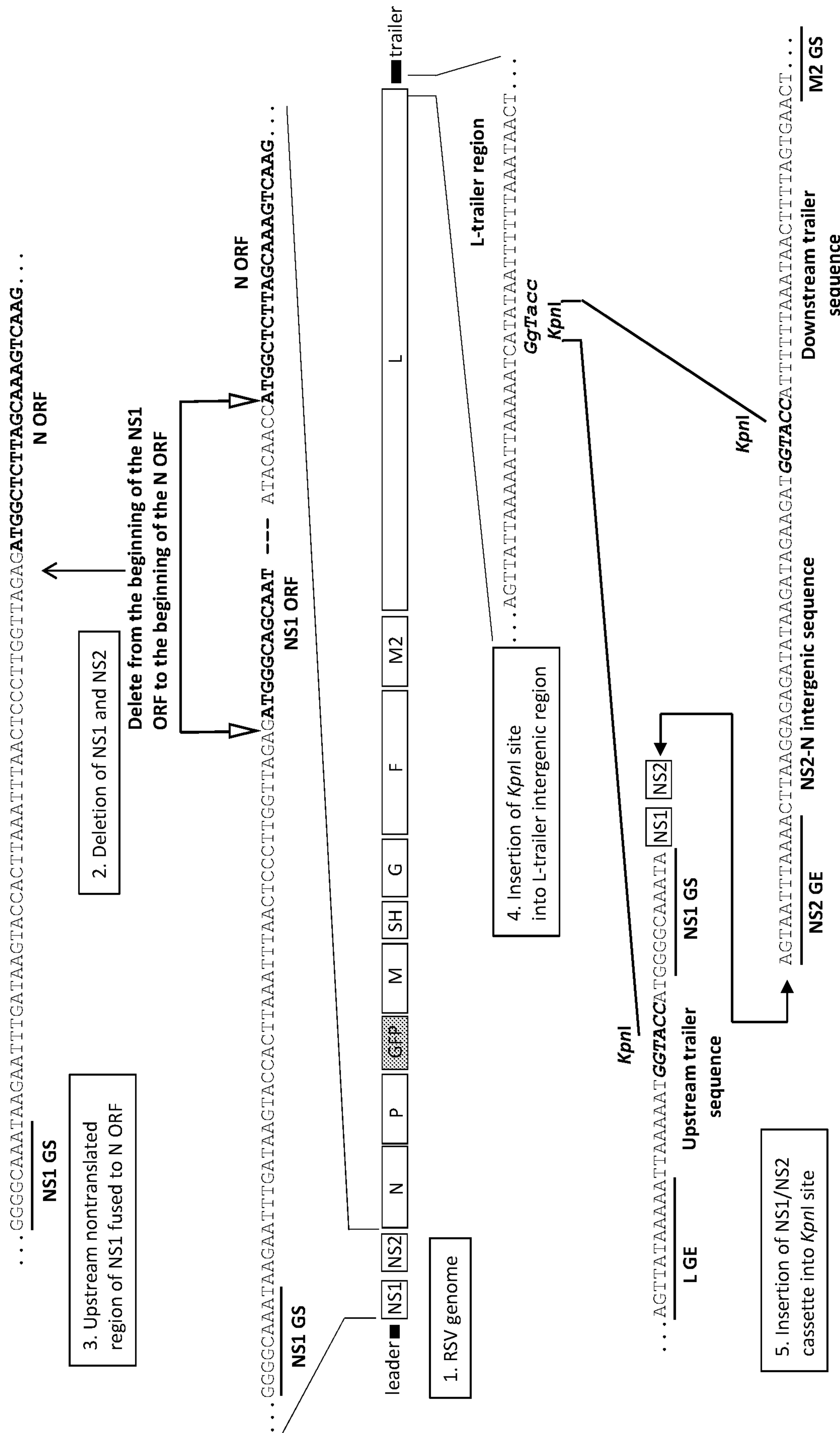
34. The method of any of claims 29-33, wherein the subject is seronegative for RSV.
35. Use of the recombinant RSV of any of claims 1-22 or claim 27 to elicit an immune response to RSV in a subject.

FIG. 1



Generation of the **RSV 6120/NS12FM2/GFP** virus, in which the NS1 and NS2 genes are shifted from their native positions 1 and 2 in the genome to positions 7 and 8

FIG. 2



Multicycle replication in Vero cells of RSV with NS1-NS2 between F-M2

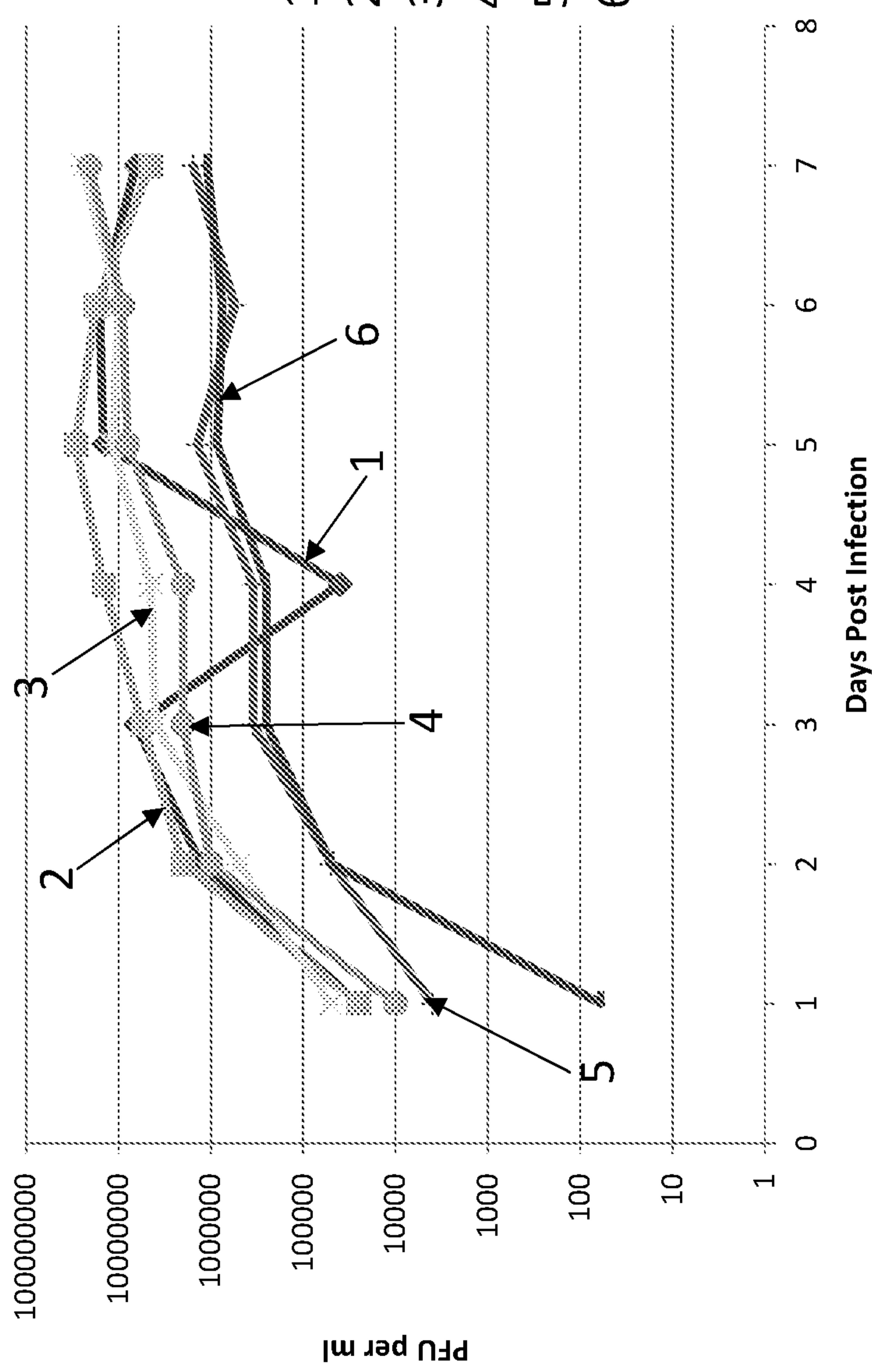


FIG. 3

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FIG. 4

Multicycle replication in A549 cells of RSV with NS1-NS2 between F-M2

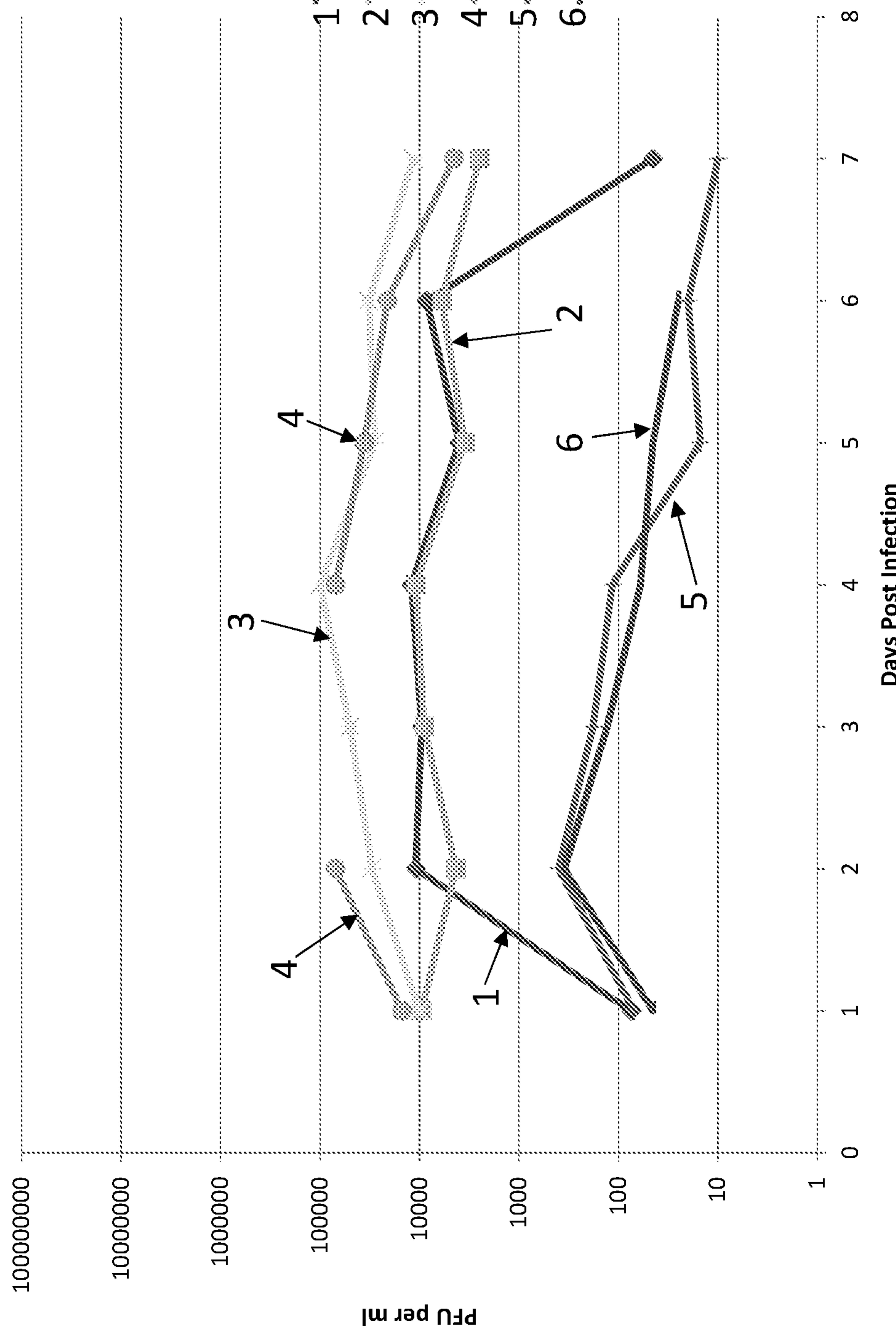


FIG. 5
Multicycle replication in Vero cells of RSV with NS1-NS2 between L-trailer

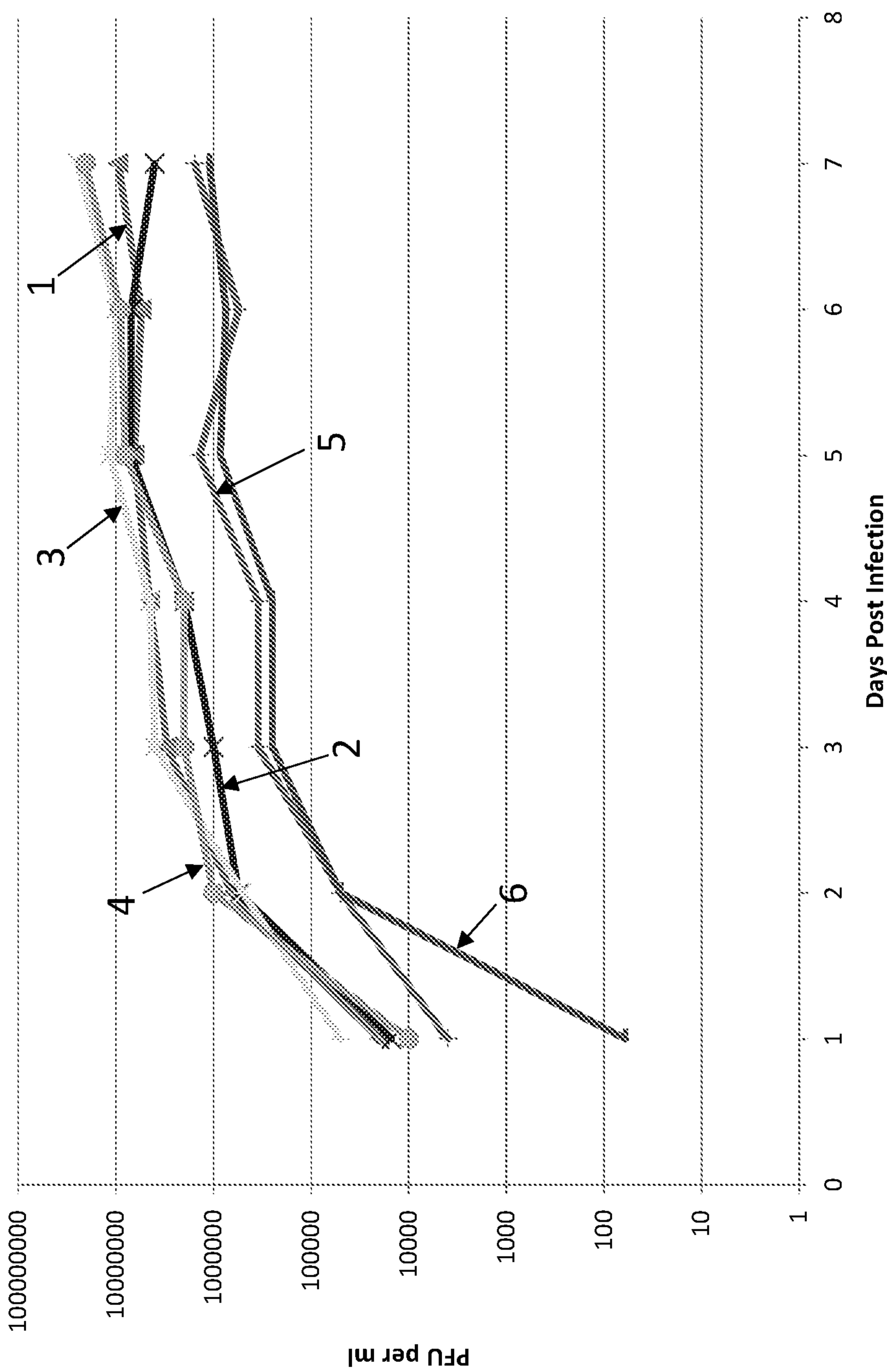


FIG. 6

Multicycle replication in A549 cells of RSV with NS1-NS2 between L-trailer

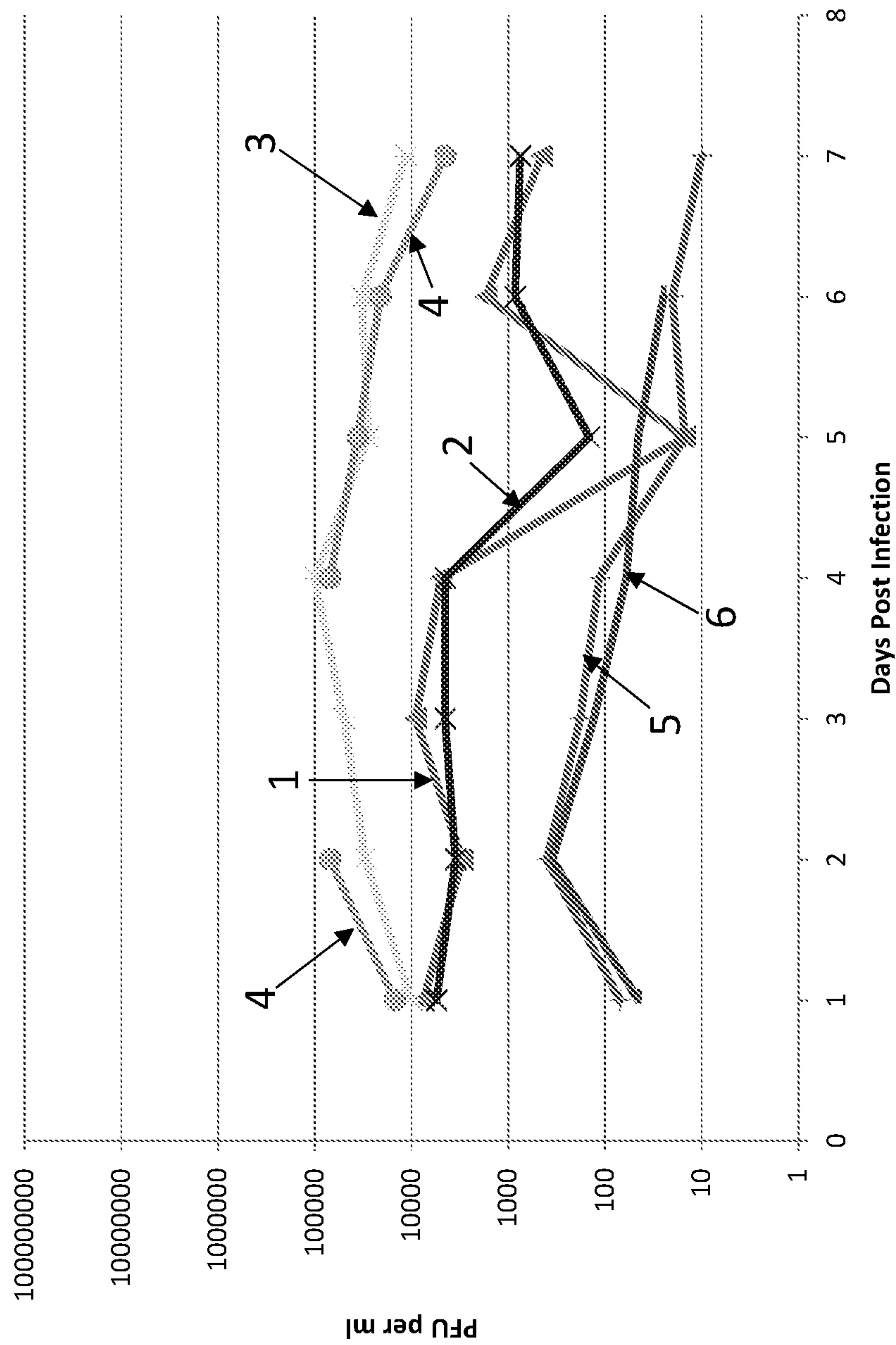
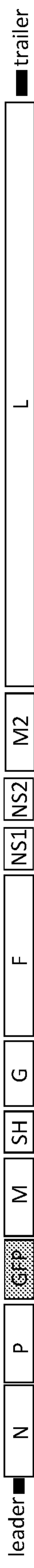
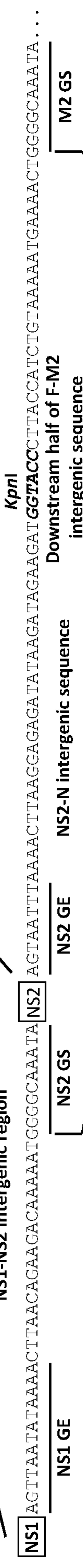
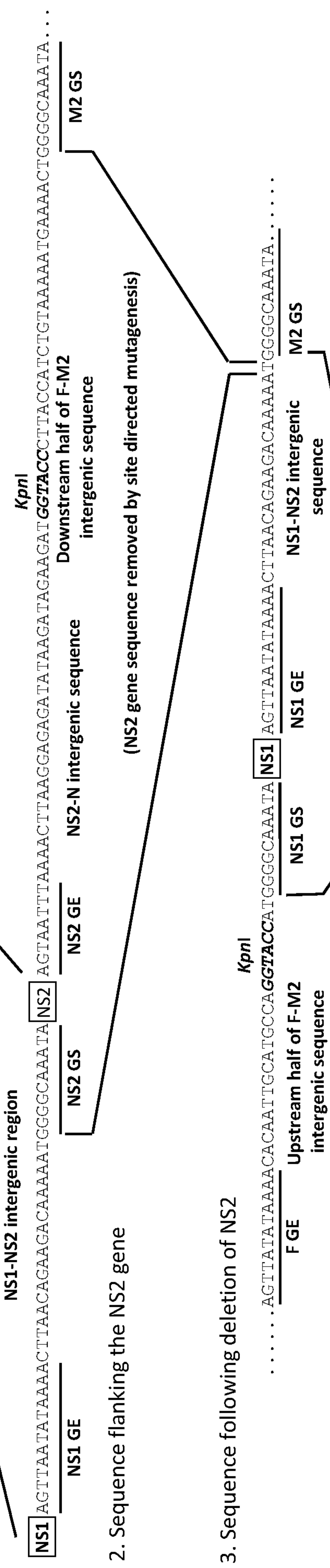
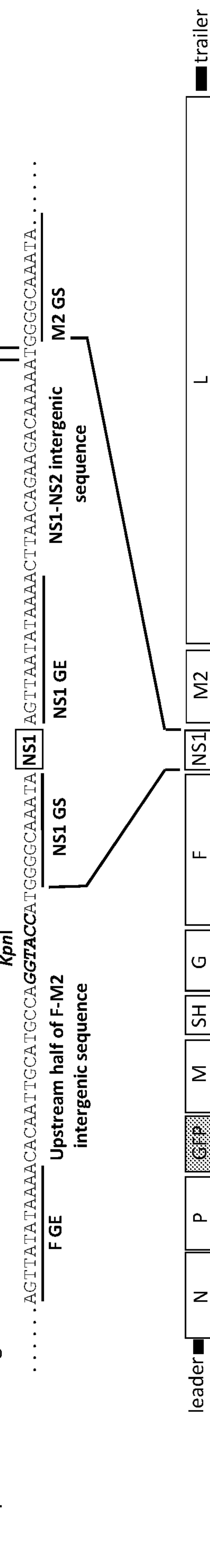


FIG. 7**1. RSV 6120/NS12FM2/GFP virus****NS1-NS2 intergenic region****2. Sequence flanking the NS2 gene****NS2-M2 intergenic region****(NS2 gene sequence removed by site directed mutagenesis)****3. Sequence following deletion of NS2****4. RSV 6120/NS12FM2/ΔNS2/GFP virus**

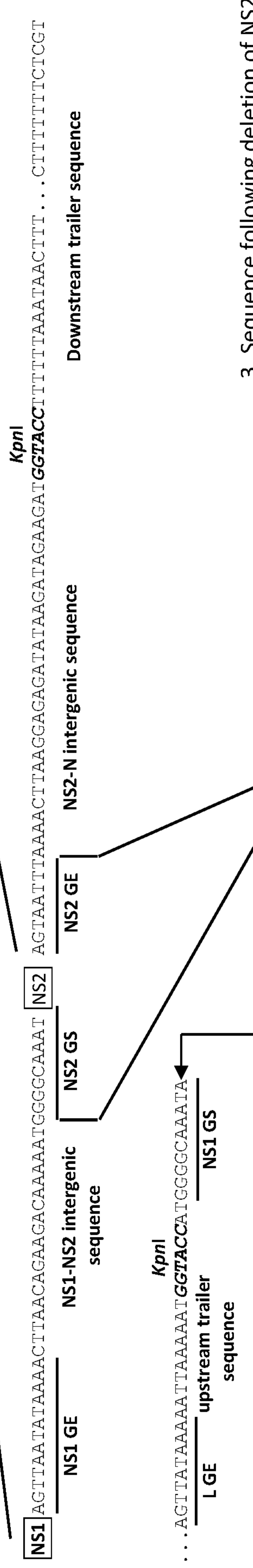
Deletion of the NS2 gene from RSV 6120/NS12FM2/GFP to create RSV 6120/NS12FM2/ΔNS2/GFP

FIG. 8

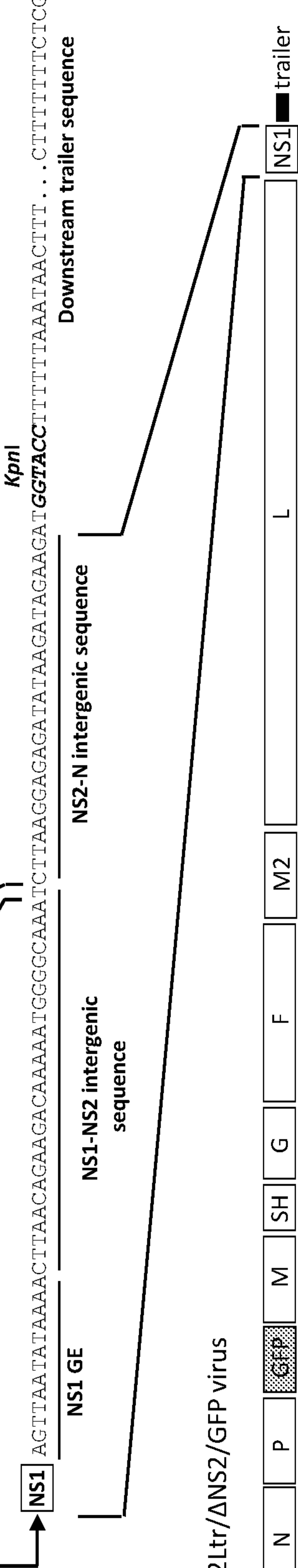
1. RSV 6120/NS12Ltr/GFP virus



2. Sequence flanking the NS2 gene



3. Sequence following deletion of NS2



4. RSV 6120/NS12Ltr/ΔNS2/GFP virus



Deletion of the NS2 gene from RSV 6120/NS12Ltr/GFP to create RSV 6120/NS12Ltr/ΔNS2/GFP

Multicycle replication of RSV 6120/NS12FM2/ΔNS2 in Vero cells

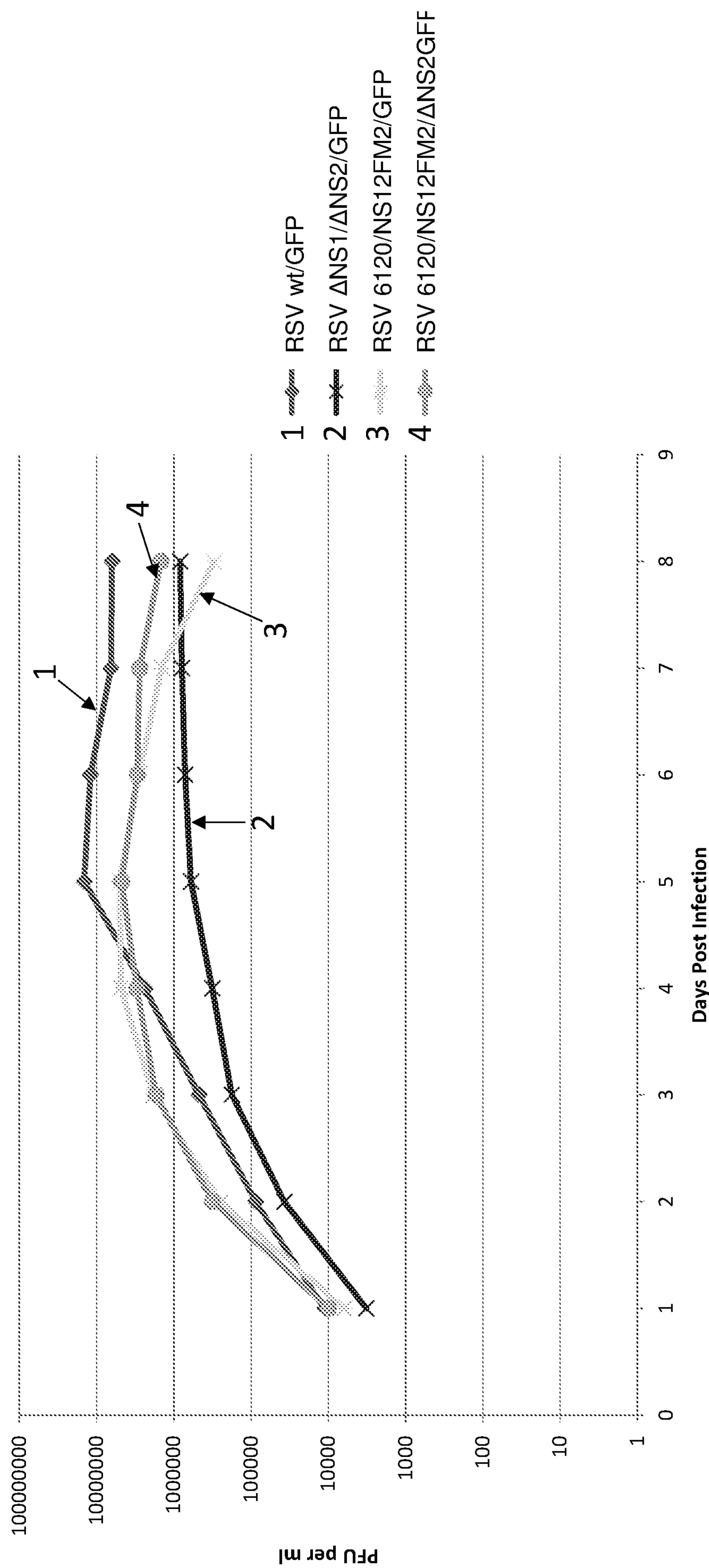


FIG. 9

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FIG. 10

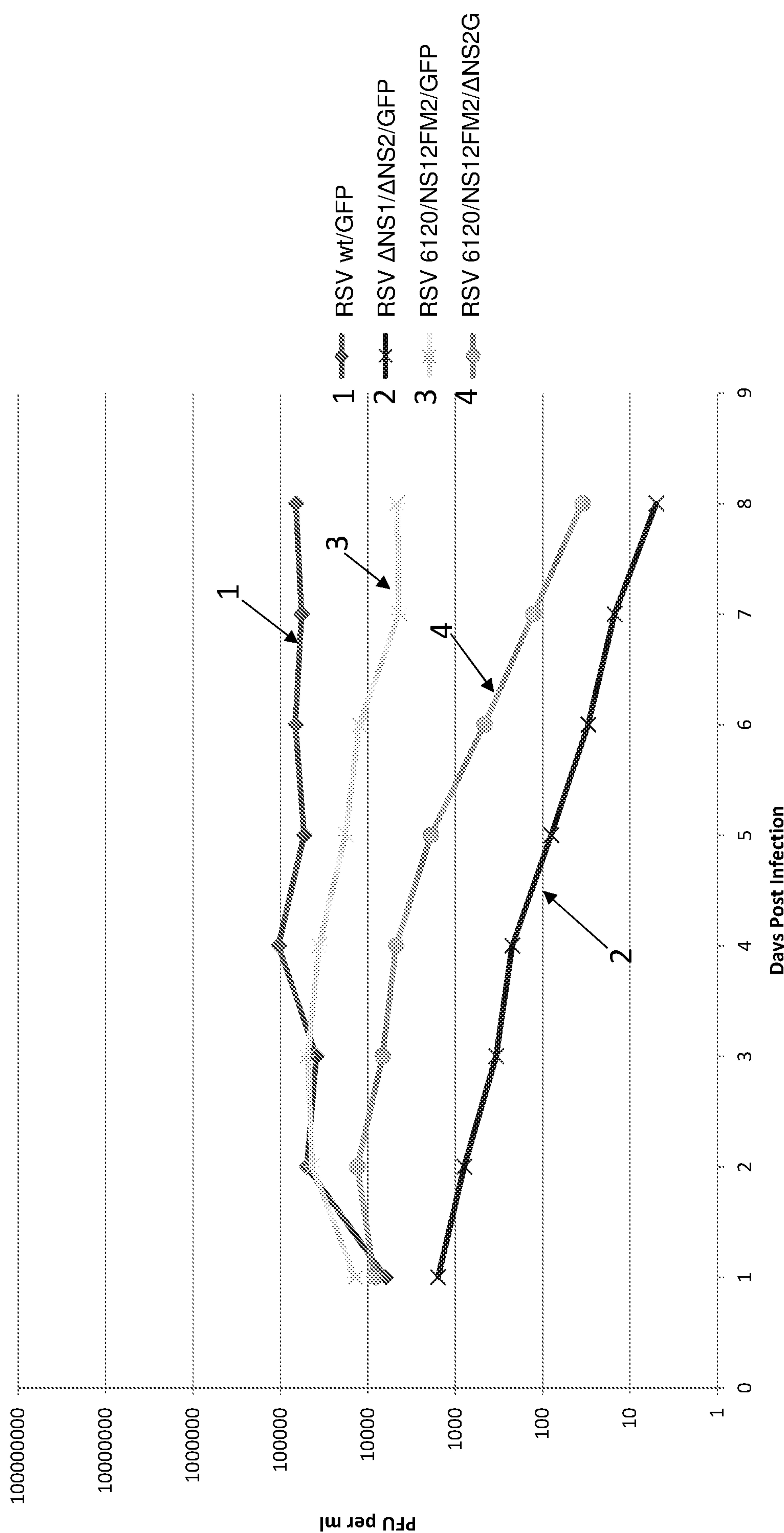
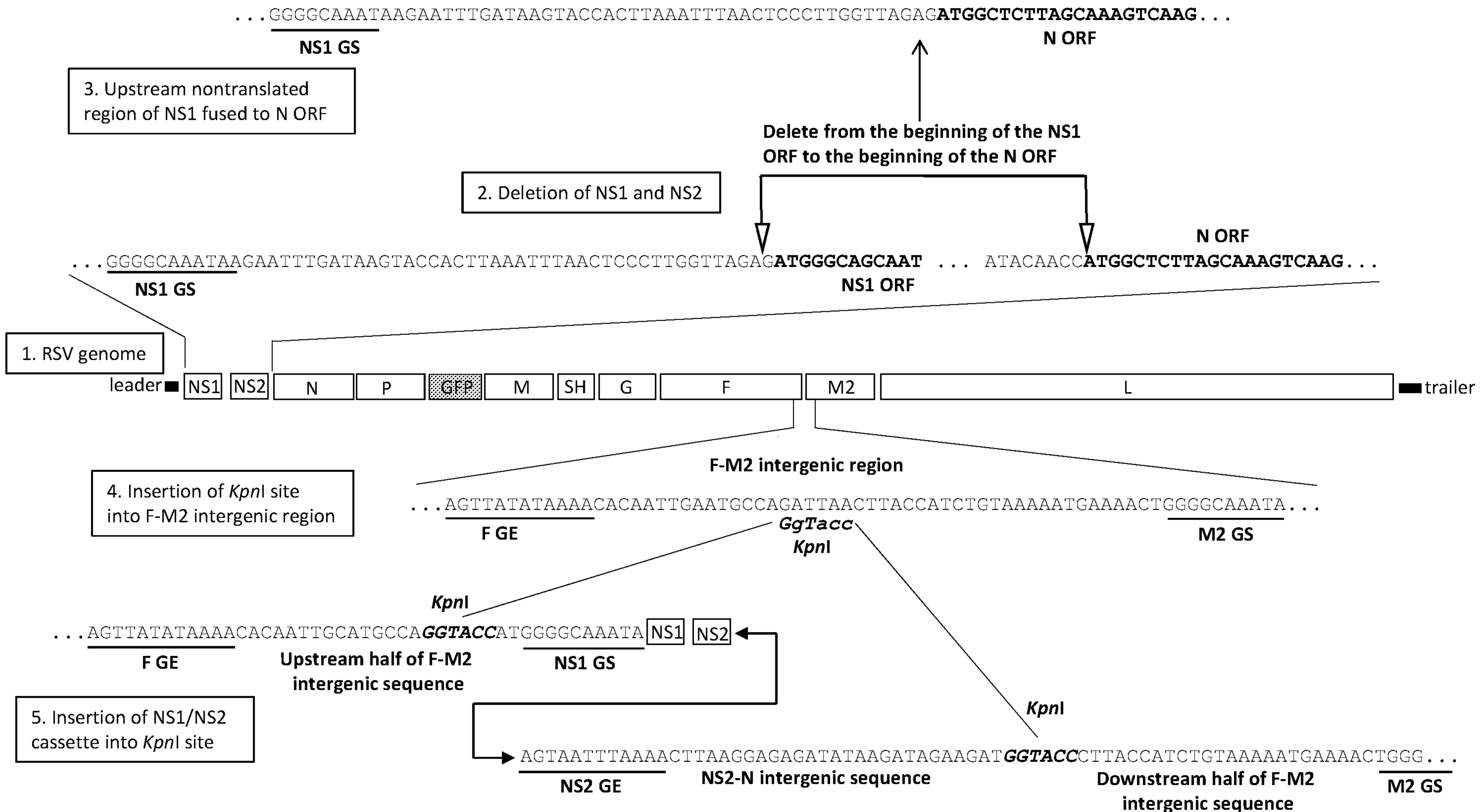
Multicycle replication of RSV 6120/NS12FM2/ Δ NS2 in A549 cells

FIG. 1



Generation of the **RSV 6120/NS12FM2/GFP** virus, in which the NS1 and NS2 genes are shifted from their native positions 1 and 2 in the genome to positions 7 and 8